

***PRESTIGE* OIL SPILL. RESULTS OF BIOREMEDIATION ASSAYS ON SUPRA-TIDAL ROCKS OF SÁLVORA ISLAND (GALICE, SPAIN)**

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**ABSTRACT**

The conditions in which the *Pestige* oil spill (November, 2002) took place, caused a coastal pollution up to tidal levels reached only very few times per year. This circumstance made necessary to apply to the rocks, after the manual or mechanical collection procedures were no longer useful, cleaning methods with pressurized water and, in difficult accessible areas, bioremediation treatments. To verify the efficiency of different bioremediation approaches, a comparative assay of several formulations was started in March 2003 on a highly affected rocky area of Sálvora island (National Park of the Atlantic Islands, Galicia, Northwest Spain). In this work the results of the more effective procedures are presented, and some general aspects of the bioremediation on rocky substrates are discussed.

## INTRODUCTION

### *Evaluation of bioremediation processes. Discussion of some usual problems.*

To evaluate the effectiveness of a bioremediación procedure in a rocky coast presents two main difficulties. The first one is the great heterogeneity of the space, the irregular surface of which promotes strong variations in the thickness of the fuel layers and in its capacity to retain the spilled materials (different textures, ruggedness, degree of exposition to the atmospheric agents). Under these conditions (figure 1A), experimental plans as those based on orthogonal designs or latin squares are not very realistic, and the minimal area or the sample size necessary to obtain an acceptable standard error are of magnitudes not very viable in practice.

The second difficulty is the evaluation criterion. Although the best option is to compare the total fuel remaining in treated and untreated (control) areas, heterogeneity also “contaminates” this procedure, so it is frequent to use the relationships between oil components with very different degradation rates. This way, the value obtained in a concrete sample would be, in principle, satisfactorily representative of a natural area, with independence of its heterogeneity. In laboratory studies it is common to use hopanes as internal standards with practically null degradation rates. In such a case as the one described here, hopanes can be substituted by asphaltene fraction (operationally defined as

the pentane-insoluble fraction in the fuel), the degradation rate of which is negligible compared to those of alkanes or even PAHs.

This way, a relationship as:

$$Ra = \frac{\text{total fuel} - \text{asphaltenes}}{\text{total fuel}} = \frac{\text{deasphalted fraction}}{\text{total fuel}}$$

could solve the two referred difficulties. Moreover, if it is accepted that only the de-asphalted fraction ( $D$ ) is biodegradable, the asphaltenes remaining for a long time in the initial value  $A_0$ , it can be defined a normalized index  $R_t$  ( $t$  being a time subindex and  $T$  being the total fuel) that would reflect acceptably the time-course of the degradation process:

$$R_t = \frac{(T_t - A_0)/T_t}{(T_0 - A_0)/T_0} 100 = \frac{D_t/T_t}{D_0/T_0} 100 \quad [1]$$

It is necessary, however, to keep in mind another problem. If, as usual, the degradation proceeds (as average) through a first order kinetics with constant rate  $r$ , we can write:

$$D_t = D_0 \exp(-rt) \quad [2]$$

And, therefore, the value of the normalized index  $R_t$  will obey the equation:

$$R_t = \frac{D_t/T_t}{b_0} 100 = \frac{100}{b_0} \frac{D_0 \exp(-rt)}{A_0 + D_0 \exp(-rt)} ; \text{ where } b_0 = D_0/(D_0+A_0) \quad [3]$$

An expression that can be used, if we have a series of  $R_t$  values, to estimate the degradation rate  $r$ , as well as the half life ( $t_{1/2}$ ), or time necessary so that the initial value of the index ( $R_{t=0}=100$ ) descends in half. Indeed, making  $R_t=50$  in [3]:

$$50 = \frac{100}{b_0} \frac{D_0 \exp(-rt)}{A_0 + D_0 \exp(-rt)} ; \text{ we obtain:}$$

$$t_{1/2} = \frac{1}{r} \ln \left[ \frac{D_0(2 - b_0)}{A_0 b_0} \right] \quad [4]$$

Nevertheless, it should be kept in mind that, although useful as an instrument for smoothing the experimental error, the  $R_t$  index is less discriminative than the value directly derived from quantification of total fuel, since  $R_t$  descends along the time less markedly than the degradable fraction. Figure 2 illustrates this problem by means of a simulation of the variation with time of  $T_t$  and  $R_t$  (assuming  $A_0=14$  and  $D_0=86$ , which are the approximate values corresponding to the fuel from the *Prestige* deposited in the rocks<sup>1</sup> at the beginning of the assay, and an arbitrary value  $r=0.02$  for the rate constant). On the other hand, the precedent conclusions are very sensitive to the supposition of a single first order kinetics for the degradation of the de-asphalted fraction (which includes components of very different stability). Finally, it is obvious that  $R_t$  loses its validity if the level of the asphaltene fraction varies with time as a direct or indirect consequence of the bioremediation process.

## MATERIALS AND METHODS

Taking into account the casuistry discussed in the preceding section, the design of the Sálvora assays tried to avoid the two types of described problems appealing to following expedients:

1) To use, for quantification of the process, panels of granitic tiles (15×15 cm) impregnated with fuel from the *Prestige* and located on the environment affected by the oil spill (figure 1B). Half of the tiles, “painted” by means of a roller, received approximately equal fuel loads, in the hypothesis that the  $R_t$  index is independent of small differences in the initial load. The other half was loaded with well-known weights (3-4 g per tile) of the same material.

2) To base the evaluation on the variation with time of the remaining total fuel determined in exhaustive extracts of impregnated tiles, both untreated (control) and subjected to the different treatments assayed. The figure 3 shows the protocol applied to the extraction and fractionation of the extracts, carried out to obtain possible complementary information from the proportions of the main fuel fractions (alkanes, PAHs, asphaltenes-resins).

Treatments began (because of bureaucratic reasons) in different moments along February-March 2003, with an approximate interval of one month between the beginning of the first one and the last one. Applications were carried out with the frequencies and under the specifications of the suppliers, and doses were similar in all the cases, to assure the maintenance of a reasonable level of bioremediation agents on the affected surfaces, by

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<sup>1</sup> Initial values (%) of the main fractions, according to data from CEDRE (France): saturated hydrocarbons  $30.5 \pm 2.4$ ; aromatic hydrocarbons  $40.1 \pm 1.2$ ; resins  $17.1 \pm 2.4$ ; asphaltenes  $12.3 \pm 1.0$ .

repeating the additions of nutrients, microbiota or both every 15-25 days. In the case of addition of allochthonous microorganisms, they were subjected to the following restrictions: not genetically modified, not photosynthetic, not spore-producing, not parasites. The following treatments were assayed:

**A:** Enrichment of the affected areas with N, P, Fe and trace levels of other nutrients present in conventional agricultural fertilizers. In a typical case, with an estimation of ~1.000 kg per Ha of present fuel, the fertilization implied the addition, every 15-25 days, of 0.5 l per m<sup>2</sup> of an aqueous solution of the following (or equivalent) commercial products:

NH <sub>4</sub> NO <sub>3</sub> (soluble; 33.5% N; Haifa Chemicals)	244 g
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> (technical; 12-61-0; Haifa Chemicals)	153 g
Libamin mix (Jisa, S.A.)	4 mL

which is equivalent to the following proportions of essential nutrients:

$$N \sim \text{Estimated fuel} \times 0,10$$

$$P \sim \text{Estimated fuel} \times 0,04$$

$$Fe > \text{Estimated fuel} \times (4 \times 10^{-5})$$

To facilitate the retention on the treated surface of the chemicals and (ambiental or specifically supplied) microbiota, 5% sepiolite was added to the nutrient solutions.

- B:** As A, but adding a bacterial consortium ( $\sim 10^{10}$  colony forming units per l) isolated from a coastal area affected by the oil spill, few days after the accident.
- C:** As B, but substituting sepiolite by bentonite, and also adding 5% of vegetable fragments.
- D:** As C, but in a commercial formulation containing 10% of natural surfactants.
- E:** As C, but adding a commercial bacterial inoculum containing three wild strains of *Pseudomonas putida*.
- F:** As A, with addition of the same commercial bacterial inoculum (*Pseudomonas putida*) used in E.
- G:** As F, with addition of 5% sepiolite.
- H:** As A, but without sepiolite and adding a commercial bacterial consortium, as well as a commercial vegetable extract containing vitamins and other micronutrients.
- I:** As H, but substituting the commercial bacterial consortium for other (autochthonous) isolated by means of similar procedures to those used in B.
- J:** The commercial formulation S-200, containing urea, a phosphoric ester and glycol ether in oleic acid. In this case, two doses of 340 ml per m<sup>2</sup> were applied.

**K:** As A, but adding a commercial bacterial inoculum containing archaea in clay as support, and additional supplements of urea, Cu, Mn, B and Mo.

## **RESULTS AND DISCUSSION**

During the first 2-3 months, the evaluation was based on the  $R_t$  index applied to the extracts of semi-quantitatively loaded tiles. In all cases a decrease of  $R_t$  was detected, that could be interpreted in terms of a first order kinetics with a rate of disappearance of the de-asphalted fraction ( $r$  in equations [2] and [3]) slightly higher in treated tiles than in controls. However, the fittings of the experimental data to the model [3] were not statistically significant ( $\alpha=0.05$ ), and the values corresponding to the last points only allowed to attribute a slight, although significant, advantage to the case J.

On the other hand, the formal treatment of  $R_t$  did not seem to translate the different behaviors that were observed in some cases during the fractionation with pentane (the separation of the asphaltenes-resins fraction was less clear in treated tiles than in controls). The simple visual observation suggested in those cases that  $R_t$  led to infra-estimations of effectiveness, probably due to the fact that the impregnated surfaces seemed to lose, in a certain extent, components of the asphaltenes-resins fraction.

When the period of treatments were between 68 and 83 days, the estimation of the remaining total fuel in tiles with strictly quantified initial loads produced the results shown in figure 4, which confirmed the suspicion that the  $R_t$  index led to infra-estimate the

effectiveness of the most effective treatments. It was also clear that the differences between control and treated tiles only overcame 20% in three formulations (F, I and J). The rest of the treatments were in the threshold of the statistical significance ( $n=4$ ;  $\alpha=0.05$ ), or not statistically significant at these levels. Additionally, the two more effective formulations (I, J) were, in fact, those with shorter periods of actuation. Therefore, in accordance with administrators of the National Park of the Atlantic Islands, we decided to continue only with the treatments F, I and J.

The ulterior evolution with time of remaining total fuel in these three cases, during a period of near 250 days, is shown in figure 5. Pictures in figure 6 show tiles in stages corresponding to the final points of the figure 5. In this last figure, an acceleration of the disappearance of the fuel is appreciated during the summer period, as well as a relative effectiveness of the three treatments that maintains the tendencies detected after 2-3 months, with a substantial advantage for the treatment J. The process was slightly less advanced in the natural area, fundamentally due to the accumulation of fuel residues in the concavities of the rocks (figure 7). However, the general state of this area was consistent with the evaluation derived from the results obtained in the tiles. In this way, the treatment J (S-200) was finally selected to apply to other coastal areas of similar characteristics to those of the Sálvora island.

It is expected that these new applications of treatment J provide the framework to study with more detail the bioremediation process, both from the point of view of the degradation of the different fuel fractions, and the microbiological aspects involved in bioremediation effects. However, the precedent results (derived from a simple comparison

of effectiveness directed to advise an administrative decision) already allow us to underline some conclusions. In fact, at least in a supra-tidal rocky space:

1) The key factor for the success of a bioremediation treatment seems to depend on the capacity of the formulation used to retain nutrients and environmental microbiota on the fuel layers that cover the rocks, more than on the addition of microbial reinforcements.

2) Fertilizations in aqueous solutions are not very capable to achieve the retention mentioned in the precedent point, even when adsorbent materials (clay, sepiolite, bentonite or vegetable fragments) are added.

3) On the contrary, the oleaginous formulation (J: S-200) showed to be able to mix with the fuel layers, to retard fuel hardening and to form surfaces able to retain environmental microbiota and to increase the bio-accessibility of the hydrocarbons (see figure 8). It is also possible that the fatty acids present in the formulation act as appropriate co-substrates, which facilitate the co-metabolic degradation of the most recalcitrant hydrocarbons. Finally, it seems clear that the dispersive effect of S-200, although very slow (figure 8), promotes the migration of the non-biodegradable fuel components (asphaltenes-resins), a process often considered undesirable. But this dispersion takes place in a finely divided state and with rates even inferior to those promoted, for example, by beating of the sea on the rocks, a process often considered as positive. On the other hand, it can be pointed out that S-200 was less sensitive than the other formulations assayed to the effects of both pluvial lixiviation, and high summer temperatures in rocks blackened by the fuel.

4) Although bacterial counts produced results with a great variability, in general it can be said that, when additional inocula was applied in combination with aqueous fertilizations, the number of micro-organisms (cfu) descended quickly after each application, arriving in a few days (8-12) until the levels detected in control areas. Preliminary results obtained by denaturing gradient gel electrophoresis (DGGE) indicate that the DNA band patterns from S-200 treated tiles show a smaller diversity that those from the control tiles, as well as a clearer predominance of some bands, corresponding to species not yet identified.

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### **CAPTIONS TO FIGURES**

Figure 1. A: Detail of the treated area (high tide). B: 9 of the 60 panels of granitic tiles impregnated with fuel from *Prestige* that were used for quantifications.

Figure 2. Relationships between the time-course of total fuel  $T_t$  (continuous line) and  $R_t$  index (dotted line), supposing a first order kinetics for degradation of de-asphalted fraction, and zero order kinetics for asphaltenes (see also text).

Figure 3: Treatment of the tiles and fractionation of total fuel extracts in the four main groups of components: aliphatic hydrocarbons and cycloalkanes, polyaromatic hydrocarbons, resins and asphaltenes. Raw extracts were obtained by submerging complete tiles in closed glass recipients with dichloromethane:methanol (2:1), under rotary agitation (60 rpm) during 24 hours at 20°C. The redundant pathways (as silica-gel column chromatography and isooctane/dimethyl sulfoxide/water partition) have only used as confirmatory expedients. In S-200 treatments, the saponification pathway (A1) was necessary to eliminate the formulation residue that, in A2 pathway, contaminates both the asphaltenes and de-asphalted fraction.

Figure 4: Remaining total fuel (% of the initial level) detected in tiles subjected to the different treatments assayed (0: control without treatment), at indicated times. Error bars refer to confidence intervals ( $n=4$ ;  $\alpha=0.05$ ).

Figure 5: Time-course of remaining total fuel (% of the initial level) in control tiles (0) and those treated with the three more effective formulations (F, I and J) after 2-3 months. Error bars refer to confidence intervals ( $n=3$ ;  $\alpha=0.05$ ).

Figure 6: Aspect of the contrast tiles corresponding to control and the three more effective treatments, after a 200 days period. The states in which these tiles are shown correspond with a very good approximation to those quantitatively described as the final points of the

figure 5. Notice the appearance, practically unaltered, of the control tiles (the most whitish areas that can be appreciated in some cases are excremental residues from gulls).

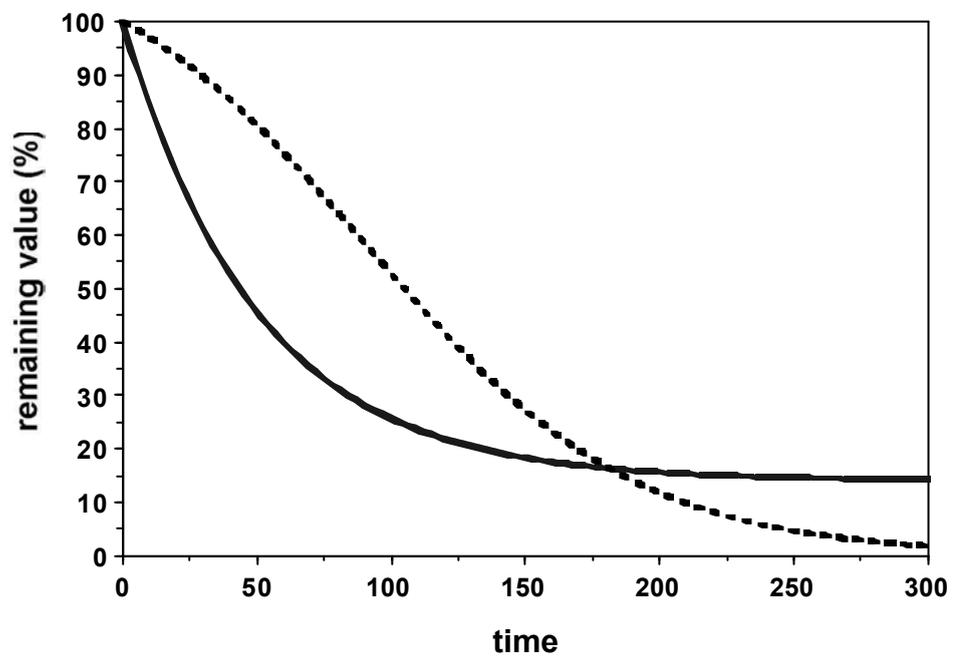
Figure 7: Effect of the S-200 treatment on some rocks after a 200 days period.

Figure 8: Granitic tiles impregnated with the same quantity of fuel from *Prestige*, after a 20 days period of exposure to the bleakness, in absence (left) and presence (right) of S-200.

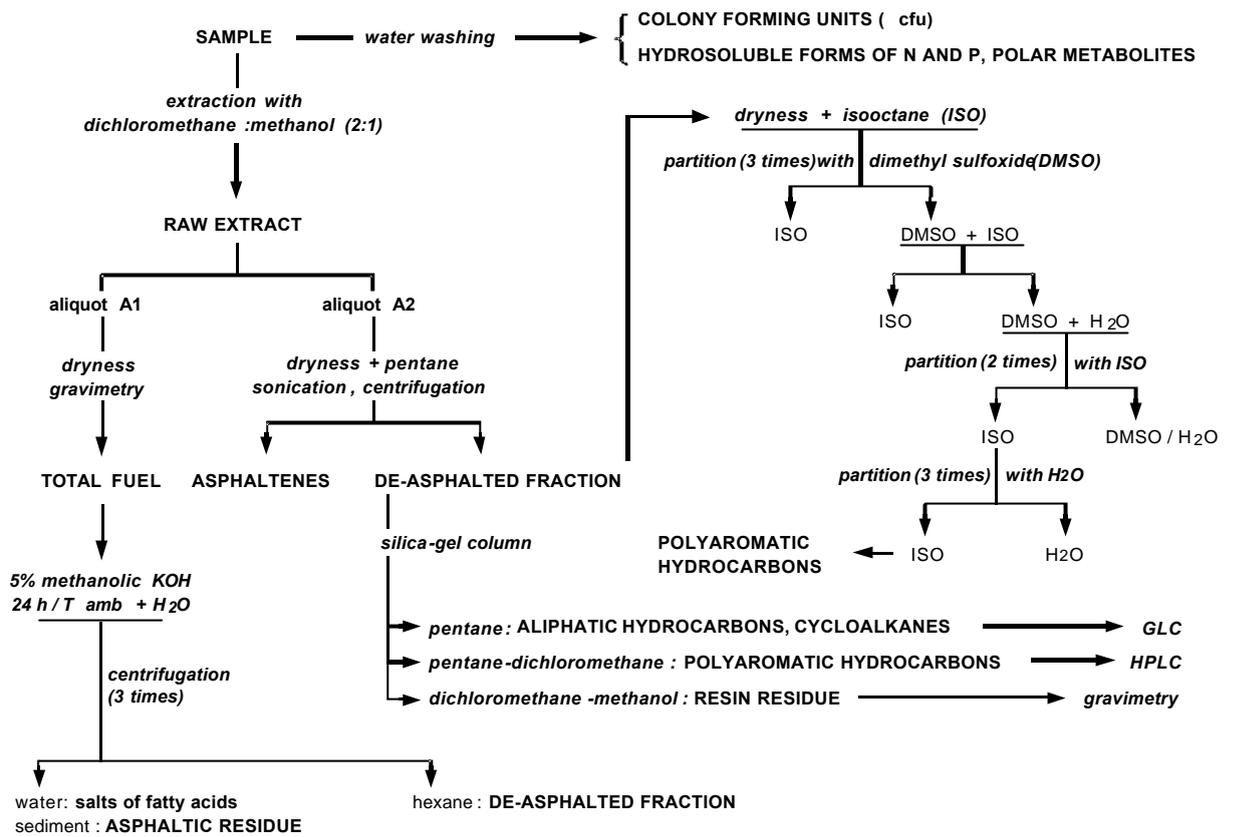
FIGURES



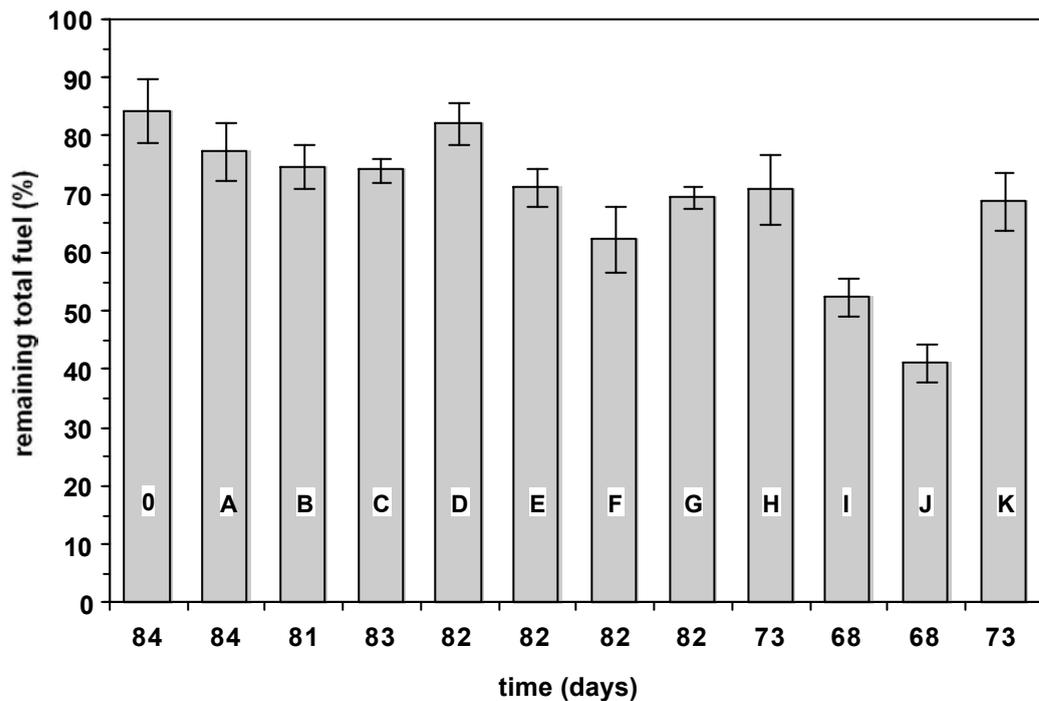
Prestige oil spill... Murado et al. Figure 1



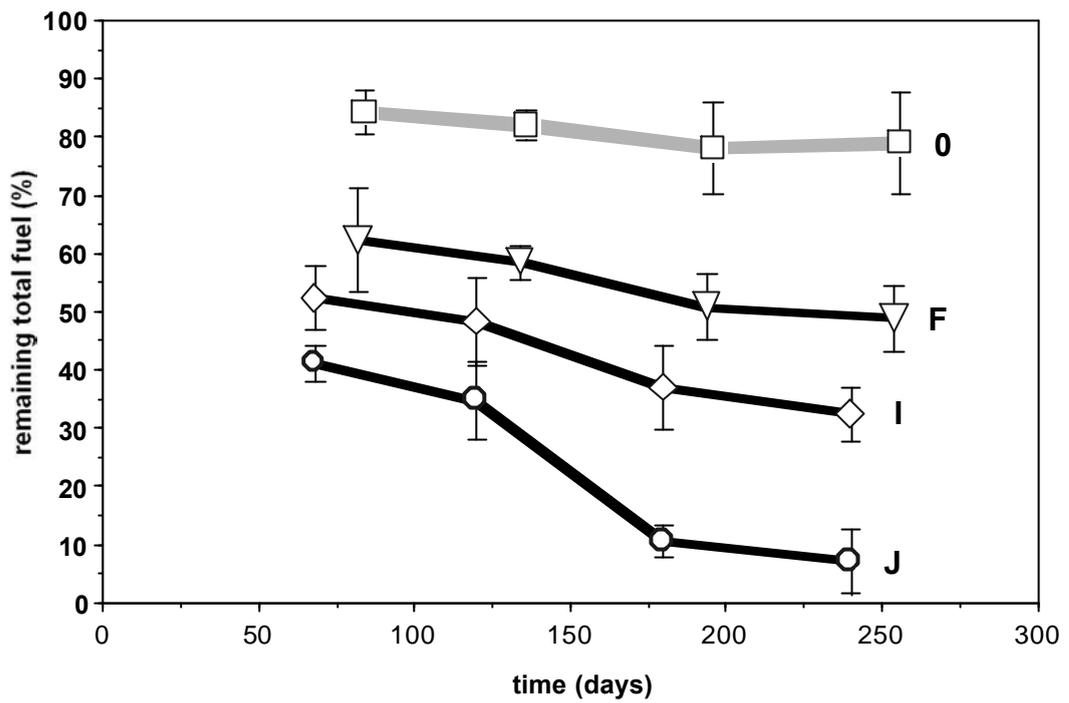
Prestige oil spill... Murado et al. Figure 2



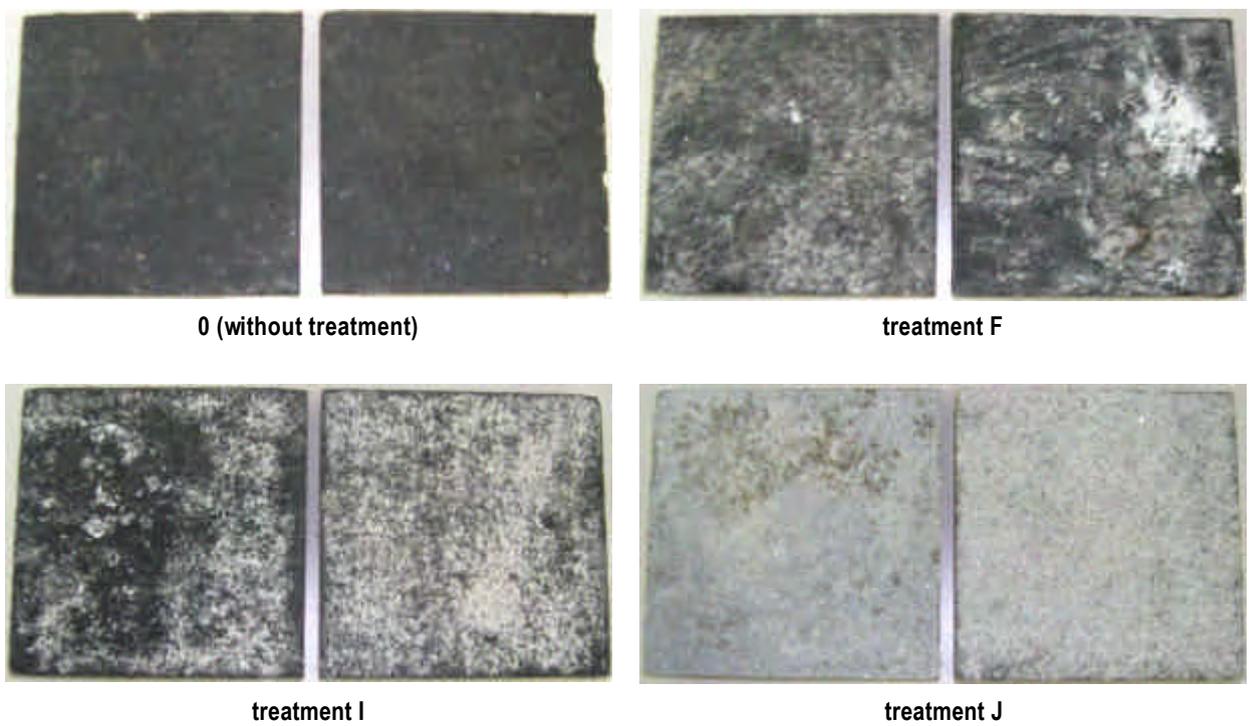
Prestige oil spill... Murado et al. Figure 3



Prestige oil spill... Murado et al. Figure 4



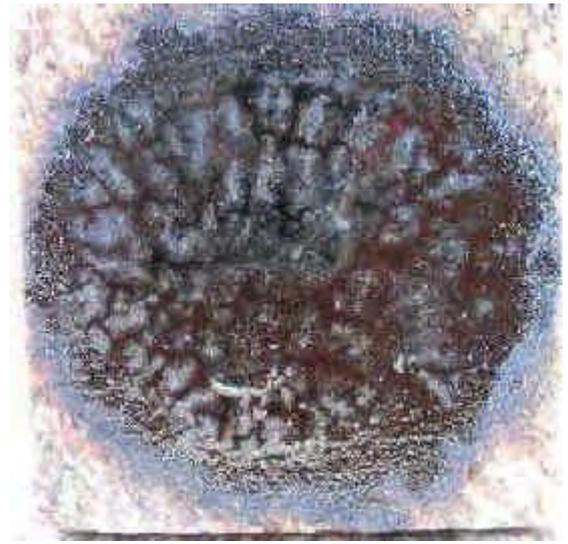
Prestige oil spill... Murado et al. Figure 5



Prestige oil spill... Murado et al. Figure 6



Prestige oil spill... Murado et al. Figure 7



Prestige oil spill... Murado et al. Figure 8