Bioremediation of the *Exxon Valdez* Oil in Prince William Sound Beaches

by

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Abstract

Lingering oil from the *Exxon Valdez* oil spill persists on some beaches in Prince William Sound, Alaska, more than 20 years after they became contaminated. Low oxygen concentrations were found to be the major factor causing oil persistence, and bioremediation through the injection of hydrogen peroxide and nutrients deep into four beaches in PWS were conducted in the summer of 2011. The bioremediation was repeated at two of these sites in 2012. The sum of the polycyclic aromatic hydrocarbons (TPAH) from sediment samples was used as the main indicator of bioremediation. The natural attenuation rate of TPAH is less than 4% per year. The TPAH biodegradation rate was found to vary from 14% to 70% at the four sites during each of 2011 and 2012. All sites manifested increases in their pore water oxygen and nutrient (nitrate and phosphate) concentrations indicating that the delivery was effective.

Key Words

Beach bioremediation; *Exxon Valdez* Oil Spill; Oil persistence; Pore Water data; Prince William Sound; Subsurface.

INTRODUCTION

The 1989 *Exxon Valdez* oil spill (EVOS) polluted around 800 km of intertidal shorelines within Prince William Sound (PWS), Alaska [*Neff et al.*; *Neff and Stubblefield*]. Studies conducted by scientists from the National Oceanic and Atmospheric Administration (NOAA) estimated that between 60 and 100 tons of subsurface oil persists in many initially-polluted beaches in Prince William Sound (PWS) [*Short et al.,* 2004; *Short et al.,* 2006]. The persistence of oil was also

noted by other studies [*Page et al.*; *Hayes and Michel*, 1999; *Michel and Hayes*, 1999; *Taylor and Reimer*, 2008; *Li and Boufadel*, 2010]. The lingering oil contains relatively high concentrations of polycyclic aromatic hydrocarbons, PAH, [*Short et al.*, 2004], which are known to be toxic to intertidal organisms [*Carls et al.*, 2001], as sea otters and harlequin ducks maybe exposed to subsurface lingering oil while foraging on the beaches of northern Knight Island [*Short et al.*, 2006].

Earlier findings based on six beaches in Prince William Sound contaminated with moderate to heavy oil residue (MOR to HOR) from the EVOS, revealed that generally the beaches consist of an upper high-permeability layer underlain by a lower layer whose permeability is two to three orders of magnitude lower than that of the upper layer [*Desai and Banat*, 1997; *Li and Boufadel*, 2010; *Xia et al.*, 2010; *Xia and Boufadel*, 2011; *Bobo et al.*, 2012]. On these beaches, the lingering *Exxon Valdez* oil was located a few inches (0.10 m) below the interface of the two layers. Oil-contaminated sediments were anoxic with a dissolved oxygen (DO) concentration around 1.0 mg/L), and had a high ratio of ammonia to nitrate as nitrogen-N [*Boufadel et al.*, 2010; *Sharifi et al.*, 2011], whereas similar oil-free sediments were oxic (DO>3 mg/L) and have a moderate ammonia to nitrate ratio (as N), suggesting that oil biodegradation is oxygen-limited in oil-contaminated sediments. In addition, the concentrations of pore water nutrients in contaminated sediments were less than 0.5 mg N/L and less than 0.04 mg P/L [*Boufadel et al.*, 2010; *Sharifi et al.*, 2011]. These values are 5 folds smaller than the nutrient concentrations that support maximal rates of oil biodegradation; they are $\geq$ 2 mg-N/L [*Venosa et al.*, 1996a; *Boufadel et al.*, 1999; *Du et al.*, 1999], and $\geq$ 0.2 mg-P/L in phosphate, with an approximate

optimal ratio of N/P around 10 [*Atlas and Bartha*, 1972; *Venosa et al.*, 1996a; *Wrenn and Venosa*, 1996; *Smith et al.*, 1998].

A laboratory study by *Venosa et al.* [2010] found that within six months, around 80% of the PAHs in Exxon Valdez oil obtained from three beaches in PWS biodegraded. The oil they used was already 70% weathered [*Atlas and Bragg*, 2007]. However, the *Venosa et al.* [2010] study required disturbing the oiled sediments to place them in microcosm, and it was argued by *Atlas and Bragg* [2009a]; *Atlas and Bragg* [2009b] that oiled areas are sheltered hydraulically from input of dissolved oxygen and nutrient. For this reason, it was important to evaluate whether biodegradation of the lingering oil could be enhanced in situ through the injection of amendments into the subsurface of polluted beaches in Prince William Sound.

In any beach subjected to tide, the net (or time-averaged) movement of pore water is seaward throughout the beach with the exception of the region near the high tide line where the flow is usually landward and downward [*Boufadel et al.*, 2006a; *Li et al.*, 2007; *Li and Boufadel*, 2010]. Therefore, water solutions applied onto the beach surface would tend to move seaward after they percolate into the beach [*Boufadel et al.*, 2006b]. Due to the two-layer structure of the beaches in PWS, where the upper layer's permeability is 100 to 1,000 times that of the lower layer [*Li and Boufadel*, 2010], solutions applied onto the surface tend to dilute with pore water and wash out to sea much more rapidly than they can be transported into the contaminated layer [*Li and Boufadel*, 2011]. *Xia et al.* [2010] found based on numerical simulations of solution migration in a PWS beach that the nutrient concentration in the oil-contaminated sediments would be only 1% of the concentration applied onto the beach surface.

Tracer studies using a conservative tracer released directly into the lower layer of three beaches in PWS resulted in much less dilution [*Boufadel and Bobo*, 2011; *Boufadel et al.*, 2011] due to the small porosity of the lower layer. Therefore, subsurface delivery of nutrients was expected to be superior to surface application, and was selected for a pilot bioremediation study.

This paper reports the findings of an in situ bioremediation investigation at four PWS beaches (Figure 1): EL056C (Northwest Bay on Eleanor Island; 60.5506/-147.5795), LA015E (Latouche Island; 60.0596/-147.8171), PWS3A44 (Mears Point, Perry Island; 60.6567/-147.9319), and SM006B (Smith Island; 60.5278/-147.3851). Hydrogen peroxide and nitrate and phosphate solutions were injected into the beaches to enhance microbial growth and subsequently oil biodegradation. The focus was on the biodegradation of the polycyclic aromatic hydrocarbons due to their toxicity and their persistence in the oiled beaches. Surrogate measurements, including the concentration of dissolved oxygen and nutrient in pore water, and the concentration of microorganisms is also reported.

MATERIALS AND METHODS

Hydrogen peroxide and nutrients were diluted in seawater and released at metered flows into the estimated depth of the lower layer of the beaches. One layout was used for EL056C in 2011 (Figure 2a), where the injection flow rate was 1.0 liter/hour/well, and another was used for the remaining beaches in 2011 (Figure 2b) where the injection flow rate was 0.20 L/hr/well. In 2012, the treated area of EL056C was increased from approximately 32 m^2 to 110 m^2. The treated area

for SM006B remained the same as in 2011, but the individual injection flow rate was increased 1.0 L/hr/well.

Hydrogen peroxide was selected as the source of oxygen for this study because it is an efficient, water-soluble oxygen source that decomposes to oxygen and water as the only products [*Watson et al.*, 2002]. Hydrogen peroxide was provided as a concentrated (35%, w/w) solution and was diluted in saltwater to have a solution of 100 mg of peroxide/L of water. The hydrogen peroxide concentration was constrained by the maximum solubility of oxygen in seawater (about 40 mg/L at 15^{o}C; Metcalf and Eddy, 1991), as higher concentrations could lead to the formation of bubbles of oxygen gas that could reduce the permeability of the formation [*Wang et al.*, 1998; *Wang et al.*, 2001]. Hydrogen peroxide has been widely used to provide oxygen to support bioremediation of hydrocarbon-contaminated groundwater and subsurface sediments [*Watson et al.*, 2002], but this is the first time it is used in a beach.

A concentrated nutrient solution was prepared to obtain solutions of nitrate and phosphate. The nitrate was obtained from lithium nitrate ($LiNO_3$) in 2011, and from sodium nitrate ($NaNO_3$) in 2012. The phosphate was obtained from sodium tripolyphosphate ($Na_5P_3O_{10}$), (STPP). Concentrated solutions of approximately 100g $LiNO_3$/L or NaNO3/L and 8 g STPP/L were obtained using freshwater, and these solutions were blended inline with seawater prior to injection to produce a nutrient concentration of 20 mg N/L and 2 mg P/L as STPP, which are sufficiently large and at the proper ratio to support a maximum rate for hydrocarbon biodegradation [*Bragg et al.*, 1994; *Venosa et al.*, 1996b; *Boufadel et al.*, 1999; *Garcia-Blanco et al.*, 2007].

The performance of the bioremediation systems was monitored using both sediment and groundwater (pore water) samples, but the oil concentrations from the sediment samples were the primary measurements. Sediment samples were collected from each of the treatment zones of the beaches (Figure 2). In 2011, each plot was divided into four treatment zones, each 2 m by 4 m (Figure 2). Two samples were collected from two locations predetermined randomly (using a uniform distribution) in each treatment zone. The initial (i.e., pretreatment) samples were collected after the injection wells were installed but before the systems were turned on, and the post-treatment samples were collected after the systems had been operating for about one month (i.e., in August) and two months (i.e., in September). Due to the larger treatment area used at EL056C in 2012 in comparison to 2011, the plot was divided into 10 zones (Figure 2). The five zones on the right-hand side (looking landward) of the plot were sampled in June 2012 before treatment began, and then at 4-week intervals until the system was shut off in early September. The five zones on the left-hand side of the plot were sampled before treatment began (June) and just before it was stopped (September). This is because most of this area was treated in 2011, and it was expected that its concentration to be lower than the rest of the beach. The plot design at SM006B in 2012 was the same as used in 2011 (Figure 2) but the flow rate was increased to 1.0 L/min/well.

Sediment samples were collected by digging pits at the predetermined locations to depths of about 0.6 m below the ground surface or to the maximum depth that could be achieved, if it was not possible to reach the full 0.6-m depth. The depth of maximum oil contamination was identified visually, and sediment samples were collected from the walls of the pit. The oil

samples were collected in 125-ml glass sample bottles that were cleaned according to EPA procedure for semivolatiles. They were frozen as soon as practical after collection, usually within about 2-3 hours, and were kept frozen during storage and shipment. They were analyzed by the National Oceanic and Atmospheric Administration (NOAA) Auke Bay Lab using Gas Chromatography Mass Spectrometer (GC-MS).

Water samples were collected from multilevel sample wells (see Li and Boufadel, 2010) that were installed at the locations shown in Figure 2, and from single-level wells that were installed at the locations from which the initial sediment samples were collected. These water samples were used to measure oxygen and nutrient concentrations and salinity. The multilevel wells consisted of stainless steel casing (3/4-inch to 1-inch diameter) with holes drilled at 24- or 30-cm intervals. Small stainless steel tubes (1/8 inch to ¼ inch) were welded to each of the holes in the casing wall to create sample ports. The hole was covered with a stainless steel screen to keep the sample-port tubing from becoming clogged by sediment. The multilevel wells allowed collection of samples at several depths below the beach surface, and thus they provide a three-dimensional "picture" of the distribution of oxygen and nutrients. The single-level wells consisted of small (1 inch by 0.5 inch), cylindrical, porous stainless steel inlet filters (20-µm pore diameter; IDEX Health & Science LLC, Oak Harbor, WA) that were connected to the surface through ¼-inch Tygontubing. The single-level wells were installed within the sampling zones in the vicinity of the oil layer (usually about 20-30 cm beneath the beach surface).

In 2011, the multilevel wells were installed at the edges of the expected treatment zone (Figure 2). Eight single-level wells were installed in each plot in 2011; two single-level wells were

installed in each of the four zones. In 2012, twenty single-level wells were installed at EL056C (i.e., two wells per zone on both sides of the plot at the locations of the initial sediment samples), and eight new single-level wells were installed at SM006B. In addition to the new single-level wells, several single-level wells were used at both 2012 sites.

Water samples were collected using disposable 60-ml polypropylene syringes (Becton Dickinson, Franklin Lakes, NJ), and used for measurement of nutrients, lithium (used as a conservative tracer in 2011), and dissolved oxygen. Four syringe volumes were discarded prior to taking the fifth syringe for oxygen measurement using the Hach High-Range DO assay (Hach Company, Loveland, CO). The syringes were stored in the dark until the samples could be analyzed, which occurred usually within 2-3 hours of being collected.

Nutrient samples were frozen as soon as possible, and kept frozen during storage and shipment. Nutrients were analyzed colorimetrically using an AutoAnalyzer3 (Seal Analytical, Mequon, WI) [*Grasshoff et al.*, 1999]. The frozen samples were thawed and stored at 4 °C until they were analyzed. Before analysis, the samples were shaken by hand for 15 s, and filtered through 0.45-μm PTFE membrane filters (Puradisc,Whatman, Florham, NJ) into the AutoAnalyzer3 cups. Ammonia in seawater was measured using the Berthelot reaction, and the colored reaction product was measured at 660 nm [*Lenore et al.*, 1998]. Nitrate in the samples was reduced to nitrite by a copper-cadmium reactor column, and the nitrite reacted with sulfanilamide under acid condition to form a purple azo dye that was analyzed at 550 nm [*Lenore et al.*, 1998]. Phosphate was measured using theascorbate-antimony-molybdatemethod [*Murphy and Riley*, 1962; *Lenore et al.*, 1998]. The blue complex was analyzed at 880 nm wavelength.

The sediment samples for microbiology were collected using aseptic protocols (e.g., sterile sample containers, alcohol-rinsed and flamed spatulas, alcohol-rinsed vinyl gloves). Sediment samples were processed as soon as possible, usually within a few hours. In cases where the time of processing could not happen within hours, but within days, the samples were stored in water coolers with cold packs until they could be processed.

Biodegradation of oil is carried out by hydrocarbon-degrading bacteria, and in this study, the concentration of these bacteria was measured using 96-well plate most-probable-number (MPN) methods that select for either alkane-degrading or PAH-degrading bacteria [*Wrenn and Venosa*, 1996]. Heterotrophic bacteria were also enumerated by modifying the microtiter-plate MPN procedures for hydrocarbon degraders to select for bacteria with the ability to grow on non-hydrocarbon substrates, such as glucose, peptides, and the components of yeast extract.

The alkane-degrader MPN method uses hexadecane as the selective growth substrate followed by detection of growth-positive wells based on reduction of iodonitrotetrazolium (INT) whose color is violet to an insoluble formazan whose color is red. The PAH-degrader method uses a mixture of phenanthrene, fluorene, and dibenzothiophene as the selective growth substrates followed by growth detection based on the formation of colored byproducts of PAH metabolism.

The heterotrophic bacteria enumeration method uses either dilute (1:20) PTYG (peptone-Trypticase-yeast extract-glucose) medium [*Balkwill and Ghiorse*, 1985] or by growth on the yeast extract component of the PAH-degrader medium. Both methods detect growth-positive

wells based on reduction of INT, but a mixture of easily degradable electron donor substrates-glucose, succinate, and pyruvate [*Johnsen et al.*, 2002] was added to the PAH-degrader medium with the INT to increase the amount of formazan that was formed. PTYG medium was used exclusively to enumerate heterotrophs in 2011, but both methods were used in 2012. Only the results of the PAH-degrader/INT/electron-donor substrate method are reported for the 2012 study, but this method always gave bacterial concentrations that were equal to or greater than the values obtained using PTYG medium.

Note that the concentration of heterotrophic bacteria may include some hydrocarbon degraders, but it is unlikely that the overlap would be complete. So, hydrocarbon-degrading and heterotrophic bacterial MPN methods should be considered to provide independent estimates of different bacterial groups. In particular, it is possible for the concentration of hydrocarbon-degrading bacteria to be higher than the concentration of heterotrophic bacteria. Note also that the MPN procedure suffers from the same limitations as all growth-based enumeration methods, especially the limitation that only organisms that can grow well from very low initial concentrations (ideally, from a single cell) under the specific culture conditions are counted. In addition, not all microorganisms in the field can grow well in the lab. The population obtained based on cultivation in the lab is in general between 5% and 50% of the total population in the field [*Herbert*, 1990; *Wrenn and Venosa*, 1996; *Harris et al.*, 1998; *Rittman and McCarty*, 2001]. Nevertheless, the MPN was conducted herein to provide a holistic evaluation of oil biodegradation.

RESULTS

Figure 3 reports the plot-averaged concentration of total extractable material (TEM) per kg of sediments in the four beaches in 2011 and 2012. The maximum plot-averaged oil concentration at EL056C and at SM006B was approximately 6 and 10 g of oil/kg of sediment, respectively. That at LA015E and PWS3A44 was less than 2.0 g/kg of sediment. Figure 3 shows large temporal variability including large increases at some sampling events (see for example, EL056C in August 2011 or SM006B in July 2012). These increases can be only interpreted by the heterogeneity in the spatial oil distribution as no oil was added to beach. But also the absence of a decreasing trend suggests that the oil was not displaced out of the plot (conforming to the design).

Figure 4 reports the concentration of various TPAHs at various degrees of alkylation; both the number of benzene rings and degree of alkylation increase going to the right. Site PWS3A44 exhibited the largest decrease in its concentrations up to C4-phenanthrene. In general, the relative decrease in naphthalene and dibenzothiophene (at various degrees of alkylation) was largest with respect to the rest. The relative decrease in fluorene (at various degrees of alkylation) was considerable at all sites except LA015E and SM006B in 2011. Compounds larger than phenanthrene (to the right of it) manifested some biodegradation, but their concentrations were low in comparison with smaller compounds. The biodegradation decreased in general with the degree of alkylation. There were some salient exceptions; at SM006B in 2011 and 2012 C3-phenantrene biodegraded more than C2-phenanthrene, and at both EL056C and SM006B in 2012 at both sites, where C4-naphtalene biodegraded more than C3-naphtalene, C3-fluorene

biodegraded more than C2-fluorene, and C2-dibenzothiophene biodegraded more than C1-dibenzothiophene.

Figure 5 reports the plot-averaged concentration of TPAH as function of time, where one notes a general decrease with time indicating biodegradation. Note that the natural biodegradation of TPAH with time was estimated to be about 1% per year [*Atlas and Bragg*, 2007]. The percent degradation of TPAH at various sites is reported in Table 1. The largest significant biodegradation is noted at PWS3A44, where the decrease in TPAH concentration due to treatment was around 70% at $p < 0.001$, and at EL056C in 2011 where the decrease was 35% at $p < 0.006$. For SM006B in 2011 and 2012, the decrease was approximately 13% at $p < 0.13$, and around 20% at $p<0.13$, respectively. The lower significance of the decreases at SM006B were due to the small number of samples and variability between samples from the same plot at the same sampling event.

Table 2 reports the beach-averaged dissolved oxygen concentration pre-treatment and post-treatment. One notes that the pretreatment values in 2011 varied from less than 1.0 mg/L to around 2.7 mg/L, which was due to averaging values larger than 3.0 mg/L with values that are less than 2.0 mg/L at various locations (measurements per locations are not shown for brevity). In 2012, the average of pretreatment values was larger than 4.0 mg/L. However, there were values smaller than 2.0 mg/L at some locations. Table 2 also shows percent increases varying from 30% to 250% in 2011. In 2012, there was essentially no change at EL056C and an increase by 17% at SM006B.

Table 3 reports the pore water concentration of nutrient (nitrate, ammonia) and phosphate before and after treatment in 2011 (note that lithium nitrate was added as a nutrient). Table 3 shows that the nitrate concentration increased at all beaches, and the smallest increase was 40% at PWS3A44. In contrast, the ammonia concentration did not show any pattern. When the two compounds are added, one notes around 200% increase in the nutrient N concentration at EL056C and SM006B, and essentially no change at the other beaches. The concentration of phosphate (Table 3) shows increases ranging from 43% to more than 600%. The P/N ratio was around 1/10 at LA015E and PWS3A44, and around 1/100 at EL056C and SM006B.

The plot-averaged microbial count (MPN) did not show any temporal trend. For this reason, we report in Figure 6 the MPN values as time-averages for each plot, where one notes that the number of the alkane degraders is comparable to the number of heterotroph. The TPAH degraders were always one to two orders of magnitude less than the other groups. Site SM006B appears to be an outlier where the MPN for each microbial group was always lower than the corresponding MPN at other sites, sometimes by several orders of magnitude. Note that this site has the largest mass of oil per beach, around 20% of the total EVOS oil [*Michel et al.*, 2009].

DISCUSSION

Numerous studies of beach bioremediation have been conducted on Prince William Sound beaches [*Bragg et al.*, 1994; *Röling et al.*, 2002] where amendments (nutrient and/or microorganisms), were applied onto the beach surface at low tide, and resulted in accelerated

biodegradation of shallow subsurface oil. However, it was found that dissolved oxygen and not nutrient was the limiting factor for the biodegradation of deep (within the beach) EV oil [*Boufadel et al.*, 2010; *Sharifi et al.*, 2011]. In this case, application on the beach surface became an obsolete option (there is plenty of dissolved oxygen in seawater that covers the beach twice daily). Therefore, there was a need to deliver oxygen deep into the beaches of PWS.

The delivery of amendment deep into aquifers for the purpose of bioremediation has been conducted on various sites [*Schincariol et al.*, 1994; *Essaid et al.*, 1995]. But none of these sites include a tidally influenced beach. Further complexity was brought by the large tidal range, 5.0 m, and by the complex two-layer geomorphology of the beaches. Achieving a successful design required understanding beach hydrodynamics [*Guo et al.*, 2010; *Li and Boufadel*, 2010], and quantifying the blowout pressures and the pathways of amendment at these beaches [*Boufadel and Bobo*, 2011; *Boufadel et al.*, 2011]. The significance and challenges of the findings are discussed next.

The absence of a trend of decrease of the TEM concentration over time (Figure 3) indicates that the engineering design was adequate; that the injection flow rate was not too large as to mobilize the oil out of the plot. This was supported by the absence of sheen on the water surface following the initiation of bioremediation. The TEM-normalized concentration of PAH and dibenzothiophene (TPAH) was initially 6 to 9 mg of TPAH/kg of sediment and decreased with time for all sites (Figure 5), and the percent decrease varied from 13% to 70% per year (Table1). The decrease of TPAH concentration due to bioremediation at EL056C was 35% and decreased to around 14% in 2012, which could suggest that the bioremediation rate decreased with time.

However, TPAH decrease at SM006B was around 13% in 2011 and increased to 19% in 2012 when the flow rate was increased from 0.2 L/min to 1.0 L/min. Thus, it is possible that increasing the flow rate reached microscopic sites in 2012 that were not reached in 2011. It is also possible that oil trapped in dead-end pores was released to the large pores of the beach during September 2011-June 2012 (i.e, before the 2012 bioremediation). The higher degradation of more highly alkylated compounds, for example in 2012, C4-naphtalene in comparison to C3-naphtalene (Figure 4) could reflect new microbial pathways that were created due to the amendment.

Beach-averaged dissolved oxygen concentrations (Table 2) showed considerable increases (more than 30%) in 2011 at all sites. But in 2012, the increase was 17% at SM006B, and the concentration was essentially unchanged at EL056C. Thus overall, the results of Table 2 reflect a success in the delivery of oxygen to the beach through the hydrogen peroxide solution.

Nutrient injection in 2011 appears to have been effective in increasing the nitrate and phosphate concentration at all sites (Table 3). The nitrogen (nitrate+ammonia) concentration increased greatly at sites EL056C and SM006B, and was essentially unchanged at sites LA015E and PWSA44. These two sites had large ammonia-N to nitrate-N ratio (especially LA015E) relative to EL056C and SM006B. This was also reported by [*Boufadel et al.*, 2010] at anoxic locations (dissolved oxygen around 1.0 mg/L). It is likely that the created aerobic conditions at anaerobic locations within beaches caused nitrification of ammonia. It is also possible that direct uptake of the ammonia by aerobic microorganisms took place [*Wrenn et al.*, 2006].

This study showed that bioremediation of hydrocarbon contaminated beaches through deep injection is feasible, and accelerates the natural rate of biodegradation. Such a study would be directly applicable to oil pollution at high latitudes, which could occur due to exploration in the Arctic. Lessons from this study could be used to bioremediate submerged coastal systems polluted with PAH, such as Department of Defense sites.

For oil analysis, a total of 24 sediment samples were collected from each beach in 2011, giving a total of 24 x 4 = 96 samples. In 2012, 60 samples were collected from EL056C and 32 samples were collected from SM006B. In 2011, sediment samples were collected from two locations in each of four zones (Figure 2) on three occasions: immediately after installing the injection wells (initial), about 3 weeks after starting the bioremediation systems (August), and about 7 weeks after system startup (September). In 2012, sediment samples from EL056C were collected on four occasions (in June before turning on the system, and at a four-week interval thereafter). The sediments were taken from two randomly preselected locations in each of the five zones on the right-hand side (looking landward) of the plot at EL056C (Figure 2). The same spatial approach was adopted on the left side of the beach, but samples were only collected in June and at the end of the study (in September). At SM006B sediment samples were collected in 2012 from two locations in each of four zones (Figure 2) before turning on the injection pumps and then at three 4-week intervals thereafter.

The oil amount was estimated as the total extractable material (TEM) using dichloromethane (DCM), and it was measured gravimetrically (i.e., by weight). Our goal was to evaluate the biodegradation of the polycyclic aromatic hydrocarbons (PAH) and dibenzothiophenes due to their toxicity (the two are labelled TPAH herein). Hopane is often measured in oiled sediments to provide a means of estimating the extent of oil degradation. As the more labile components of oil are lost through weathering losses, the concentration of hopane remaining in the oil residue usually increases because hopane is more resistant to these weathering losses [*Bragg et al.*, 1994; *Venosa et al.*, 1996a]. However, after prolonged oil weathering, hopane itself may be degraded [*Atlas and Bragg*, 2007], and if extensive losses occur, then hopane may no longer provide a reliable index of weathering extent. In particular, hopane concentrations (mass of hopane per mass of oil) that are markedly lower than in the un-weathered oil indicate extensive microbial degradation of the oil, and oil that has already undergone extensive biodegradation (i.e., 95%) is unlikely to degrade much further following addition of nutrients to stimulate microbial activity. These highly weathered samples may be identified on the basis of their hopane and TPAH concentrations. The mean concentration of hopane in 20 un-weathered Alaska North Slope reference oil samples analyzed at the Auke Bay Laboratory was found to be 0.163 mg/g of TEM (Figure 7). In the field samples analyzed in this project (Figure 7), the hopane concentration exceeded 0.300 mg/g of TEM, reflecting weathering losses of more labile components. Corresponding TPAH concentrations were 24.5 mg/g of TEM for the reference oil samples, and less than 16 mg/g in the field samples (Figure 7), reflecting lower TPAH concentrations and higher hopane concentrations, as expected during initial weathering stages. The most extensively degraded field samples had hopane concentrations of 0.100 mg/g or less, and corresponding TPAH concentrations were less than 1.25 mg/g, indicating more than 95% degradation of the

TPAH (Figure 7). For these reasons, field samples that had concentration of hopane less than 0.100 mg hopane/g and TPAH concentrations less than 1.25 mg/g of TEM (i.e., less than 5% of the TPAH concentration in the unweathered oil) (see Figure 7) were excluded from the statistical analysis to evaluate the efficacy bioremediation.

The effect of the treatment is evaluated herein by comparing the average concentration of TPAH (mg of TPAH/g of TEM) per beach before treatment to that after treatment. Traditionally this question is evaluated with a Student's *t*-test, but this approach assumes normality of the data distribution [*So and Young*, 2001]. An alternative approach that does not make an assumption on the underlying distribution is known as the randomization test (RT). RTs are based on expectations derived from a null hypothesis that data groups are samples of the same underlying distribution of data [*Snoeyink and Jenkins*, 1980]. The overall study design consisted of sampling stratified by tidal elevation at each beach, with samples collected from pre-designated locations before and after treatment. However, due to large spatial variability, we report herein beach-averaged results.

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Table 1: Mean total PAH (mg/g of Total Extractable Material) from sediments collected from the study sites in Prince William Sound, Alaska. Numbers in parentheses after TPAH concentrations are the number of samples contributing to the reported mean. The "P" value represents the probability that the difference between pre-treatment and post-treatment is due to randomness. The smaller the value of "P" the less likely the difference is random.

Mean TPAH Concentration, mg/(g of TEM)				
2011				
Station	EL056C	LA015E	PWS3A44	SM006B
Pre-treatment Average	9.00 (*11*)	2.87 (*14*)	9.53 (*16*)	7.05 (*16*)
Post-treatment Average	5.84 (*9*)	2.12 (*15*)	2.62 (*16*)	6.15 (*16*)
Difference (Initial minus Final)	3.16	0.75	6.91	0.90
Percent decrease	35.1%	26.2%	72.5%	12.8%
p	0.006	0.192	<0.001	0.126
2012				
Station	EL056C			SM006B
Pre-treatment Average	4.52 (*15*)			5.86 (*8*)
Post-treatment Average	3.91 (*30*)			4.76 (*23*)
Difference (Initial minus Final)	0.61			1.1
Percent ***decrease***	13.6%			18.7%
p	0.121			0.131

Table 2: Mean DO (mg/L) from water samples collected from the study sites in Prince William Sound, Alaska. Numbers in parentheses following DO concentrations are the number of samples contributing to the reported mean. The "P" value represents the probability that the difference between pre-treatment and post-treatment is due to randomness. The smaller the value of "P" the less likely the difference is random.

Mean DO Concentration (mg /L)				
2011				
Station	EL056C	LA015E	PWS3A44	SM006B
Pre-treatment Average	2.72 (*21*)	0.89 (*9*)	2.57 (*22*)	1.34 (*14*)
Post-treatment Average	4.41 (*24*)	3.15 (*55*)	3.40(*59*)	3.4 (*29*)
Difference (Initial minus Final)	-1.69	-2.26	-0.83	-2.07
Percent Increase	62.0%	254.4%	32.2%	154.8%
p	0.02	0.002	0.056	<0.001
2012				
Station	EL056C			SM006B
Pre-treatment Average	5.11 (*34*)			4.3 (*14*)
Post-treatment Average	4.71 (*127*)			5.03 (*85*)
Difference (Initial minus Final)	0.39			-0.73
Percent Increase	-7.7%			17.1%
p	0.76			0.08

Table 3: Mean nutrient (Nitrate+ammonia) (mg-N/L) and phosphate (mg-P/L) from water samples collected from the study sites in Prince William Sound, Alaska. Numbers in parentheses following concentrations are the number of samples contributing to the reported mean. The "*p*" value represents the probability that the difference between pre-treatment and post-treatment is due to randomness. The smaller the value of "*p*" the less likely the difference is random.

(nitrate+ammonia) mg-N/L				
Station	EL056C	LA015E	PWS3A44	SM006B
Pre-treatment Average	0.23+0.18=0.4 (18)	0.63+1.42=2.05 (7)	0.35+0.34=0.69 (7)	0.45+0.15=0.60 (8)
Post-treatment Average	0.78+0.12=0.9 (6)	1.15+0.70=1.85 (7)	0.49+0.13=0.63 (5)	1.53+0.13=1.7 (7)
Difference of N (Initial minus Final)	-0.5	0.2	0.06	-1.10
Percent Increase	120%	-9%	-9%	183%
p	<0.01	0.65	0.55	< 0.01
Phosphate (mg-P/L)				
Station	EL56C	LA015E	PWS3A44	SM006B
Pre-treatment Average	0.007 (22)	0.024 (7)	0.01 (6)	0.007 (8)
Post-treatment Average	0.01 (6)	0.177 (7)	0.052 (5)	0.014 (7)
Difference (Initial minus Final)	-0.003	-0.153	-0.042	-0.007
Percent Increase	43%	640%	420%	100%
p	0.26	< 0.06	< 0.01	< 0.025

Figure 1: Locations of beaches where the bioremediation pilot studies were conducted. Map is also showing Bligh Reef, location where the tanker Exxon Valdez ran aground in 1989.

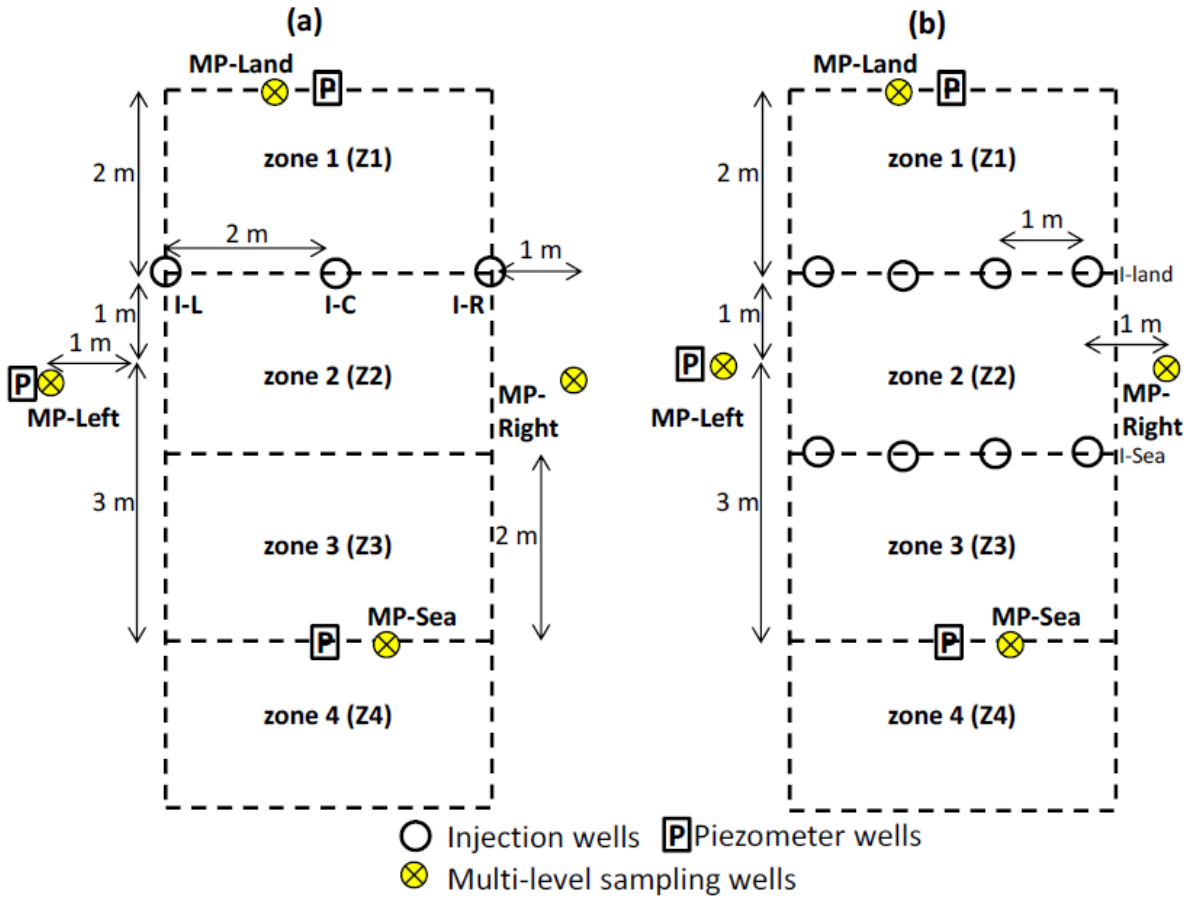


Figure 2: Plot layout for system that was used in 2011 at (a) EL056C and (b) LA015E, PWS3A44, and SM006B. The top of the diagram corresponds to the landward direction and the bottom is seaward. Sediment samples were collected from randomly predetermined locations within Zones 1-4. The plot design at SM006B in 2012 was the same as in 2011. Layouts are not to scale. The plots were in the vicinity of the mid-intertidal zone. They were exposed to the atmosphere during the low lowest tide, but they got completely submerged during high tides. The maximum water depth (submergence depth) at MP-Land at various sites/years varied from 0.6 m to 1.8 m. The maximum water depth at MP-Sea at various sites/years varied from 1.7 m to 2.5 m.

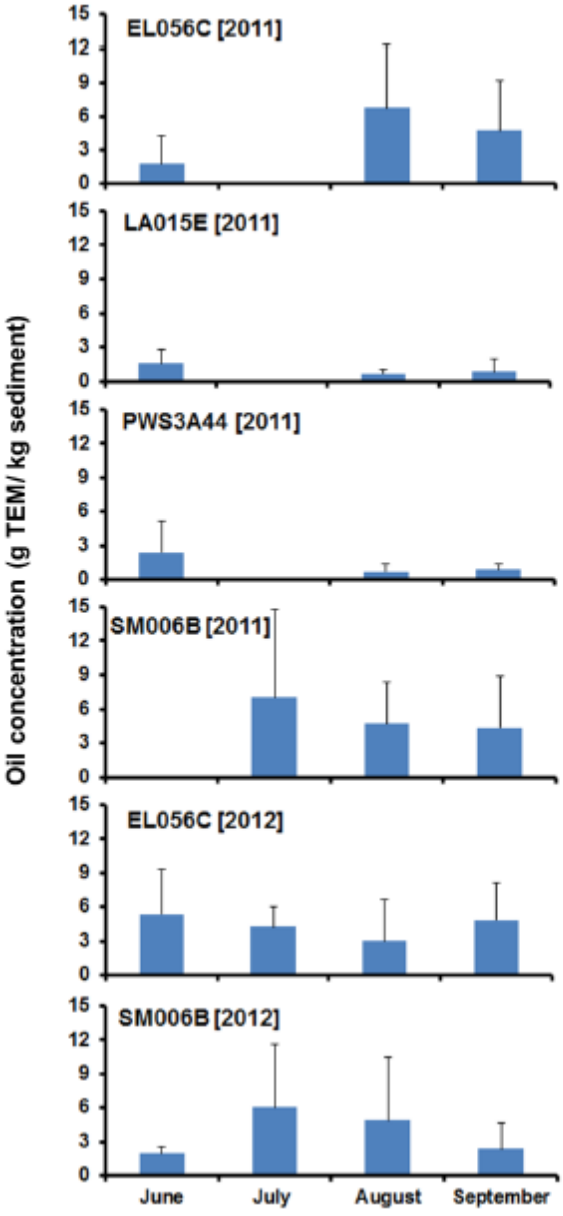
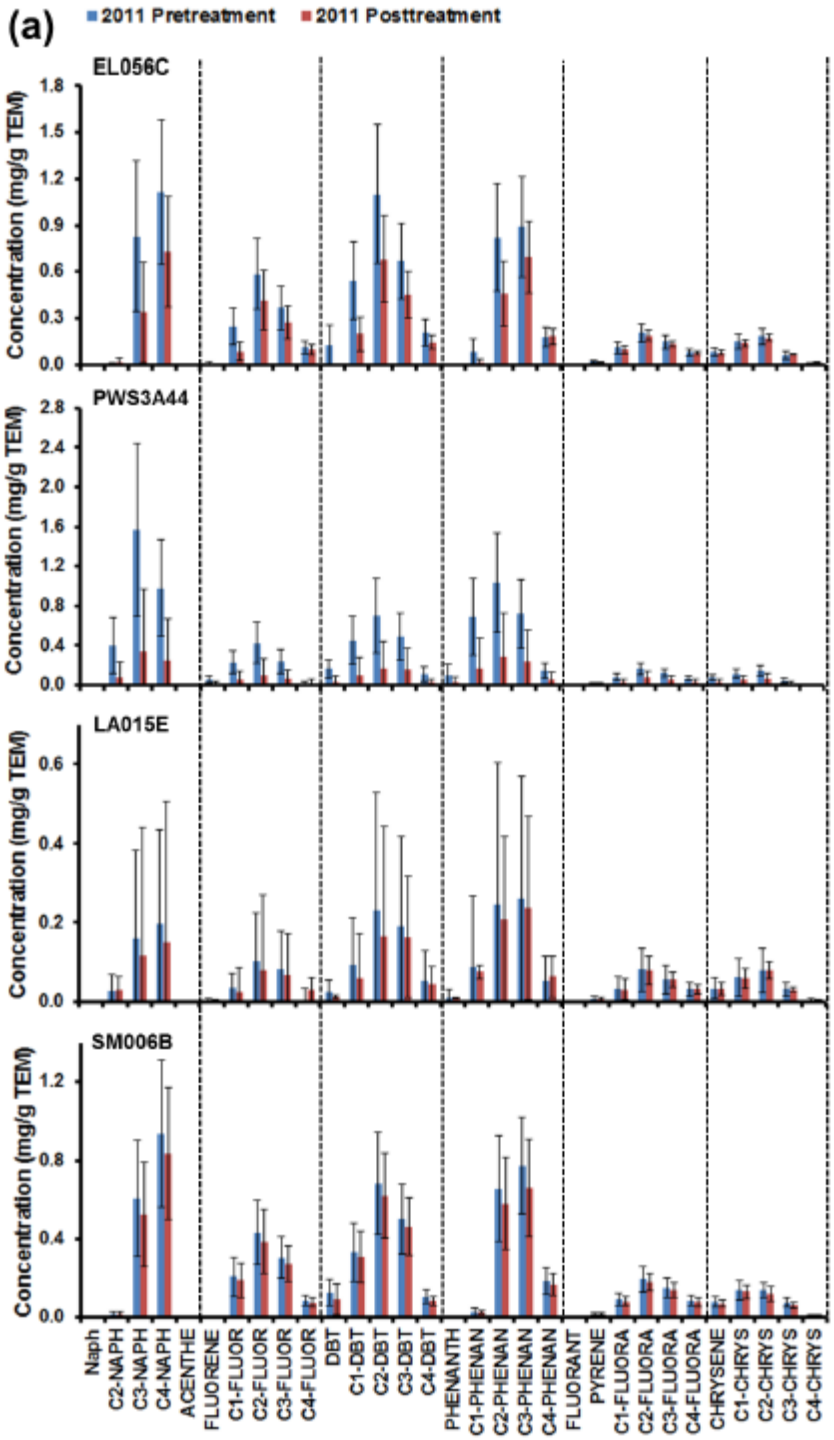


Figure 3: Concentration of TEM (Total Extractable Material) per kilogram of sediment for all the beaches (plots) as a function of time in 2011 and 2012. The TEM represents oil. Results are averaged over the whole plot (see Figure S1). No particular temporal pattern is noted.


(a)
2011 Pretreatment
2011 Posttreatment
EL056C
PWS3A44
LA015E
SM006B
Concentration (mg/g TEM)
Naph
C2-NAPH
C3-NAPH
C4-NAPH
ACENTHE
FLUORENE
C1-FLUOR
C2-FLUOR
C3-FLUOR
C4-FLUOR
DBT
C1-DBT
C2-DBT
C3-DBT
C4-DBT
PHENANTH
C1-PHENAN
C2-PHENAN
C3-PHENAN
C4-PHENAN
FLUORANT
PYRENE
C1-FLUORA
C2-FLUORA
C3-FLUORA
C4-FLUORA
CHRYSENE
C1-CHRYS
C2-CHRYS
C3-CHRYS
C4-CHRYS

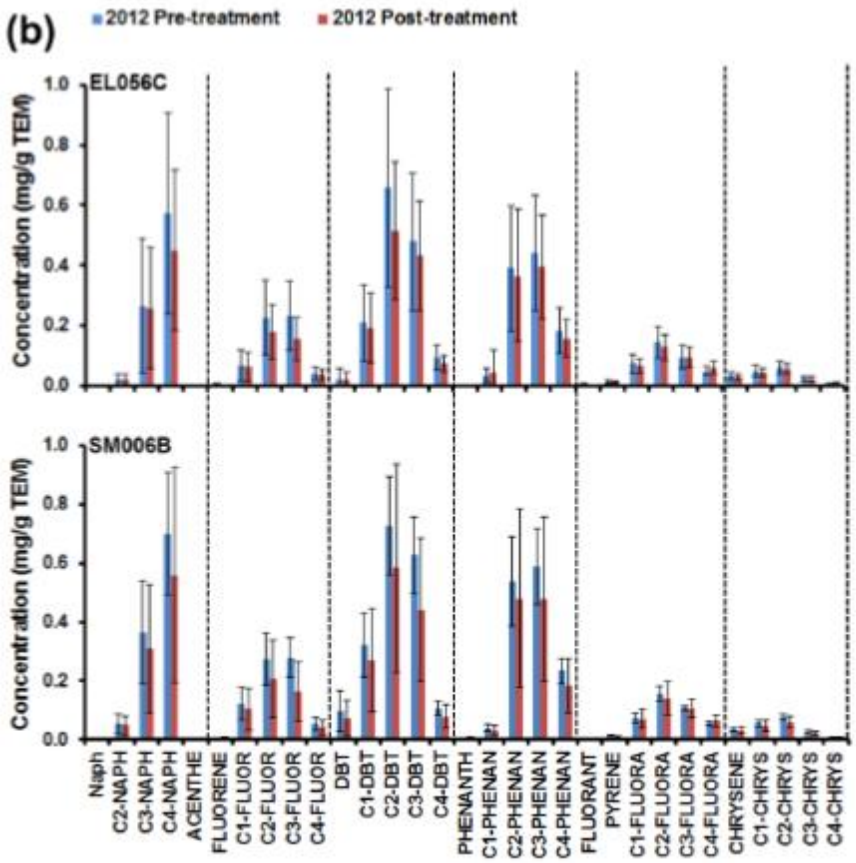


Figure 4: Comparison of the individual TPAH concentrations before and after treatment in (a) 2011 and (b) 2012. Note that individual TPAH are reported by increased degree of alkylation. Note that the vertical scales are different to highlight changes due to treatment.

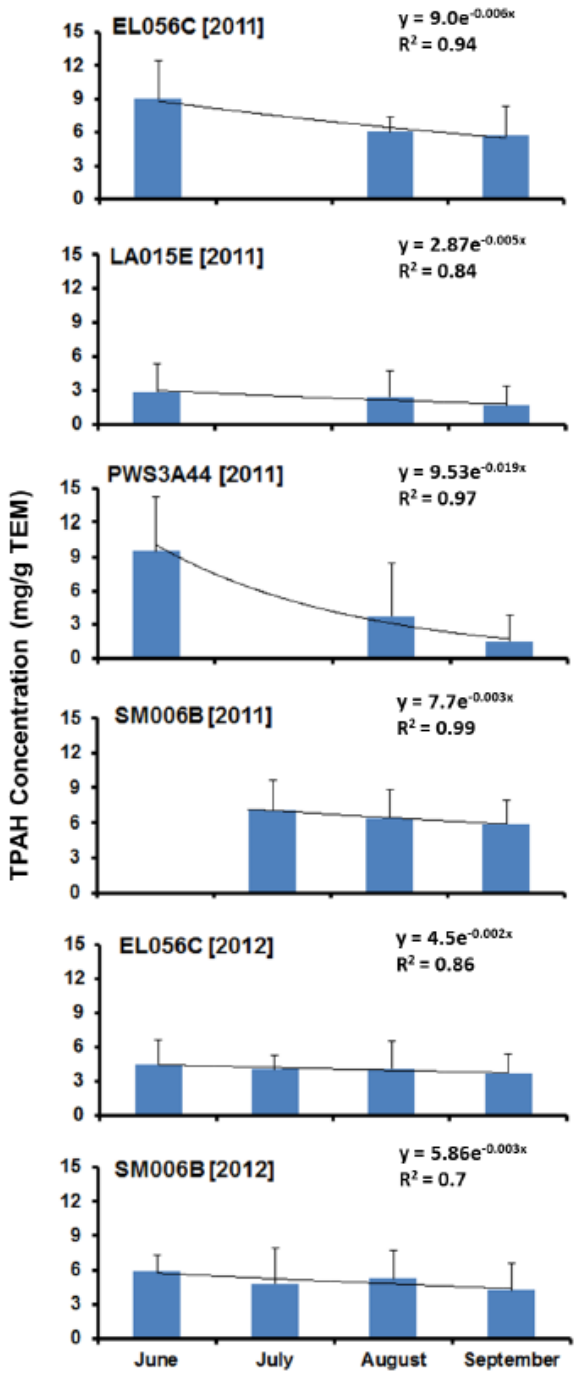


Figure 5: Concentrations of total PAH (polycyclic aromatic hydrocarbons+dibenzothiophene) per TEM (total extractable material) in each beach as function of time in 2011 and 2012. Exponential decay curves were fit from the initial time to the final sampling time. The unit of time "x" for the fitted equations is day. Error bars represent one standard deviation. See Table 1 for percent biodegradation.

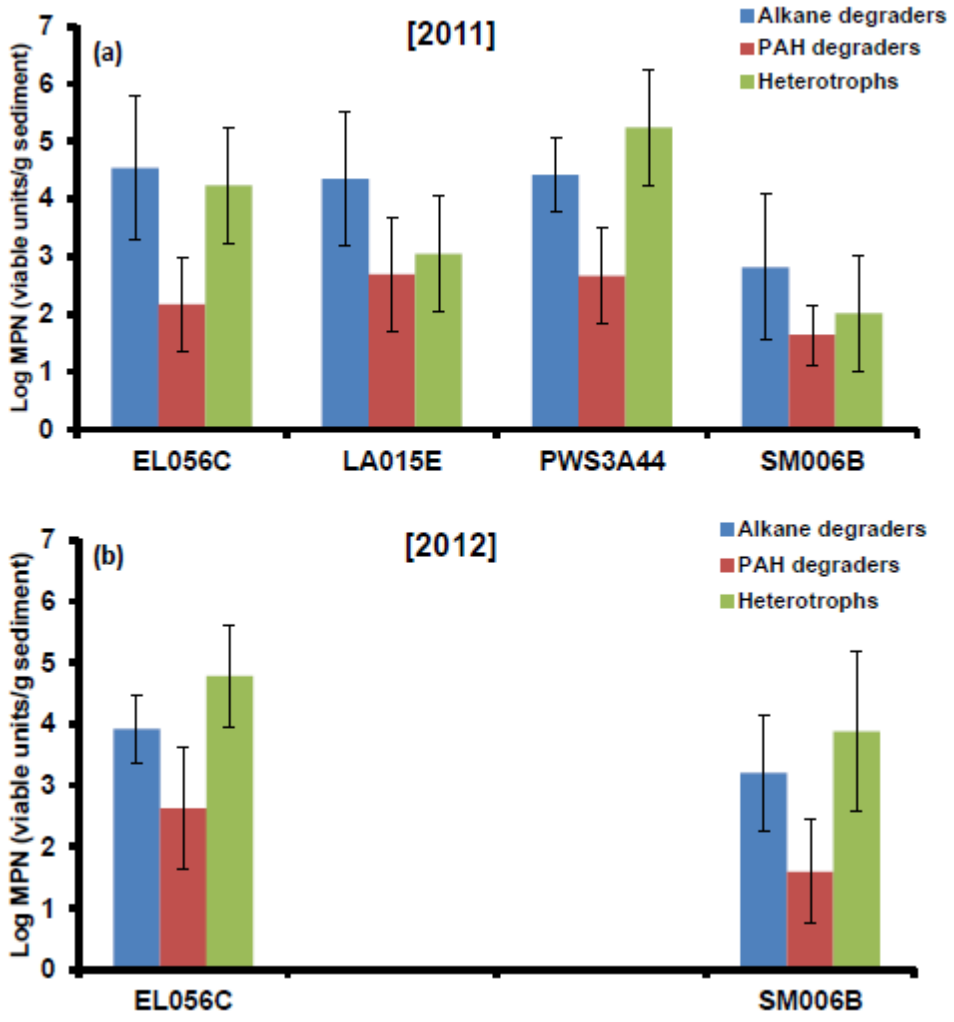


Figure 6: Most-probable-number (MPN) concentrations of hydrocarbon-degrading and heterotrophic bacteria at both beaches investigated during the 2011 (a) and 2012 (b) study. Error bars represent one standard deviation. Bars labeled with asterisks designate concentrations at SM006B that are significantly lower than concentrations of the same type of bacteria at EL056C. Comparisons were performed between beaches for each bacterial type; different types of bacteria were not compared between or within beaches (i.e., alkane degraders at EL056C were not compared to PAH degraders at EL056C or SM006B). Note that the y-axis (MPN) is in logarithmic scale. The reported values represent the average of samples collected along the four months span of each year.

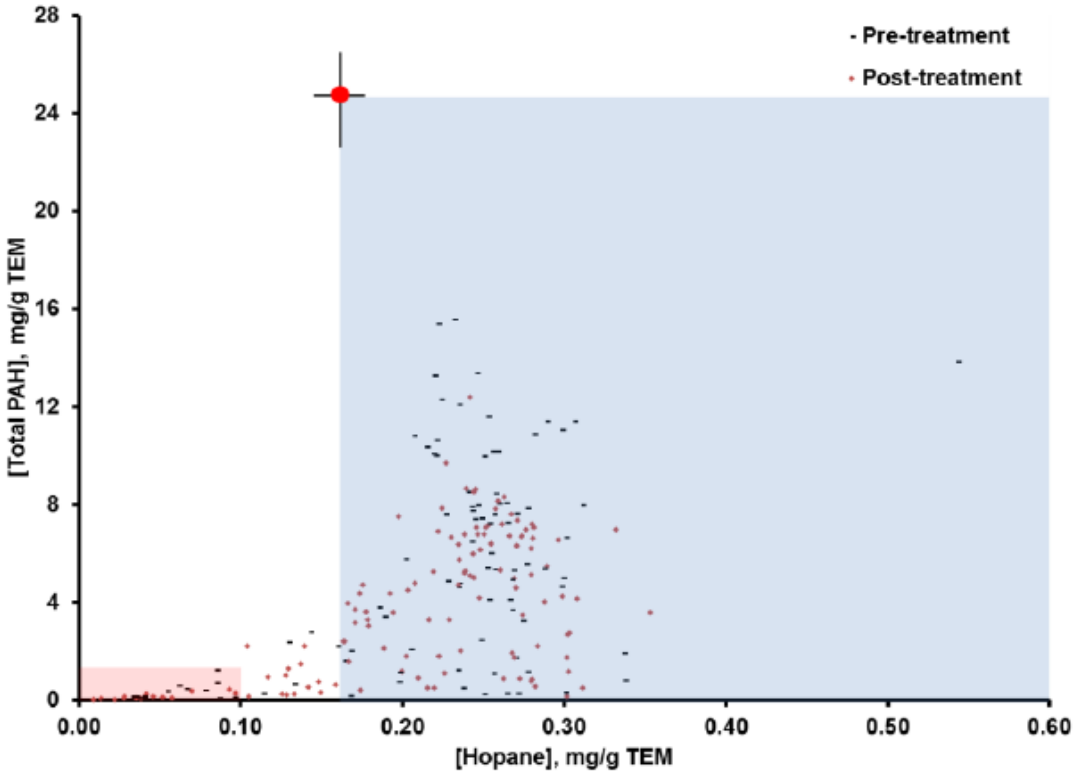


Figure 7: Comparison of hopane and total polycyclic aromatic hydrocarbon (TPAH) concentrations in total extractable material (TEM) in a) pre-treatment samples and b) all 223 field samples collected from Prince William Sound beaches during 2011 and 2012. The red dot (top of the figure) indicates the mean value of 20 analyses of un-weathered Alaska North Slope crude oil reference samples, and the associated horizontal and vertical black bars indicate 2 standard deviations above and below the mean hopane and TPAH values. Blue shading indicates field samples in which weathering increased hopane concentrations and decreased TPAH concentrations. Pink shading indicates extensively weathered field samples containing degraded hopane concentrations and associated highly degraded TPAH. Samples in the pink shaded area were considered in this study too degraded for bioremediation to be effective and/or statistically detectable, and therefore were excluded during the assessment of bioremediation.