

Bioremediation of an Experimental Oil Spill on the Shoreline of Delaware Bay

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In the summer of 1994, a field study was undertaken in Delaware in which light crude oil was intentionally released onto plots to evaluate bioremediation. The objectives were to obtain credible statistical evidence to determine if bioremediation with inorganic mineral nutrients and/or microbial inoculation enhanced the removal of crude oil contaminating a sandy beach and to compute intrinsic and enhanced biodegradation rates. Biodegradation was tracked by GC/MS analysis of selected components, and the measured concentrations were corrected for abiotic removal by hopane normalization. A randomized block design was used to assess treatment effects. Three treatments were evaluated: a no-nutrient addition control, addition of water-soluble nutrients, and addition of water-soluble nutrients supplemented with a natural microbial inoculum from the site. Although substantial hydrocarbon biodegradation occurred in the untreated plots, statistically significant differences between treated and untreated plots were observed in the biodegradation rates of total alkane and total aromatic hydrocarbons. First-order rate constants for the disappearance of individual hopane-normalized alkanes and PAHs were computed, and the patterns of loss were typical of biodegradation. Significant differences were not observed between plots treated with nutrients alone and plots treated with nutrients and the indigenous inoculum. The high rate of oil biodegradation that was observed in the untreated plots was attributed to the background nitrogen that was measured at the site. Even though oil loss was enhanced by nutrient addition, active bioremediation in the form of exogenous nutrient addition might not be appropriate in cases where background nutrient levels are already

sufficiently high to support high intrinsic rates of hydrocarbon biodegradation.

Introduction

A number of studies of oil spill bioremediation on marine shorelines have been conducted (1-13). These studies have concluded that bioremediation enhances the removal of crude oil several times more than the intrinsic rate. Much skepticism remains in the field, however, because data from all of these investigations have been equivocal to some extent. One reason for the uncertainty is a fundamental flaw in the experimental design of many of these studies. That is, the studies usually used pseudoreplication to test for treatment effects, in which either the treatments were not replicated or replicates were not statistically independent (14). The pseudoreplicate designs usually resulted from intense subsampling of unreplicated plots (1, 2, 8-11, 13). Without full replication and random interspersal of treatments, it is impossible to ascribe statistically significant differences in the response variable(s) to the treatments (14). The reason is simply that unknown, uncontrollable variables (unidirectional longshore currents, spatially distinct underground flows, prevailing winds, etc.) may exist in different parts of the same experimental area that may impart bias to one of the treatments. The only way to control this is to replicate the treatments and to randomly locate and interdisperse them on the experimental plane. Then, if systematic geomorphological or other physical biases exist in certain areas, the statistical analysis can easily account for them. Two studies that used proper replication and randomization to demonstrate significant treatment effects were conducted in terrestrial environments rather than on marine beaches (15, 16). Since the physical factors affecting oil and nutrient removal, as well as the persistence of introduced microorganisms, are so different in terrestrial and beach ecosystems, it is not possible to extrapolate from these studies to conclude that bioremediation will also be effective on oil-contaminated coasts.

Another problem common to many field studies is the differences in the way the treated and control plots are manipulated (1, 2, 8-11, 13). It is important to keep experimental manipulation of the treatments identical except for the one variable being tested, otherwise differences cannot be attributed unambiguously to the process being evaluated. For example, in one of the studies (8), the control plot was unamended and untouched whereas the treated plot was watered and tilled daily in addition to the nutrient and microbial amendments. This confounds conclusions regarding the cause of any observed differences.

Two recent reports have pointed to the need for more controlled studies of bioremediation for cleanup of oil-contaminated beaches (17, 18) because the current database is inadequate to provide guidance to spill responders. In particular, the relative advantages of biostimulation (i.e.,

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addition of nutrients alone) and/or bioaugmentation (addition of nutrients plus hydrocarbon-degrading bacteria) need further study (17). The goals of this project were to quantify the effectiveness of intrinsic biodegradation due to natural levels of background nutrients already present in the Fowler Beach area of Delaware Bay, to demonstrate the effectiveness of biostimulation and/or bioaugmentation, to determine the extent of any resulting rate enhancement, and to provide guidelines that can be used by spill responders and on-scene coordinators for the effective bioremediation of oil-contaminated sandy shores. Other goals were to quantify the biological effects of the oil and of the bioremediation treatments using various types of toxicity bioassays. Preliminary results of the toxicology work have been published elsewhere (19).

Since huge quantities of crude oil and refined petroleum products are transported through Delaware Bay each year, the State of Delaware is concerned about the ecological impact of a large oil spill in the area. Large quantities of migratory birds feed on the eggs deposited by horseshoe crabs (*Limulus polyphemus*) in the intertidal sands of the Delaware and New Jersey shores. Thus, an oil spill that occurred during spawning season could have catastrophic effects on these bird populations as well as future generations of horseshoe crabs.

Experimental Section

Approach. The null hypothesis was that biostimulation and/or bioaugmentation will not increase the rate or extent of removal of measured oil components over background. A randomized complete block design with repeated measures was used to assess treatment effects. Five areas of beach were selected based on the depth to a layer of peat below the surface of the sand that extended from a saltwater marsh several hundred meters west of the beach area. Each area ("block") was large enough to accommodate four experimental units or test plots. The blocks were situated in a row on the beach parallel to the shoreline. Three treatments were tested on oiled plots: a no-nutrient addition control, addition of water-soluble nutrients, and addition of water-soluble nutrients supplemented with a natural microbial inoculum from the site. A fourth treatment, an unoiled and untreated plot, served as a background control for the biological recovery studies. The four treatments were randomized in each of the five blocks.

Experience from previous studies (20–22) suggested that, to minimize edge effects in a small plot study such as this, oil should be applied near the spring high tide line. A schematic diagram of the block layout is summarized in Figure 1. The five blocks of four treatments were located on one long beach as shown. Each treatment plot measured 36 m² in area (4 m × 9 m). The distance between plots within a block was 10 m. The minimum distance between blocks was 10 m, although 104 m separated blocks 1 and 2 and 154 m separated blocks 4 and 5. The plots were laid out using standard surveying equipment. The top of each plot was positioned at the same elevation, measured relative to benchmarks (fence posts) placed on the high dune area, so that all plots would experience the same levels of submersion and exposure. Three steel fence posts were driven in the middle of each plot on a line bisecting the longitudinal axis of the plot. These were used to monitor the change in beach topography with time (by measuring the distance from the tops of the posts to the sand surface) as tide and wave action caused erosion and accretion of

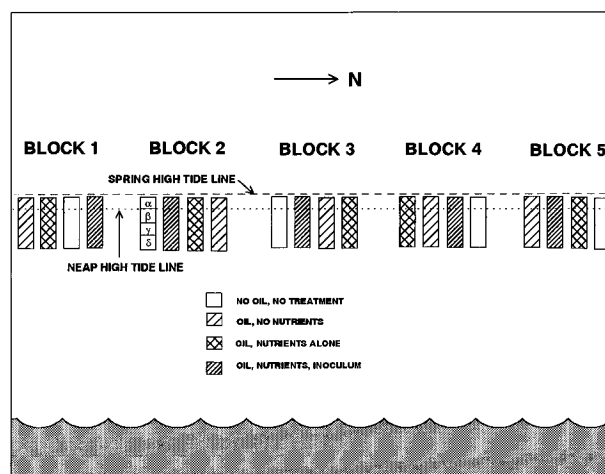


FIGURE 1. Layout of the randomized complete block design showing actual random order of plots on Fowler Beach.

the sand within the plots. Such topographical alterations could artificially affect oil concentrations measured.

Detailed Description of Blocks. Each block was equipped with its own gasoline-powered generator to supply power to four electric pumps, one for each of the four plots. The pumps supplied seawater with or without bioremediation amendments to their respective plots. Adjacent to each pump was a polyethylene reservoir holding 800 L of seawater containing the appropriate amendments for daily application to the plots. Slickbar booms were used to contain the oil and free-moving oiled sand within the plots. These booms were 30 m in length and contained 0.6 m long plastic floats riveted into a Kevlar skirted sheet extending about 0.6 m below the water surface. The booms were held in place between pairs of steel pipe driven approximately 1 m below the surface of the sand and spaced every 2.3 m around the perimeter of each plot. The booms floated on the water during flooding tides to contain floating oil and served as dams containing oiled sand when the tide ebbed. A sorbent boom was placed around the Slickbar boom as an added safety precaution in the event of an accidental release beyond the primary containment. The sorbent boom was removed several days after application of the oil, when floating oil was no longer observed. Length measurements were taken from the tops of the inner steel posts retaining the booms to the sand surface to supplement the beach topography data.

Application of Oil to Plots. The crude oil used was Bonny Light (Escravos), which is imported from Nigeria. It had a specific gravity of 0.848, API gravity of 35.3, Reid vapor pressure of 6.9, viscosity of 5.6 CST at 21 °C, a pour point of −3 °C, and a sulfur content of 0.11% by weight. It was weathered prior to application by placing about 3 m³ in a 3.6 m diameter plastic tank, connecting a pump and hose, and continuously spraying and recirculating the oil within the tank for 2 days to evaporate the light fraction. Following this, the weathered oil was placed into 210-L drums (136 L/drum) to await application. Oil was applied manually by two four-membered teams on July 1, 1994. Two persons held onto each end of a 4 m length of pipe connecting a hose to a pump that led into the 210-L drum containing the oil. The oil was pumped through the hose out of four atomizer nozzles attached to the pipe manifold. The persons applying the oil walked slowly up and down the plot while a third person lifted the trailing hose over the steel pipe holding the containment booms in place. The

fourth person held a stopwatch and timed the walking pace of the applicators. The 136 L of oil was applied uniformly in 14 sweeps of the plot over a period of about 7 min. A total of 2040 L of crude oil was released onto the 15 plots.

Nutrient Application. The minimum nitrate concentration needed to support the maximum growth rate of alkane degraders under continuous flow conditions ranges between 0.5 and 2.5 mg of N/L (23). Since nitrate in the interstitial pore water is quickly diluted to background levels whenever the incoming tide completely submerges the plot (24), we applied nutrients every day. To achieve the target 1.5 mg/L average interstitial pore water concentrations, we assumed a 100-fold safety factor to account for dilution. Thus, the nutrients added to each of the 10 designated reservoirs consisted of 2 kg of technical grade sodium nitrate (330 g of nitrogen) and 128 g of sodium tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), which is more soluble in seawater than orthophosphate. The nutrients were added to seawater in the appropriate reservoirs and dissolved by recirculating immediately prior to application to the plots. Once a week, 30 L of a suspended mixed population of hydrocarbon-degrading bacteria was also added to the inoculum plots (see below).

The sprinkler system used for supplying the nutrients consisted of Schedule-40 PVC pipe (3.8 cm i.d.) connected to six Maxi-Bird impact sprinkler heads per plot (Rainbird, Inc., Glendora, CA), each rated at 6 L/min. Electric pumps (rated at 70 L/min) were connected to the PVC sprinkler manifold via 15 m $\times$ 1.9 cm diameter garden hoses. Seawater was applied to all plots regardless of whether they received nutrients, inoculum, or no amendment at all.

Inoculum Preparation. The indigenous inoculum was grown for 2 weeks in two 210-L stainless steel drums. To allow weekly inoculation with fresh 2-week cultures, each drum was offset in time from the other by 1 week. The drums contained 170 L of seawater from Delaware Bay, the weathered Bonny Light crude oil (600 mL) as the sole carbon source, and the same nutrients used on the beach. The original culture consisted of a mixed consortium isolated from the same beach several months prior to the experiment and grown on the same Bonny Light crude oil. The organisms were not characterized as to genus and species. The number of alkane and aromatic degraders measured in the drums were $1.9 \times 10^5/\text{mL}$ and $2.5 \times 10^4/\text{mL}$, respectively. The oil in the drums became emulsified within 1 day following each inoculation, signifying actively metabolizing cultures.

Sample Size, Frequency, and Handling. Sand samples were collected every 14 days from the 15 oiled plots. Each plot was divided into four equal sectors (labeled α , β , γ , and δ , landward to seaward, respectively). Subsamples were collected from each sector to determine if differences existed in oil biodegradation according to the length of time under water. Two subsamples from each sector were collected according to a predetermined random number sequence that disallowed subsequent resampling from the same hole, composited into one sample, frozen, and shipped to Cincinnati for extraction and analysis by GC/MS. Thus, 60 samples, stratified within plots according to location within the intertidal zone and spread among 15 independent plots, were collected at each sampling event.

The subsamples consisted of two cores, each 7 cm in diameter $\times$ 14 cm deep, from one hole and two identical cores from another hole from the same plot sector. The samples were composited and mixed by a trowel in a

galvanized bucket and placed in a pre-labeled 3.8-L paint can. After the composited samples were thoroughly mixed, subsamples were taken from the mixture for other types of analyses (microbiological and toxicological). A portion of each composited sample was frozen and archived in the event that a re-analysis was needed at a later time.

Microbiological Analysis. Subsamples from each sector of each plot, including the five un-oiled plots (80 samples altogether), were placed in Whirlpak bags, brought back under ice to the on-site mobile laboratory trailer, and immediately processed for most probable number (MPN) analysis of alkane- and PAH-degrading bacteria (25). Approximately 10 g wet weight (exact weight was recorded after weighing on a top-loading balance) was placed in a dilution bottle containing 90 mL of sterile detachment solution (1 g/L disodium pyrophosphate and 20 g/L NaCl) and shaken for 1 h at 300 rpm. The samples were then placed onto a Beckman Biomek 1000 Laboratory Workstation for automated serial 10-fold dilutions in 96-well microtiter MPN plates. The growth medium was Bushnell-Haas (26) supplemented with 2% sodium chloride.

Oil Chemistry. Frozen sand samples from the field were shipped to Cincinnati for processing. Either 100 or 500 g of sand was mixed with an equal volume of anhydrous Na_2SO_4 . This mixture was extracted by sonicating three times for 10 min each with 150 or 450 mL of dichloromethane (DCM), respectively. This extract was poured through a funnel packed with anhydrous Na_2SO_4 into a tared round-bottom flask. The extract was then concentrated to dryness with a rotary evaporator. The flask was reweighed to determine the total DCM-extractable organic material (EOM). The residue was redissolved in DCM and diluted to a volume based on the amount of oil present. The final DCM extract was then solvent-exchanged to hexane. A 1.0- μL aliquot of the hexane extract was injected into a Hewlett-Packard 5890 Series II gas chromatograph equipped with an HP 5971A mass selective detector (MSD). The MSD was operated in the selected ion monitoring (SIM) mode for quantifying specific saturated hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), and sulfur heterocyclic constituents.

The GC was equipped with a DB-5 open tubular column (30 m long, 0.25 mm i.d., and 0.25 μm film thickness) and a split/splitless injection port operating in the splitless mode. Operating conditions for the GC were as follows: injection port, 290 $^\circ\text{C}$; transfer line, 320 $^\circ\text{C}$; initial GC oven temperature, 55 $^\circ\text{C}$ held for 3 min; first temperature ramp rate, 5 $^\circ\text{C}/\text{min}$ to 280 $^\circ\text{C}$, held for 5 min; second temperature ramp rate, 3 $^\circ\text{C}/\text{min}$ to 310 $^\circ\text{C}$, held for 10 min. The total run time was 73 min. All analyte data were normalized to the conservative, nonbiodegradable biomarker $\text{C}_{30-17}\alpha\text{-(H),}21\beta\text{(H)-hopane}$ (27, 28).

Nutrient Analysis. Concentrations of nitrate-N in the interstitial pore water of oiled plots were measured each day by collecting a sand core from each of the four sectors of a randomly selected test plot and the longitudinal midpoint of a nonnutrient control plot [to prevent interference with samples for oil analysis, the nutrient samples were all collected at the edges of the plots (i.e., within 30 cm of the boom)]. Each of the five samples was extracted with 1.0 L of deionized water on a shaker (the water had been previously acidified by adding 1.0 mL of concentrated H_2SO_4 to 500 mL of deionized water). Nitrate concentrations were quantified on an autoanalyzer after the pH was neutralized to 7.0 with 10 N NaOH. The method used was

based on cadmium reduction of nitrate to nitrite (29). Results were reported as milligram of nitrate-N/liter of interstitial water after determining the water content of the sample gravimetrically.

Laboratory Analysis of Biodegradation. To determine the rate of biodegradation of oil components in closed laboratory flasks for comparison to field observations, 500-mL respirometer flasks (30) were partially filled with 250 mL of artificial seawater, 5.0 g/L weathered crude oil was added to each flask, and the reactors were inoculated with a mixed culture of oil-degrading bacteria isolated previously from Slaughter Beach, DE, which is located about 1 mi north of the Fowler Beach area. The flasks were sacrificed periodically, and the entire contents was extracted with DCM followed by solvent exchange into hexane for GC/MS analysis of the PAH components of the oil.

Statistical Analysis and First-Order Hopane-Normalized Biodegradation Model. Oil constituents can be lost from a beach by physical washout, dissolution, volatilization, and biodegradation. An underlying assumption of this work is that a nonbiodegradable constituent of oil (namely, hopane) can be used to estimate the first three loss rate mechanisms and that the actual biodegradation rate of an analyte can be estimated from the difference between its total loss rate and its physical loss rate as estimated from the hopane loss rate. For this assumption to be true, the physical washout rate of the oil must be dominant, and the individual physical loss removal mechanisms of dissolution and volatilization must be negligible. Furthermore, due to the lack of more information about the mechanism of biodegradation of each analyte, the rate of biodegradation is assumed to be first order. Based on the aforementioned assumptions, the total loss rate of an analyte, $((dA/dt)_t)$, the total loss rate of hopane, $(dH/dt)_t$, and the biodegradation rate of the analyte, $-kA$, are related by the following equation:

$$\left(\frac{dA}{dt}\right)_t - \frac{A}{H}\left(\frac{dH}{dt}\right)_t = -kA \quad (1)$$

where A is the concentration of an analyte, H is the concentration of hopane, and k is the first-order biodegradation rate constant for an analyte. Using the definition of the derivative of a quotient, eq 1 can be rewritten as

$$\frac{d\left(\frac{A}{H}\right)}{dt} = -k\left(\frac{A}{H}\right) \quad (2)$$

Integrating eq 2 yields the following first-order relationship:

$$\left(\frac{A}{H}\right) = \left(\frac{A}{H}\right)_0 e^{-kt} \quad (3)$$

where (A/H) is the time-varying hopane-normalized concentration of an analyte, and $(A/H)_0$ is the value of that quantity at time t_0 .

Nonlinear regression analysis was used to estimate the first order rate of oil biodegradation for each of the three treatments and for comparison of the treatments. The coefficients of determination (r^2), which were derived from all the data (i.e., five replicates per sampling event $\times$ eight sampling events), were determined by the adjustment method of Kvalseth (31). Rate coefficients and intercepts of the three treatments were compared for statistical differences. Statistically valid variability estimates and

power curves were obtained to aid the design of future bioremediation studies.

Results

Visual Observations. The crude oil was applied at low tide on the morning of July 1, 1994. The application was quite uniform, as the coarse sand absorbed the oil almost instantly as it was applied. The waves were mildly rough during the first high tide several hours later as winds picked up from the southeast. Waves breaking on the plots caused some oil to be lifted over the containment booms, and visible sheens were observed floating on the water. The sorbent booms were slightly discolored by the escaped oil. The amount of oil that actually escaped, however, was negligible. The mean concentration of total petroleum hydrocarbons (gravimetric residue weight) in samples collected from all 15 plots 4 days after application was 4.74 g/kg, which is within 5% of the amount of oil (5.0 g/kg) originally calculated to be applied.

During the course of the experiment, significant movement of sand took place within the plots due to tide and wave action. This was especially evident at the periphery of the plots because of the damming effect caused by the skirted containment booms. The three steel fence posts driven in the middle of each plot along with the steel posts retaining the boom that fell on the same transect served as a means of measuring the resultant change in topography.

Physical Loss of Oil. To distinguish physical loss from biodegradative loss of oil, the concentration of hopane in the sand was quantified in each sample. The measured overall means and standard deviations of the hopane concentrations at T_0 , (i.e., the day nutrients and inoculum were first applied) according to position within the intertidal zone were α -sector, 3.58 ± 1.82 mg/kg; β -sector, 3.73 ± 1.56 mg/kg; γ -sector, 2.90 ± 1.05 mg/kg; and δ -sector, 1.38 ± 1.05 mg/kg. Knowing that the concentration of hopane in Bonny Light crude oil was 597 mg/mg oil (from GC/MS analysis), we computed the theoretical hopane concentration in the sand at T_0 to be 2.83 mg/kg sand (assuming that we sampled the entire oiled depth). This agrees well with the measured overall mean hopane concentration, which was 2.90 ± 1.07 mg/kg (averaged over all sectors). Note that the hopane concentrations were higher in the α - and β -sectors of the plots and lower in the δ -sector. This was caused by the breaking waves churning the oiled beach matrix and carrying it landward, which is typical of the oil deposition pattern in an actual spill event (20, 21).

Figure 2a summarizes the overall disappearance of hopane with time from each of the three oiled treatments. The data, which were averaged over all intertidal sectors, represent plot mean hopane losses. First-order nonlinear regression curves for each treatment were fitted to the data, and no differences in rate coefficients or y-intercepts were found. Thus, an overall first-order curve was plotted through the pooled data, and a hopane half-life of 28 days was calculated. This was interpreted to represent physical loss of crude oil due to wave action and tidal inundation, since hopane was assumed to be nonbiodegradable in the time period of this experiment (1, 27, 28, 32). Figure 2b is a similar plot showing the temporal loss of total extractable organic material from the plots. The half-life was 20 days. The EOM first-order rate coefficient was significantly higher than the hopane disappearance rate. The difference in loss rates (and half-lives) between hopane and EOM is attributed to biodegradation because EOM includes both

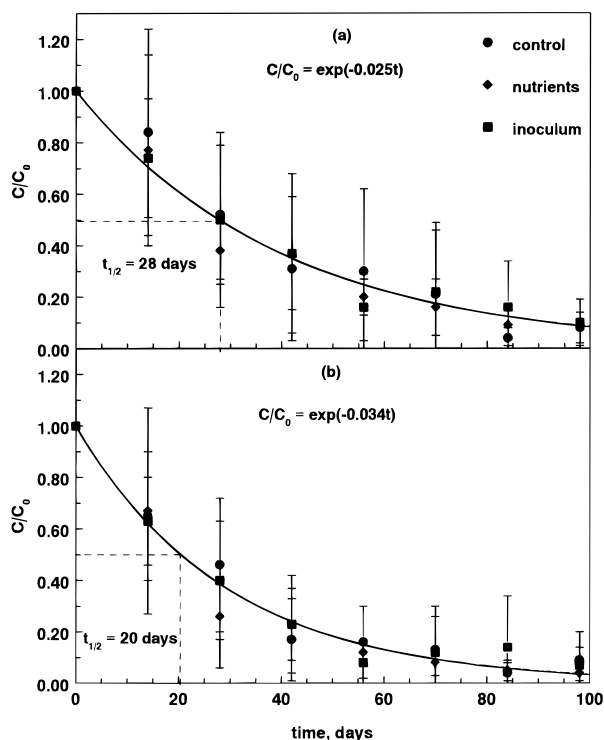


FIGURE 2. First order decay of (a) hopane and (b) total extractable organic matter during the experimental time period.

TABLE 1

Summary of Nitrate Concentrations by Block (mg of Nitrate-N/L of Interstitial Water)

block	control		nutrients		inoculum	
	mean	SD	mean	SD	mean	SD
1	0.7	0.7	3.9	1.4	2.6	2.3
2	0.8	0.9	9.5	3.3	3.9	3.2
3	1.3	1.1	6.2	3.8	1.1	0.5
4	0.6	0.5	3.6	2.1	5.5	5.5
5	0.8	1.3	8.6	7.9	4.2	2.4
total	0.8	0.3	6.4	2.7	3.5	1.7

biodegradable and nonbiodegradable components. However, EOM is not a sensitive enough indicator to discern treatment differences.

Nutrient Persistence. Table 1 summarizes the daily nitrate data from the oiled plots by block. The control plots receiving only seawater with no nutrients had measurable concentrations of nitrate (mean of 0.82 mg/L), which were approximately half the 1.5 mg/L target level desired for maximum biodegradation. The concentrations in the nutrient and inoculum treated plots were substantially higher. The Fowler Beach area of Delaware Bay was close to farm land, where runoff could easily account for the high background levels found. Samples along the shoreline of Delaware Bay were collected late in the project at considerable distances south and north of our experimental location and even in the Chesapeake Bay area, and the same high nitrate levels were measured. High nutrient levels are common in this coastal area (38).

Microbiological Analysis. Figure 3 summarizes the results of the alkane and aromatic degrader population data for all sampling events. No statistically significant differences were evident among the three oiled treatments at any sampling event. The populations in all the oiled plots

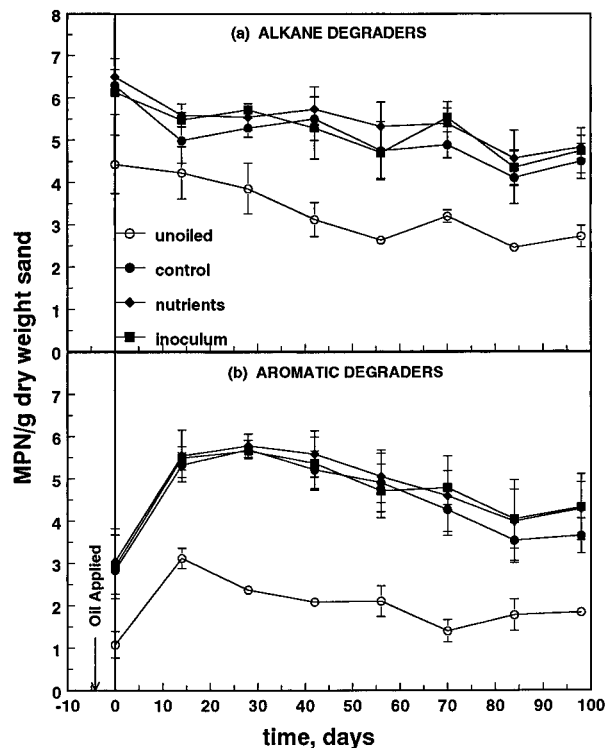


FIGURE 3. Growth of (a) total alkane degraders and (b) total aromatic degraders in treated, untreated, and unoiled plots.

were significantly higher than the unoiled plots. Even though the treatment differences were not statistically significant, the populations in the nutrient-amended plots were always greater than the nonamended (oiled) controls by about half an order of magnitude, especially in the latter weeks of the study. Note that the populations of alkane and aromatic degraders in the oiled plots were 2 orders of magnitude higher than those in the unoiled controls at T_0 , which was 4 days after oil was applied. This suggests that considerable and rapid increases in the microbial populations occurred within 4 days due solely to exposure to crude oil and the natural background levels of nutrients present. The number of alkane degraders in both unoiled and oiled plots slowly decreased over time, while aromatic degraders increased almost 3 orders of magnitude in the first 2 weeks, leveled off, and then decreased. The aromatic degraders even increased in the unoiled plots, suggesting that enough oil may have initially escaped from the oiled plots onto the unoiled plots to cause a stimulation in growth. Note, however, that the amount of oil required to stimulate growth to approximately 1000 PAH degraders/g of dry sand is extremely low (<1 g of oil/plot) relative to the amount of oil added to each of the oiled plots (approximately 110 kg of oil/plot). One plausible explanation that could account for the observed lack of change in the alkane degrader populations was that they were already at their maximum field capacity at T_0 .

Total Target Analytes. Results from the biweekly samplings are summarized in Figure 4a,b which shows the hopane-normalized concentrations of total target alkanes (i.e., the sum of all alkane analytes from n -C₁₀ to n -C₃₅ plus pristane and phytane) (Figure 4a) and total target aromatics (i.e., the sum of all groups of PAHs and sulfur heterocyclics analyzable by GC/MS and their alkyl-substituted homologues) (Figure 4b) in the nutrient-treated, inoculum-treated, and control plots as a function of time. Each data

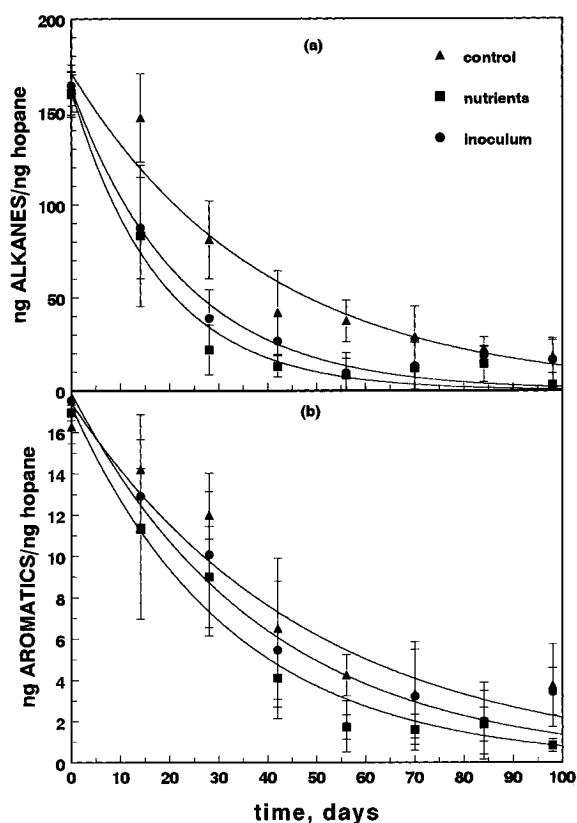


FIGURE 4. First-order declines in (a) total target alkanes (nC_{10} – nC_{35} plus pristane and phytane) and (b) total target aromatics, including alky-substituted homologues (error bars represent $\pm$ one standard deviation unit).

TABLE 2

Summary of Rate Coefficients, Y-Intercepts, and Coefficients of Determination for the Total Analyte Biodegradation Data

treatment	alkanes			aromatics		
	intercept	k , day ⁻¹	r^2	intercept	k , day ⁻¹	r^2
control	171.1	-0.026	0.879	17.4	-0.021	0.839
nutrients	161.8	-0.056 ^a	0.901	17.3	-0.031 ^a	0.886
inoculum	163.0	-0.045 ^a	0.911	17.9	-0.026 ^a	0.829

^a Rate coefficient significantly different from control ($p < 0.05$).

point is the mean of five independent replicates, and the error bars represent one standard deviation unit. The y-intercepts of the three treatments were not significantly different but the first-order rate coefficients were. Both the alkane and the aromatic biodegradation rates in the nutrient- and inoculum-treated plots were significantly greater than the control. Table 2 summarizes the intercepts, rate coefficients, and coefficients of determination (r^2) for the first-order relationships.

Individual Analytes. Figure 5 summarizes for each of the treatments the first-order biodegradation rate constants of individual alkanes calculated by nonlinear regression. The estimated initial concentrations (y-intercepts) were equivalent for all treatments, but the first-order rate coefficients were significantly different among the treatments for all of the analytes. The hopane-normalized loss rates in the nutrient- and inoculum-treated plots were significantly greater than the unamended controls. Hopane-normalized rate constants for nutrient plots averaged approximately 2.3-fold higher than control plots for normal

TABLE 3

Comparison of First-Order Rate Constants of Homologous PAHs Determined in the Laboratory and the Field

PAH	k , day ⁻¹	field		laboratory	
		k relative to highest substituted homologue	k , day ⁻¹	k relative to highest substituted homologue	ratio, $k_{\text{field}}/k_{\text{lab}}$
nap	0.302	13.727	0.734	4.170	3.29
C ₁ -nap	0.108	4.909	0.603	3.426	1.43
C ₂ -nap	0.045	2.045	0.340	1.932	1.06
C ₃ -nap	0.031	1.409	0.227	1.290	1.09
C ₄ -nap	0.022	1.000	0.176	1.000	
phe	0.046	3.067	0.365	3.614	0.85
C ₁ -phe	0.030	2.000	0.212	2.099	0.95
C ₂ -phe	0.020	1.333	0.162	1.604	0.83
C ₃ -phe	0.015	1.000	0.101	1.000	
flu	0.049	2.722	0.367	2.603	1.05
C ₁ -flu	0.029	1.611	0.246	1.745	0.92
C ₂ -flu	0.022	1.222	0.171	1.213	1.01
C ₃ -flu	0.018	1.000	0.141	1.000	
dbt	0.043	3.308	0.322	3.320	1.00
C ₁ -dbt	0.023	1.769	0.196	2.021	0.88
C ₂ -dbt	0.019	1.462	0.147	1.515	0.97
C ₃ -dbt	0.013	1.000	0.097	1.000	

alkanes and about 1.6-fold higher for the isoprenoid hydrocarbons. The latter result was expected, since branched alkanes are known to be more resistant to biodegradation than their straight-chain counterparts (33). Rate differences were somewhat lower for the inoculum and control plot comparisons, the inoculum plots yielding about 1.8-fold higher rate constants than the control plots for normal alkanes and 1.5-fold higher for the isoprenoids.

Figure 6 summarizes the comparative hopane-normalized first-order biodegradation rate constants of the individual PAH groups. Figure 6a compares the nutrient plots to the controls, while Figure 6b compares the inoculum plots to the controls. The rate constants for the nutrient-treated plots were significantly higher than the control for 16 of the 27 PAHs measured, whereas the rate constants for the inoculum-treated plots were higher for only 4 of the 27 compounds. The overall patterns of decay for all treatments were indicative of biologically mediated degradation reactions (34, 35), i.e., the rate constants were higher for all parent compounds except pyrene and chrysene and progressively lower as more alkyl groups appeared on the ring structure. Rate constants for the parent and monomethylated naphthalene group were equal to or even greater than those of the lowest molecular weight alkanes in the nutrient-treated plots. Rate constants for the three-ring polycyclics were approximately equivalent to those of the intermediate carbon number alkanes in the unamended control plots or the high carbon number alkanes of the nutrient-treated plots. The naphthobenzothiophenes had the highest rate constants among the four-ring PAH groups. Concentrations of the pyrene and chrysene groups in this particular crude oil were close to the detection limit of the instrument, so calculated biodegradation rate constants are less accurate than for other compounds.

Table 3 compares the first-order biodegradation rate constants of the hopane-normalized two- and three-ring PAH compounds measured in the field to the rate constants measured in sealed laboratory respirometer flasks. Also shown in Table 3 are the rates of the hopane-normalized

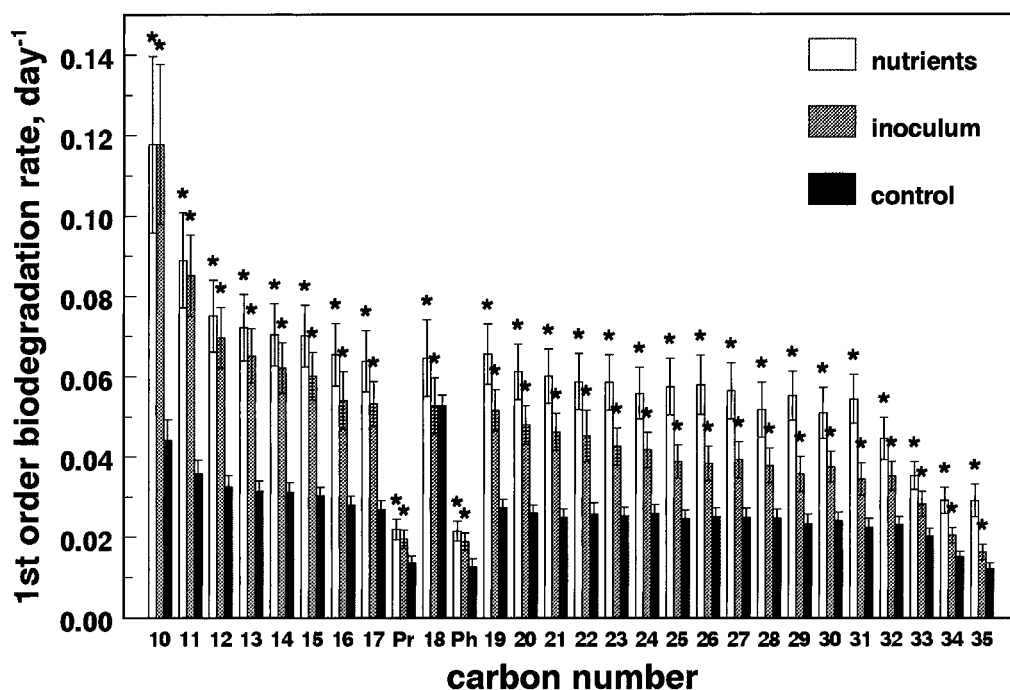


FIGURE 5. First-order biodegradation rates of all individual normal and branched alkanes computed by nonlinear regression of the data over the entire 14-week experimental time period [error bars represent $\pm$ one standard deviation unit, and asterisks identify significant differences from untreated control plots ($p < 0.05$)].

parent and substituted homologous PAHs relative to their respective highest and least volatile substituted homologue. For all compounds except naphthalene and C1-naphthalene, the agreement in biodegradation rates of the substituted homologues relative to their respective highest substituted homologue was nearly identical between laboratory and field, i.e., ratios ranged from 0.83 to 1.09 (last column, Table 3). The reason why the agreement broke down for naphthalene and C1-naphthalene was that the latter compounds are significantly more volatile than the others; consequently, rate constants of these two compounds were greater in the field. These results verify our interpretation that the pattern of loss of homologous PAHs measured in this field study are indicative of biodegradation as opposed to mere physical loss or solubility differences. Losses measured in the field represent losses due to washout, dissolution, volatilization, and biodegradation, whereas losses measured in a sealed flask can only be biodegradative. Since the relative rates measured in the field closely approximated those measured in the laboratory, differences in the dissolution and volatilization rates among homologues were negligible relative to differences in the biodegradation rates. Hopane normalization, which accounts for losses due primarily to washout, eliminates the uncertainty caused by all the physical factors acting on the oil components.

Positional Effects within the Intertidal Zone. Figure 7 summarizes the hopane-normalized first-order rate constants of the three treatments as a function of the plot sectors (i.e., α , β , γ , and δ) from which the samples were taken. The greatest differences between treated and control plots for both the saturate and the aromatic fractions of the oil were evident in the α -sector of the plots, i.e., the location closest to the high dune area of the beach. Somewhat lower rates occurred in the β -, γ -, and δ -sectors. These data suggest that more biodegradation occurred in the upper intertidal zone than in the lower intertidal area. This upper area of the beach is submerged the least and is thus likely

to be exposed to higher aerobic conditions for a longer time period. It is also less subject to churning action of breaking waves, which occurs more on the lower portion of the beach. Thus, nutrients added for bioremediation purposes would persist longer in the higher intertidal zone and would be more available for continued biostimulation. To provide further support for this conclusion, we estimated the average time of submersion for each sector from available tide tables. The α -sector was under water for approximately 0.5 h/day, the β -sector for 2.5 h/day, the γ -sector for 6.5 h/day, and the δ -sector for 11 h/day. Submersion times were estimated independently for each tidal cycle. These time estimates begin when the tide reached the midpoint of each sector on the rising tide and when it reached the same point on the subsequent falling tide. These submersion times are based on average tidal elevations to the midpoints of the four sectors and represent the average time of submersion from rising tide to falling tide.

Statistical Power Analysis. A statistical power test was performed to determine the number of replicates that would be needed in future studies where the investigator wished to determine differences among treatments, given similar experimental conditions and variability as experienced in this study. Figure 8a presents power curves for the saturate fraction (similar power curves, not shown due to space limitations, were generated for the aromatic fraction) for a varying sample size (number of replicate plots) and a constant variance (σ). Figure 8b presents similar power curves for a constant sample size but varying σ . For a power of 80% (i.e., the probability that significant differences between two or more treatments are detected when indeed they exist), the difference in hopane-normalized alkanes between treated and untreated plots at any sampling time would have to be >120 at two replicates, 63 at three replicates, 47 at four replicates, 39 at five replicates, 31 at seven replicates, and 25 at 10 replicates to be statistically detectable at the variance experienced (Figure 8a). Actual

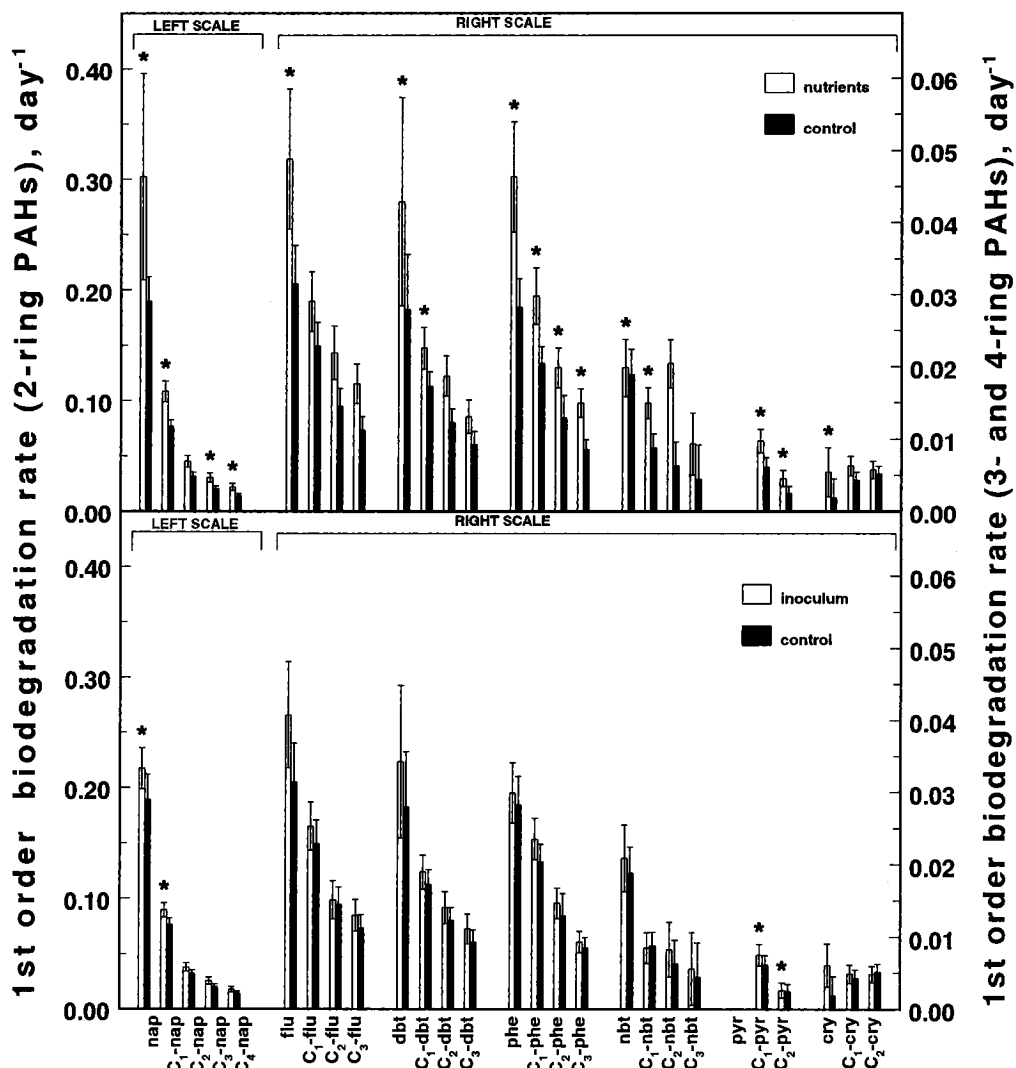


FIGURE 6. First-order biodegradation rates of all aromatics and their alkyl-substituted homologues. (a) Comparison of nutrient and control plots; (b) comparison of inoculum and control plots [error bars represent $\pm$ one standard deviation unit, and asterisks identify significant differences from untreated control plots ($p < 0.05$)].

observed differences averaged over weeks 4, 6, and 8 were 41 ng of alkanes/ng of hopane. Had only four replicate plots been used, the difference of 41 ng of alkanes/ng of hopane between the nutrient-treated plots and the control plots would not have been detectable. If the difference had been greater than 63 at any of those sampling events (i.e., due to a lower intrinsic biodegradation rate), we would have been able to detect such a treatment difference with as little as three replicates of each treatment.

For a power of 80% with a constant number of replicate plots (five), the difference in hopane-normalized alkanes between treated and untreated plots would have to be 20 at a variance of 8.3, 39 at a variance of 16.3 (the actual variance encountered in this study), 61 at a variance of 25.3, and 85 at a variance of 35.3 to be statistically detectable. Thus, if the expected variance in a future field study is 50% higher than that encountered in Delaware, a difference of 61 ng of alkanes/ng of hopane would have to exist to be statistically detectable with five replicates.

Topographical Changes. The plot-averaged change in elevation of beach sand relative to initial sand elevations determined at the time of the initiation of the experiment was computed for every sampling event. This calculation was performed along a transect bisecting the plot perpen-

dicular to the water edge. Each plot had three internal posts and two edge posts along the transect. Depth measurements from the top of a post to the surface sand elevation were used for the calculations. The plot-averaged change in elevation was calculated from the changes in elevations measured along the posts using the trapezoidal rule according to the equation:

$$\Delta h = \frac{1}{\sum_{i=1}^4 \Delta x_i} \sum_{i=1}^4 (\Delta h_i + \Delta h_{i+1}) \frac{\Delta x_i}{2}$$

where Δh is the plot-averaged change in elevation (cm); Δh_i is the change in elevation at post_{*i*} (cm); and Δx_i is the distance between post_{*i*} and post_{*i+1*} (cm).

These Δh data computed by the above procedure indicate very little change in average plot elevation over time: the average change was 1.1 ± 2.9 cm for the oiled control plots, 0.4 ± 2.5 cm for the nutrient plots, -2.4 ± 2.3 cm for the plots receiving both nutrients and inoculum, and -3.4 ± 4.6 cm for the unoiled control plots. It is clear from these data that little net sand movement occurred between the plots and the surrounding beach. Further-

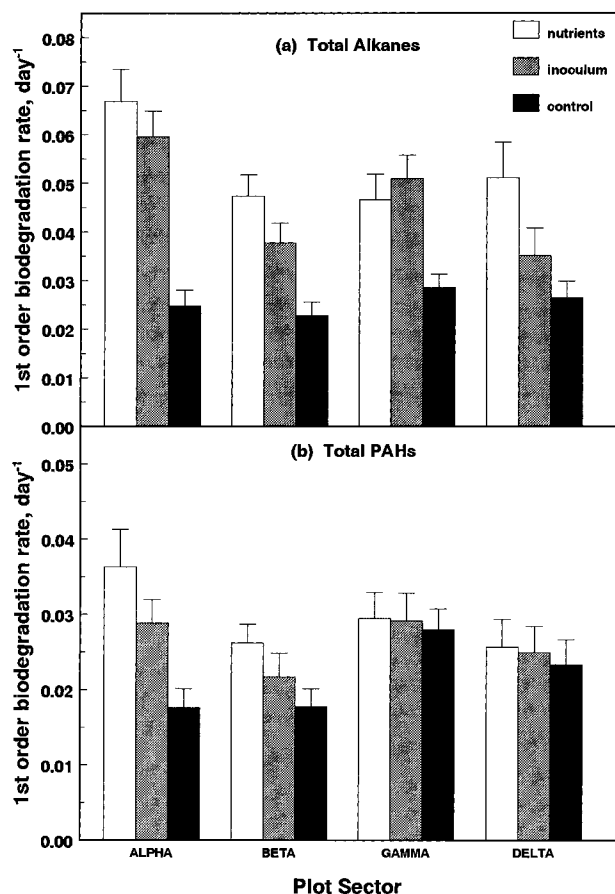


FIGURE 7. First-order biodegradation rates of (a) total target alkanes and (b) total target aromatics according to location within the intertidal zone (α -sector is closest to the high dune area; δ -sector is closest to the water) (error bars represent $\pm$ one standard deviation unit).

more, no meaningful difference existed between the boomed plots and the unboomed, unoiled plots relative to net changes in sand elevation. Although no net change in elevation occurred in the unboomed portions of the beach, we observed considerable movement of sand with rising and falling tides throughout the experimental period. This type of dynamic sand movement is exactly what would happen with oiled sand in a real spill where the whole beach is contaminated. However, in the latter instance, sand movement will not result in a major change in oil concentration in any one location because the whole beach is oiled. The fact that the boomed plots were able to retain the oiled sand for the duration of the study implies that results observed from this bioremediation study should closely simulate results expected from a study where the entire beach is oiled.

Discussion

In designing bioremediation experiments on a sandy beach, one has to contend with the fact that sand is transported into, out of, and along a beach by several mechanisms. Some of the mechanisms responsible for the movement of sand are tidal action, wind action, long-shore currents, animal and human activity, and storms. Since our permit limited the application of oil to 4×9 m plots, any movement of sand might have resulted in oiled sand leaving and fresh sand entering the plot. If the whole beach were contaminated, this movement of sand would not result in any major experimental problems since both the sand entering and

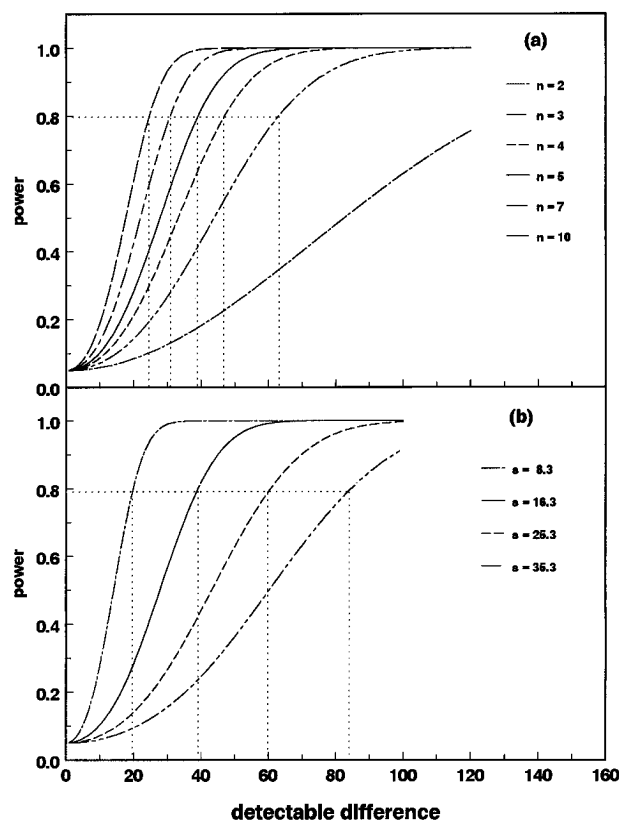


FIGURE 8. Statistical power curves for hopane-normalized total alkanes (a) for varying n and constant σ of 16.3 and (b) for varying σ and constant n of 5.

leaving the plot would be contaminated; however, when only the plots are contaminated, any movement of sand will result in artificially accelerated dilution of the oiled sand. The use of hopane as a biomarker provides a procedure for overcoming this problem so that losses due to biodegradation can be isolated. However, dilution may result in toxicity reduction for which hopane normalization cannot account.

The two previously reported studies, which used replication and randomization to support the demonstration that significant treatment effects occurred, were conducted in terrestrial environments rather than on marine beaches (15, 16). However, the abiotic loss mechanisms that act upon both the petroleum substrate and the nutrient amendments are substantially different in a beach environment as compared to a soil environment. The intertidal zones of beaches have much higher inputs of energy and flow-through of materials. The huge influx of seawater from tidal inundation can bring about substantial losses of hydrocarbons due to dilution. Surface waves as well as the interaction of tides and underground water flow are extremely important transport mechanisms for nutrients and bacteria and will affect the ability of exogenously introduced microorganisms to survive and colonize successfully. Consequently, the experimental design in a beach remediation field study is critical for providing the information needed to support objective conclusions on treatment effects. A randomized complete block design is ideally suited to test the biostimulation/bioaugmentation hypothesis.

Results presented in this paper were derived from five independent replicates of three separate and distinct oiled treatments. The reason why randomization was so im-

portant was to enable inferences on treatment effects over the whole experimental plane, not just the plots of interest. If a researcher has conducted a pseudoreplicate experiment and has assumed that no other effects except a treatment effect are present, then the inference will be limited to just the two plots of beach used in the experiment rather than the whole beach area, since a random sample of the plots, not the whole beach, was used. This has been the case in the vast majority of field studies reported (1, 2, 8–11, 13). In this study, we did not want to limit our conclusions to the experimental plots; rather, we wanted to be able to infer treatment effects over the entire beach. The only way to accomplish this objectively was to sample randomly over the whole beach or a representative section of it.

The information from the statistical power analyses is useful for designing future research experiments involving three treatments in a randomized block design. If an experimenter has good reason to assume a certain variance and an expected treatment difference after a specified time period, the power curves from Figure 8 can be used as an aid in designing the experiment.

Clearly, bioremediation of crude oil spilled on a sandy beach in the temperate climate of the east coast can take place extremely rapidly. The nitrogen concentrations naturally present along the coast of Delaware Bay were high enough to sustain rapid intrinsic rates of biodegradation without human intervention. Although biostimulation with an exogenous source of inorganic mineral nutrients did indeed significantly accelerate the rate of hydrocarbon biodegradation, the incremental increase in biodegradation rate over the intrinsic rate (i.e., slightly greater than 2-fold for the alkanes and 50% for the PAHs) (Table 2) might not have been high enough to warrant a recommendation to actively initiate a major, perhaps costly bioremediation action in the event of a large crude oil spill in that area. This finding suggests that, in the event of a catastrophic oil spill impacting a shoreline, the first task is to measure the natural nutrient concentrations in that environment to determine if they are already high enough to sustain significant intrinsic biodegradation. If they are high enough, the next task is to determine if such a nutrient loading is typical for that area and season (i.e., determine the impact of chronic runoff from nearby agricultural practices or pollution from an upstream wastewater treatment plant or industrial discharge). Continuously renewable concentrations approaching 1–2 mg of nitrogen/L of interstitial pore water should support near optimum hydrocarbon biodegradation activity. The decision to supplement natural nutrient levels with an exogenous source should be based on how high the natural levels are relative to this threshold. If we had conducted this study on a beach with a similar temperature regime but substantially lower background levels of nitrogen and had encountered the same biodegradation rates in the nutrient-treated plots, the rate enhancement factor would have been considerable because the background rate would likely have been substantially lower than that observed. In such a case, the decision to implement bioremediation would be unambiguous.

With respect to bioaugmentation as a means of enhancing cleanup of an oil spill, results from this study suggest that supplementation of natural populations with indigenous cultures from the same site may not result in further enhancement over and above simple nutrient addition. This conclusion could be extended to include allochthonous sources of microorganisms because if in-

digenous cultures that are adapted to the environmental conditions of the site do not accelerate the degradation rates, organisms enriched from different environments, grown in the laboratory, and not acclimated to a particular climatic or geographical location should be even less able to compete with the natural populations. The reason for this is that hydrocarbon degraders are ubiquitous in nature (36), and when an oil spill occurs at a given site, the large influx of biodegradable carbon will cause an immediate response in the abundance of hydrocarbon-degrading populations (37). If nutrients are limiting, however, the rate of oil biodegradation will be less than optimal. Supplying nutrients will enhance the succession initiated by the spill, but supplying added microorganisms will not because they still lack the necessary nitrogen and phosphorus to support growth on the oil carbon.

Perhaps of even more importance are the factors that determine the carrying capacity for hydrocarbon degraders in a marine beach environment. The most important influence is likely the physical removal rate caused by scouring of biomass when breaking waves tumble sand grains. If that is the case and indigenous bacteria are present, then they can grow to the carrying capacity rapidly if sufficient nutrients are present. Adding exogenous hydrocarbon degraders cannot increase the population density because physical removal will control it. In the present study, if nutrient supplementation was sufficient to bring the background biodegradation rate to near its maximum, bioaugmenting the natural populations is unlikely to stimulate the rate further. Of course, this assumes that what we observed was very near the maximum biodegradation rate and that such a rate was determined by something other than the growth rate of hydrocarbon degraders (e.g., oxygen concentration, maximum bacterial population density, surface area available for attachment). If this is the case, then we cannot assume that bioaugmentation is always unnecessary. This study suggests that, in addition to surveying the background nutrient levels at a spill site, the background hydrocarbon-degrading populations should also be determined as part of the site assessment. These levels should then be compared to some standard to determine if bioaugmentation is necessary. Such a standard does not exist at this time, but future research that would define the carrying capacities of various environments and the mechanisms that control them could be fruitful in this regard.

The differences that we observed in the rates of disappearance of homologous PAHs cannot be explained by differences in solubility or volatility. The biodegradation rates observed in sealed respirometer flasks showed nearly identical relative disappearance rates to those observed in the field. This lends credence to our conclusion that the patterns that we observed in the field were indicative of biodegradation, because losses due to volatilization and dissolution cannot occur in a closed system. Normalizing all measured analyte concentrations to hopane markedly facilitates conclusions regarding analyte biodegradability in the field. One must assume that hopane does not biodegrade at rates approaching the other analytes and that hopane is subject to the same physical loss factors to which all other oil components are subject. Based on the experience of this and other studies (1, 27, 28, 32), the first assumption is well justified. The second assumption, however, is less certain. Hopane, which is a saturated, high molecular weight alkane, behaves like one with respect to

physical/chemical loss mechanisms. Despite this weakness, hopane normalization is useful as a first approximation for monitoring the progress of biodegradation in the field. No truly universal surrogates are available, and the extreme biological recalcitrance of hopane places it among the most reliable of available biomarkers. Used in combination with biodegradability differences within homologous series, hopane normalization can provide strong evidence in support of a biological removal mechanism during remedial operations.

In summary, we have shown that significant intrinsic biodegradation of petroleum hydrocarbons can take place naturally if sufficient nutrients already exist in the impacted area. We have also demonstrated statistically significant rate enhancement even in the presence of an already high intrinsic rate by supplementing natural nutrient levels with inorganic mineral nutrients. We affirmed that the decision to apply nutrients and microorganisms should depend on the background concentrations available at the contaminated site. We have shown that bioaugmentation will likely not significantly contribute to cleanup of an oil spill. We have developed for the first time from field data first-order biodegradation rate constants for the resolvable normal and branched alkanes and the important two- and three-ring PAH groups (and at least one four-ring PAH group) present in light crude oil. We have shown that the relative biodegradation rates of homologous PAHs measured in the field are close to those measured in the laboratory, thus corroborating the rates as being due to biodegradation and not physical washout or solubility differences. We have confirmed the importance of hopane as a useful biomarker for tracking biodegradation success in the field. We have demonstrated that maintenance of a threshold concentration of about 1–2 mg of nitrate-N/L of interstitial pore water will permit close to maximum hydrocarbon bioremediation. We have shown that better hydrocarbon biodegradation takes place in the upper intertidal zone than in the lower intertidal zone. And, finally, we demonstrated that the experimental conditions imposed by the booms surrounding the plots did not diminish our ability to simulate the sand movement that takes place naturally on a beach from long-shore currents and tidal inundations.

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Literature Cited

- (1) Bragg, J. R.; Prince, R. C.; Harner, E. J.; Atlas, R. M. *Nature* **1994**, *368*, 413–418.
- (2) Halmo, G. In *1985 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1985.
- (3) Lee, K.; Levy, E. M. In *Proceedings of the 1987 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1987.
- (4) Lee, K.; Levy, E. M. In *Aquatic Toxicology and Water Quality Management*; Nriagu, J. A., Ed.; John Wiley and Sons, Inc.: New York, 1989.
- (5) Lee, K.; Levy, E. M. In *Proceedings of the 1989 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1989.
- (6) Lee, K.; Levy, E. M. In *Proceedings of the 1991 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1991.
- (7) Lee, K.; Tremblay, G. H.; Levy, E. M. In *Proceedings of the 1993 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1993.
- (8) Rosenburg, E.; Legmann, R.; Kushmaro, A.; Taube, R.; Adler, R.; Ron, E. Z. *Biodegradation* **1992**, *3*, 337–350.
- (9) Sendstad, E. In *Proceedings of the 3rd Arctic and Marine Oil Spill Program*; Environment Canada: Ottawa, 1980.
- (10) Sveum, P. In *Proceedings of the 10th Arctic and Marine Oilspill Program*; Environment Canada: Ottawa, 1987.
- (11) Sveum, P.; Ladousse, A. In *Proceedings of the 1989 International Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1989.
- (12) Pritchard, P. H.; Costa, C. F. *Environ. Sci. Technol.* **1991**, *25*, 372–379.
- (13) Pritchard, P. H.; Mueller, J. G.; Rogers, J. C.; Kremer, F. V.; Glaser, J. A. *Biodegradation* **1992**, *3*, 315–335.
- (14) Hurlbert, S. H. *Ecol. Monogr.* **1984**, *54* (2), 187–211.
- (15) Westlake, D. W. S.; Jobson, A. M.; Cook, F. D. *Can. J. Microbiol.* **1978**, *24*, 245–260.
- (16) Jobson, A. M.; McLaughlin, M.; Cook, F. D.; Westlake, D. W. S. *Appl. Microbiol.* **1974**, *27*, 166–171.
- (17) Office of Technology Assessment. *Bioremediation for Marine Oil Spills*; U.S. Government Printing Office: Washington, DC, 1991.
- (18) Science Advisory Board. *SAB Review of the Alaskan Bioremediation Oil Spill Project*; U.S. Environmental Protection Agency: Washington, DC, 1992; EPA-SAB-EPEC-LTR-92-015.
- (19) Mearns, A.; Doe, K.; Fisher, W.; Hoff, R.; Lee, K.; Siron, R.; Mueller, C.; Venosa, A. D. *Proceedings of the 18th Arctic and Marine Oilspill Program Technical Seminar*; Environment Canada: Ottawa, 1995; Vol. 2, pp 1133–1144.
- (20) Owens, E. H.; Robson, W. *Arctic* **1987**, *40* (Suppl. 1), 230–243.
- (21) Owens, E. H.; Harper, J. R.; Robson, W.; Boehm, P. D. *Arctic* **1987**, *40* (Suppl. 1), 109–123.
- (22) Hayes, M. O.; et al. In *Proceedings of the 1979 Oil Spill Conference*; American Petroleum Institute: Washington, DC, 1979; pp 192–198; API Publication 4308.
- (23) Venosa, A. D.; Haines, J. R.; Suidan, M. T.; Wrenn, B. A.; Strohmeier, K. L.; Eberhart, B. L.; Holder, E. L.; Wang, X. In *Symposium on Bioremediation of Hazardous Wastes: Research, Development, and Field Evaluations*; U.S. Environmental Protection Agency, Office of Research and Development: Washington, DC, 1994; EPA/600/R-94/075; pp 103–108.
- (24) Venosa, A. D.; Suidan, M. T.; Wrenn, B. A.; Haines, J. R.; Strohmeier, K. L.; Holder, E. L.; Eberhart, B. L. In *Twentieth Annual RREL Research Symposium*, U.S. Environmental Protection Agency: Cincinnati, OH, 1994; EPA/600/R-94/011; pp 139–143.
- (25) Wrenn, B. A.; Venosa, A. D. *Can. J. Microbiol.*, in press.
- (26) Bushnell, L. D.; Haas, F. F. *J. Bacteriol.* **1941**, *41*, 653–673.
- (27) Peters, K. E.; Moldowan, J. M. *The Biomarker Guide*; Prentice Hall: Englewood Cliffs, NJ, 1993; 363 pp.
- (28) Seifert, W. K.; Moldowan, J. M. *Geochim. Cosmochim. Acta* **1979**, *43*, 111–126.
- (29) Technicon Industrial Systems. *Industrial Method No. 158–71W*; Technicon Industrial Systems: Tarrytown, NY, 1972; 3 pp.

- (30) Venosa, A. D.; Haines, J. R.; Nisamaneepong, W.; Govind, R.; Pradhan, S.; Siddique, B. J. *Ind. Microbiol.* **1992**, 10, 13–23.
- (31) Kvalseth, T. O. *Am. Stat.* **1985**, 39, 279–285.
- (32) Butler, E. L.; Douglas, G. S.; Steinhauer, S.; Prince, R. C.; Aczel, T.; Hsu, C. S.; Bronson, M. T.; Clark, J. R.; Lindstrom, J. E. In *On-Site Bioreclamation: Processes for Xenobiotic and Hydrocarbon Treatment*; Hinchee, R. E., Offenbuttel, R. F., Eds.; Butterworth-Heinemann: Boston, 1994; p 515.
- (33) Pirnik, M. P.; Atlas, R. M.; Barth, R. J. *J. Bacteriol.* **1974**, 119, 868.
- (34) Cerniglia, C. E. *Biodegradation* **1992**, 3, 351.
- (35) Bayona, J. M.; Albaiges, J.; Solonas, A. M.; Pares, R.; Garrigues, P.; Ewald, M. *Int. J. Environ. Anal. Chem.* **1986**, 23, 289.
- (36) Prince, R. C. *Crit. Rev. Microbiol.* **1993**, 19 (4), 217–242.
- (37) Pierce, R. H.; Cundell, A. M.; Traxler, R. W. *Appl. Microbiol.* **1975**, 29 (5), 646–652.
- (38) Sharp, J. H. University of Delaware, personal communication, 1995.

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