

Investigations into the  
Persistence of Petroleum  
Contamination in Marsh  
Sediments and the  
Associated Microbial  
Community

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## INTRODUCTION

The long-term persistent nature of petroleum hydrocarbons in the marine environment is evidenced in the salt marsh of Wild Harbor, West Falmouth. This site was heavily impacted when the barge *Florida* ran aground spilling ~650 000 L of No. 2 fuel oil into Buzzards Bay. At the time of the spill, there was mass mortality of flora and fauna and concentrations of petroleum hydrocarbons were found to be as high as 12 mg g<sup>-1</sup> dry weight sediment several months after the spill (1).

Today, from an initial glance, the marsh appears to have recovered and the flora and fauna have returned. Recent studies, however, tell a different tale and oil is found to persist at depth in the marsh at concentrations of ~8 mg g<sup>-1</sup> dry weight in sediment horizons corresponding to the time of the spill (2).

It is of great interest to understand why the oil continues to persist in these sediments up to 34 years after the spill and it is likely that physical, chemical and biological factors all play a role. Once petroleum is incorporated into marine sediments, biodegradation and dissolution are the primary mechanisms by which it may be removed. At the West Falmouth site, the persistence of oil has been proposed to be due to the high organic carbon content, and anoxic conditions in the sediments that may hinder microbial degradation as well as the limited water washing that this “low-energy” environment receives thus limiting loss by dissolution (3). More recently, however, these concepts have been expanded upon and it has been suggested that the oil persists for a variety of reasons such as the lack of a necessary electron acceptor for anaerobic degradation, the structurally complex composition of the persistent, but weathered oil and a proposed limited bioavailability of the oil (2).

The persistent nature of petroleum in the marine environment is intriguing, but is heavily dependent upon the specific nature of the impacted environment as different environments experience different levels of recovery from a petroleum contamination. Rapid degradation rates of petroleum hydrocarbons, for example, have been reported in a contaminated wetland in southeast Texas. At this site, intrinsic bioremediation was observed and was attributed to conditions favorable to degradation such as elevated nutrient levels from a flood deposition, the unconsolidated nature of freshly deposited

sediment providing a nutrient rich, oxic environment and the presence of an active and capable microbial community that had been previously exposed to petroleum (4).

When comparing the fate of the West Falmouth contaminated site to the site in southeast Texas, it is clear that there are several factors that may influence the degradation of petroleum in marine sediments and for biodegradation to occur, several criteria must be met. These can be summarized as the presence of an appropriate microbial consortia that possesses the requisite catabolic ability, bioavailability of the potential substrate, and environmental parameters that may include some or all of temperature, redox potential, oxygen and nutrient availability (5).

This study aimed to promote degradation of the persistent oil from Wild Harbor by setting up sediment incubations under both oxic and anoxic conditions with additions of sulfate and nutrients (nitrogen and phosphorus). In the process of encouraging degradation of the oil it is hoped that something can be learned about why it persists. Preliminary work analyzing the diversity of the microbial population in the salt marsh environment of Wild Harbor was also performed to assess the impact that the contamination may or may not have on the structure of the community.

## **METHODS**

### **Sediment Collection**

A 10-cm diameter core was collected from the intertidal sediment in Wild Harbor, West Falmouth, MA and placed immediately on ice. The entire core was returned to the laboratory and sectioned at 2-cm intervals. The outer 1-cm of each section was discarded in order to minimize vertical contamination. 1 g was taken from six of the sediment horizons (0-2 cm, 4-6 cm, 8-10 cm, 10-12 cm, 14-16 cm and 28-30 cm) and frozen for microbial analysis. The remainder of the sample was immediately transferred to an anaerobic hood to undergo preparation for incubations.

### **Incubations**

In an anaerobic hood, sediment horizons corresponding to the area of petroleum contamination (8 through 16 cm) were combined and wet sieved with sea water filtered with a 2 micron filter that was made anaerobic by bubbling with N<sub>2</sub> and CO<sub>2</sub>. This was also performed for sediments that were un-impacted by the oil contamination (24 through

32 cm). Both the uncontaminated and contaminated homogenized sediment slurries were then each evenly divided into 5 serum bottles for the incubations. The incubation conditions for both contaminated and uncontaminated samples were as follows – 1) anaerobic with no additions (manipulation control), 2) anaerobic with no additions and sterilized by autoclaving the sample (abiotic control), 3) anaerobic with additions of nitrogen ( $\text{NH}_4\text{Cl}$   $0.25 \text{ g l}^{-1}$ ) and phosphorous ( $\text{KH}_2\text{PO}_4$   $0.2 \text{ g l}^{-1}$ ), 4) aerobic (uncapped and allowed to equilibrate with the air) with no additions and 5) aerobic (as before) with additions of nitrogen and phosphorous (as before). The incubations were removed from the hood, wrapped in foil so as to eliminate light and placed on shaker tables at room temperature. Sub-samples of the sediment slurry (5 ml) were taken at day 1, 4, 7 and 10 from all incubations to examine any change in the oil content or microbial population during this time.

#### **Total Petroleum Hydrocarbon (TPH) Analysis**

4 ml of sediment slurry taken from the incubations at day 1, 4, 7 and 10 was allowed to air-dry. It was then weighed and placed in pre-combusted glass vials and extracted with dichloromethane and methanol (9:1) in a heated sonicator for 30 minutes. The extracts were removed from the vials and filtered through glass wool (to remove any residual sediment). The extracts were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph with a cooled injection System (CIS) and a flame ionization detector (FID). A 1- $\mu\text{l}$  sample was injected into the CIS, which was temperature programmed from 40 (0.3-min hold) to 350 °C at 12 °C  $\text{s}^{-1}$  (5-min hold). Compounds were separated in a poly(dimethylsiloxane) capillary column (Chrompack CP Sil 5 CB, 60 m, 0.25-mm i.d., 0.25  $\mu\text{m}$  film), with helium carrier gas at a constant flow of 2  $\text{mL min}^{-1}$ . The GC oven was temperature programmed from 40 (1 min hold) to 120 °C at 30 °C  $\text{min}^{-1}$  and then from 120 to 320 °C at 6 °C  $\text{min}^{-1}$  (30 min hold). Response factors were generated with a standard solution of n-hexatriacontane. Total petroleum hydrocarbons were evident as an unresolved complex mixture (UCM) and were quantified by integrating the total area and using the response factor of n-hexatriacontane standard.

#### **Microbial Community Analysis**

***DNA extraction and amplification.*** 0.5 g of wet and unsieved sediment from six sediment horizons (0-2 cm, 4-6 cm, 8-10 cm, 10-12 cm, 14-16 cm and 28-30 cm) and 1

ml of sediment slurry from each incubation after 10 days was taken and added to a 2-ml bead solution tube from a MO BIO Laboratories, Inc. UltraClean™ Soil DNA Isolation Kit. The protocol (modified by Brian D. Wade) was followed and total DNA extracted from the samples. Gel electrophoresis of the DNA extract was performed to ensure isolation of DNA and to quantify the DNA prior to amplification. Polymerase Chain Reaction (PCR) amplification of DNA was then performed using 8F and 1492R primers.

***Amplified Ribosomal DNA Restriction Analysis (ARDRA).*** Purified PCR product was taken and incubated with the Msp 1 restriction enzyme (New England Biolabs, Inc) at 37°C for 3 hours before heating to 65°C for 20 minutes. Gel Electrophoresis of the digested product was performed to obtain a 16S rDNA fragment fingerprint of the microbial community in the original marsh sediment and after 10 days of incubation.

## RESULTS AND DISCUSSION

***Total Petroleum Hydrocarbons.*** The incubations containing oil-contaminated sediments were examined at days 1, 4, 7 and 10 to monitor any changes in the concentration of petroleum hydrocarbons. The results for four of the incubations are shown in Figure 1 and it is clear that no significant loss of petroleum is observed during the first 10 days of any of the incubations. A slight decrease in TPHs is observed in the aerobic incubation, but whether or not this is significant is uncertain because the loss is so small and because similar fluctuations of both loss and gain in TPHs are observed in the anaerobic plus nutrients and aerobic plus nutrients incubations. This suggests that what is seen in the first 10 days is noise associated with the sub-sampling and quantification technique, which could be reduced with more rigorous methods. It should also be noted that performing triplicates of each incubation would be necessary to obtain reproducible results. The lack of biodegradation in the incubations in this short time frame is not surprising, however, it would be of great interest to carry out these incubations for a much longer time period. If time is not a factor in the biodegradation of the oil then it may be that the oil is not bioavailable. To test this, future experiments could involve the addition of surfactants to increase the availability of the oil to the microorganisms. If biodegradation is still not observed then it may be just that either the appropriate

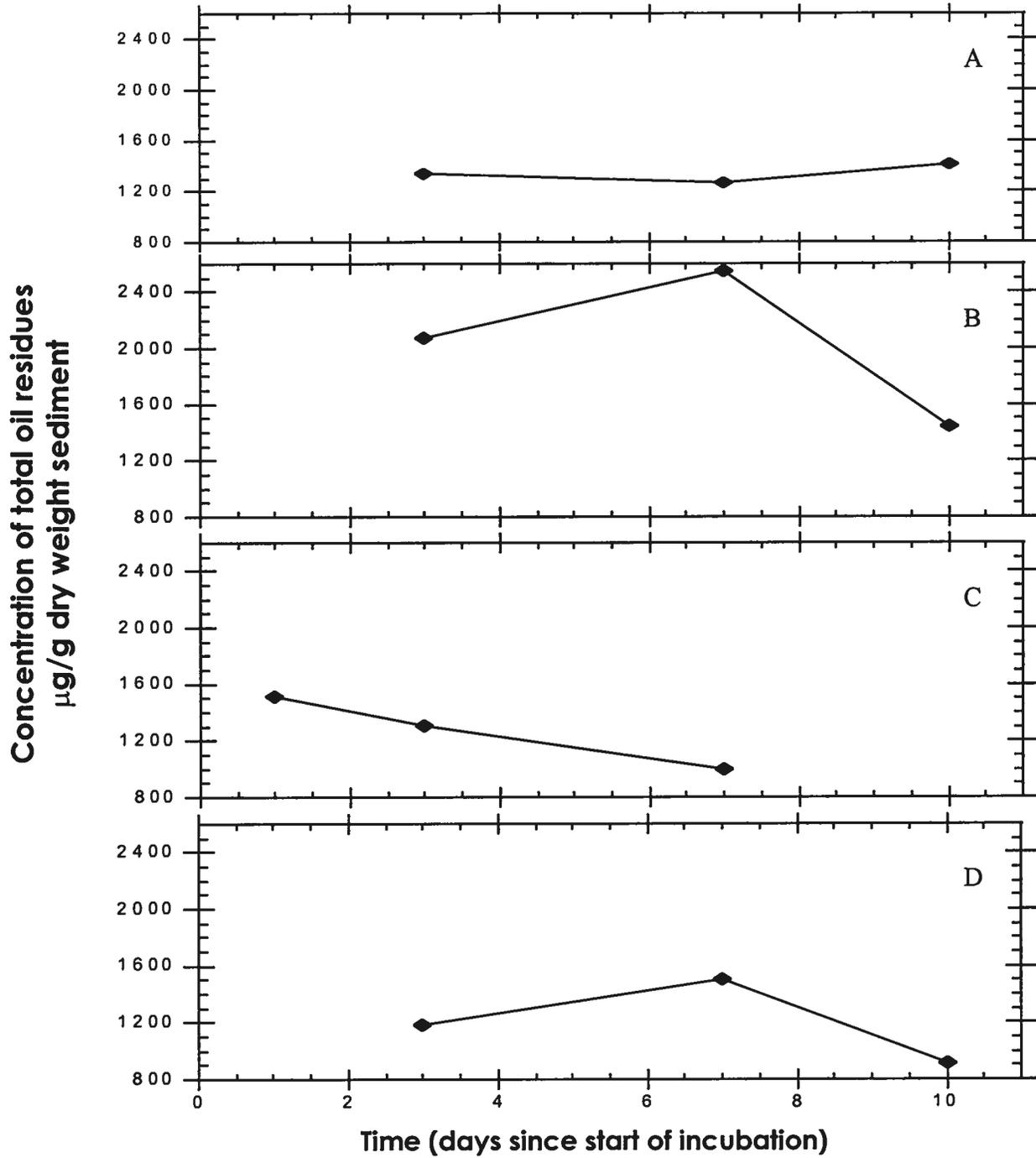
microbial community is not present or that those present would prefer to eat other natural carbon sources present in these organic rich sediments. It may be that the naturally derived carbon in the sediments is more bioavailable and more easily degraded than the weathered oil.

***Microbial Community Analysis.*** The 16S rDNA fragment fingerprints produced by ARDRA are shown in Figure 2. Examination of the 6 original salt marsh sediment horizons shows a predominant band corresponding to ~900 bp in all horizons. Two less dominant bands around 400 and 600 bp are observed in all horizons except 4-6 cm, 8-10 cm and 28-30 cm. In these horizons it may be that the band is present, but is very faint and it is difficult to conclude that they are not present from this gel. These results suggest that the microbial community is similar throughout the length of the core. This may be due to the spartina roots that penetrate the entire length of the core that may play a critical role in determining the composition of the microbial community. Analysis of the microbial community at a finer scale and with different probes may provide more insight and perhaps show differences in the sediment at finer resolution, but from this data it can be concluded that the microbial community appears to be somewhat homogeneous at this site.

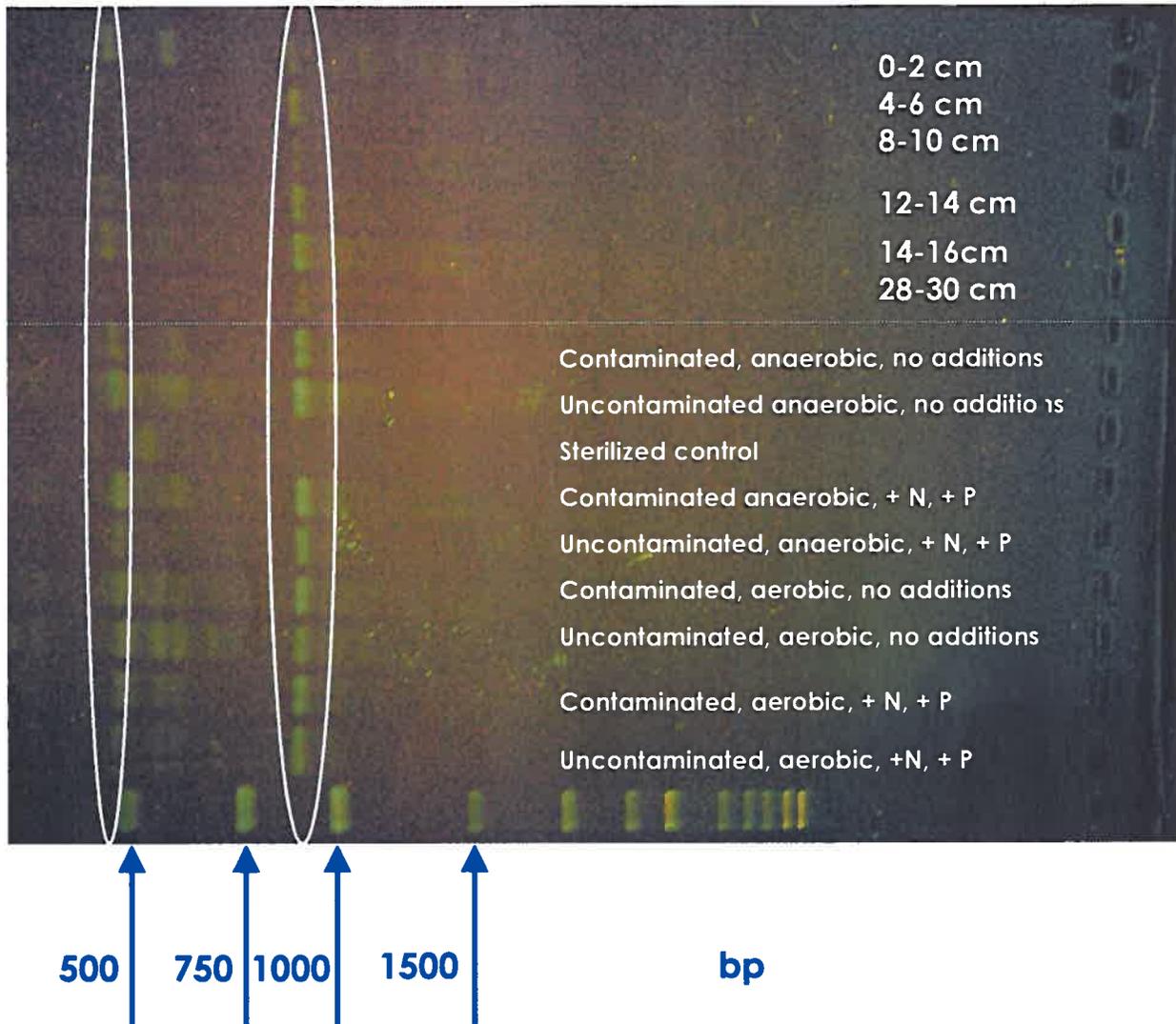
Examination of the 16S rDNA fragments from the incubations display a very similar pattern to the original sediment horizons suggesting that there has not been a significant change in the microbial community during the 10 day incubations. It appears that the factors that control the composition of the microbial community are not perturbed by any aspects of the incubations such as manipulation, replenishment of sulfate, addition of nutrients and aerobic vs. anaerobic conditions during this time. It would be of interest to extend the incubations and monitor the microbial community for a longer period of time to see if any shifts in the community occur.

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**Figure 1:** Comparison of the change in concentration of total petroleum hydrocarbons in incubations containing sediments from Wild Harbor oil-contaminated sediments. A) Anaerobic, no additions, B) Anaerobic plus nitrogen and phosphorous, C) Aerobic, no additions and D) Aerobic plus nitrogen and phosphorous.



**Figure 2.** Comparison of the 16S rDNA fragment fingerprint from oil contaminated Wild Harbor sediments from specific sediment horizons (top portion of gel as labeled) and sediments from incubation experiments after 10 days (lower portion of gel as labeled). The last lane is a 1kb DNA ladder (Promega) and base pair steps closely related to fragments of interest are labeled in blue.

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