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Use of hopane as a conservative biomarker for monitoring the bioremediation effectiveness of crude oil contaminating a sandy beach

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Much of the variability inherent in crude oil bioremediation field studies can be eliminated by normalizing analyte concentrations to the concentration of a nonbiodegradable biomarker such as hopane. This was demonstrated with data from a field study in which crude oil was intentionally released onto experimental plots on the Delaware shoreline. Five independent replicates of three treatments were examined: no nutrient addition, addition of inorganic mineral nutrients alone, and nutrient addition plus indigenous oil-degrading microorganisms from the site. Samples collected biweekly were analyzed for the Most Probable Numbers (MPNs) of alkane and aromatic degraders and oil component analysis by GC/MS. The data were normalized to either the mass of sand that was extracted or to the concentration of hopane that was measured. Hopane normalization enabled detection of significant treatment differences in hydrocarbon biodegradation that were not detected when the data were normalized to sand mass. First-order loss rates for the hopane-normalized data were lower than those for the sand-normalized data because hopane normalization accounts only for loss due to biodegradation whereas sand normalization includes all loss mechanisms. Plots amended with nutrients alone and nutrients plus the inoculum showed enhanced removal of hydrocarbons compared to unamended control plots. However, no differences were detected between the nutrient-amended plots and the nutrient/inoculum-amended plots.

Keywords: oil spills; hydrocarbons; bioremediation; hopane; biomarker

Introduction

When beaches are contaminated by nearshore marine oil spills, the complex dynamics of sand and oil movement due to the influences of tides, waves, and weather confounds objective assessments of subsequent cleanup actions. Observations made in previous spill incidences showed that beached crude oil usually is stranded in the upper third of the intertidal zone [14,15]. With time, some oil is washed away, some ebbs and flows with the tides resulting in both physical losses and reoiling, and some gets redistributed laterally along the beach according to the forces of longshore currents. Because of these complex dynamics, estimating the extent of oil loss during the course of an oil spill bioremediation experiment due solely to the bioremediation activity is a scientific challenge.

Crude oil is composed of a myriad of chemical compounds of varying molecular weight and structure. Most researchers agree that the majority of compounds fall into several primary fractions depending on their physical and chemical properties, including the alkanes (normal, branched, and cyclic), the aromatics (monocyclic and polycyclic), and polar compounds (resins; nitrogen-, sulfur-, and oxygen-heterocyclics; and asphaltenes) [11,18]. These fractions differ in their susceptibility to biological attack. The normal alkanes and low molecular weight aromatics are usually the most sensitive, followed by the branched alkanes, multi-ringed and alkyl-substituted aromatics, cyclic alkanes, and finally the polars and asphaltenes [5,10,13,16]. These differences in biodegradability have been used to help assess the extent of biodegradation in oil-contaminated environments.

For years, researchers have relied on the decrease in the ratio of normal to branched alkanes, specifically the *n*-heptadecane (*n*-C_{17})/pristane and *n*-octadecane (*n*-C_{18})/phytane ratios [1–3,12]. Recently, however, the latter ratios have not proven dependable in the field, as the branched alkanes sometimes decline at rates approaching those of the normal alkanes [4,18,19]. As a result, attention turned to the use of hopanes as the biomarker of choice for assessing the degree of temporal loss of hydrocarbons due to biodegradation [4,7]. Hopanes are polycyclic saturated ring compounds (pentacyclic triterpanes) that are structurally similar to steroids and found in all crude oils. They are highly resistant to biodegradation [17,20]. Because of this innate resistance to biological attack, the hopanes are an excellent biomarker against which all other biodegradable analytes can be normalized. The purpose of this paper is to present evidence to support the utility of hopanes, specifically C_{30}-17α(H),21β(H)-hopane, as the biomarker of choice for assessing hydrocarbon biodegradation in the field. Data were obtained from a field study conducted in the summer of 1994 involving an intentional release of crude oil onto replicated plots on a sandy beach in Delaware Bay [23].

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Materials and methods

Experimental design

A randomized complete block design was used in the setup of the field plots. Five 60-m lengths of beach were marked off, each accommodating four experimental units or test plots. Three treatments were tested on 4-m × 9-m oiled plots (the shorter dimension parallel to the shoreline): 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 microbial population control. The four plots in each block were separated from each other by a 10-m buffer zone to mitigate transfer of amendments from one plot to another. The four treatments were randomized in each of the five blocks. Each plot was divided into four equal horizontal sectors to evaluate the effect of position within the intertidal zone. 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 the sand within the plots. Slickbar™ containment booms were placed around the plots to contain the oiled sand within the plots and minimize edge effects [14]. Oil was applied on 1 July 1994, and the experiment began 4 days later (defined as day 0). Details of the block layout, nutrient and oil application methods, and sampling procedures used are reported elsewhere [23].

Mineral nutrients (sodium nitrate and sodium tripolyphosphate) were dissolved in seawater and applied daily via a sprinkler system to maintain a threshold level of nitrogen (approximately 1.5 mg nitrate-N L^{-1} interstitial pore water) [22] that would support maximal biodegradation activity at all times. The nutrients added to each of the 10 designated reservoirs consisted of 2 kg technical grade $NaNO_3$ (330 g nitrogen) and 128 g $Na_5P_3O_{10}$. Although we achieved several-fold higher levels than that on the untreated plots, the natural levels of nitrate due to agricultural runoff on the Delaware Bay shoreline were high enough (average 0.82 mg L^{-1}) to sustain a significant intrinsic rate of biodegradation [23]. Once a week, 30 L of a suspended mixed population of hydrocarbon-degrading bacteria was also added to the inoculum plots (see below). For the no-nutrient control plots, only seawater was applied through the sprinkler system. Bonny light crude oil, previously weathered by aeration for 2 days, was applied on 1 July 1994, at the rate of 136 L per plot, resulting in a calculated crude oil contamination level of approximately 5 g kg^{-1} sand (assuming a penetration depth of approximately 30 cm).

Inoculum preparation

The indigenous inoculum was grown for 2 weeks in two 210-L stainless steel drums and aerated vigorously by a diffuser attached to an air pump. 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), 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 in the laboratory on the same Bonny Light crude oil. The number of alkane and aromatic degraders measured in the drums were 1.9×10^5 ml^{-1} and 2.5×10^4 ml^{-1}, respectively. The oil in the drums became emulsified within 1 day following each inoculation, signifying the presence of actively-metabolizing cultures.

Microbiological analysis

Sediment subsamples from each sector of each plot were placed in Whirlpak bags, brought under ice to the on-site mobile laboratory trailer, and immediately processed for MPN analysis of alkane- and PAH-degrading bacteria [24]. 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^{-1} disodium pyrophosphate and 20 g L^{-1} 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 salts [6] supplemented with 2% sodium chloride. The carbon source for alkane degraders was hexadecane (2 μl $well^{-1}$), and for aromatic degraders it was a mixture of phenanthrene (10 μg $well^{-1}$), anthracene (1 μg $well^{-1}$), fluorene (1 μg $well^{-1}$), and dibenzothiophene (1 μg $well^{-1}$). Positive wells were scored by observing the pink to red color formed by the formazan precipitate following addition of iodonitrotetrazolium violet to the alkane plates and the yellow color resulting from the intermediate compounds formed by the cleavage of the aromatic ring structure of the substrate PAHs in the aromatic plates [24].

Chemical analyses

Sand samples from the field were collected every 14 days and shipped frozen on dry ice to the US EPA laboratory in Cincinnati, OH 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 it 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 using 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 specific 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. Operating conditions of the GC/MS instrument have been described [23]. Nitrate

was analyzed by the cadmium reduction method using an autoanalyzer [21].

Statistical analysis

Since the plots were remeasured at prespecified sampling times during the course of the investigation, repeated measures analysis of variance (RMANOVA) [9] was used to analyze the response variables (MPN and oil analytes). When the RMANOVA indicated significant differences ($P < 0.05$), univariate ANOVAs were run on data at each time point. Where significant differences were indicated at a specific time point ($P < 0.05$), protected least significant difference (LSD) mean separations were used to assess treatment differences. Nonlinear regression analysis was used to estimate the first order rate of oil degradation for each of the three treatments. An F-test was conducted to compare each pair of intercept and slope coefficients statistically as opposed to simply computing confidence intervals for each parameter and comparing the end points of the confidence intervals to assess significant differences.

Results

Physical movement of oil

As erosion and accretion of the beach substrate occurred within the plots due to wave action, we attempted to find a correlation between changes in beach topography with the oil levels in the sand (as represented by the concentration of hopane). Figure 1 summarizes hopane concentrations after 28 days in each of the four plot sectors as a function of the measured differences in sand elevation relative to day 0. Also shown in the figure for reference purposes is the overall average concentration of hopane measured at day 0 (horizontal dotted line). Although the scatter in Figure 1 is quite wide, the general trends indicate a correlation between the hopane concentration and changes in the elevation of the surface of the beach. When the change in elevation was negative (signifying loss of sand from the area), the hopane concentrations were generally lower. The opposite was true when accretion of sand took place, ie positive changes in elevation giving rise to higher hopane concentrations. This suggested that either the churning action of breaking waves carried oiled sand from the lower intertidal zone to the upper zone or more physical washout of oil occurred in the lower intertidal zone

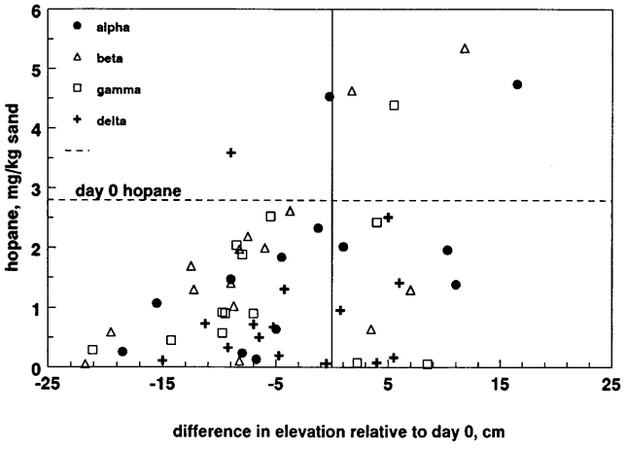

Figure 1 Correlation between the concentrations of hopane at day 28 and the differences in beach elevation relative to day 0. Greek letters alpha, beta, gamma, and delta refer to the four equal subdivisions of the plots ranging from landward to seaward, respectively.

Hydrocarbon degraders

Three assumptions have been made in support of the microbiological analysis: (1) hopane does not biodegrade (at least during the 14 weeks of the field trial); (2) the concentration of hopane is representative of the fraction of the original oil remaining at any given time; and (3) biodegradation of oil absorbed to sand particles requires adherence of degrading microorganisms to the oiled sand surface. If these three assumptions are true, then normalizing oil degrader numbers to hopane might provide a better representation of the temporal changes that occur in actively degrading populations on oiled beach sands rather than the conventional way of reporting numbers in terms of density per unit dry weight of beach material. Figures 2 and 3 summarize the MPN data for alkane- and aromatic-degraders, respectively, for all sampling events. Both figures present the data in two different ways: (1) $\log_{10}$MPN g^{-1} dry weight of beach sand (Figures 2a and 3a); and (2) $\log_{10}$MPN mg^{-1} hopane (Figures 2b and 3b).

Figure 2a shows that alkane degraders started out at high numbers ($>10^6$ g^{-1} sand), which progressively decreased over time. Linear regression analysis of the data indicated

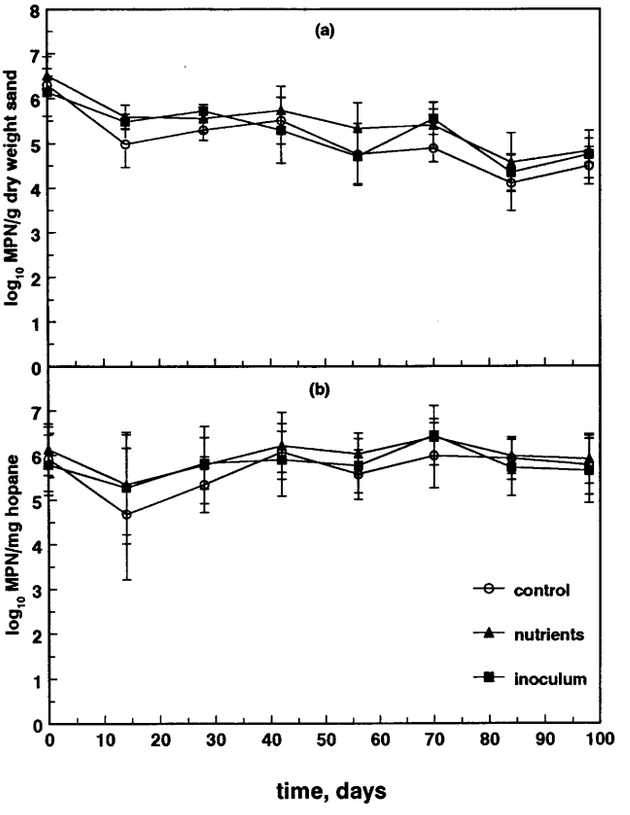

Figure 2 Most Probable Number estimates of alkane degraders per (a) g dry weight sand and (b) mg hopane. Error bars represent ±1 standard deviation unit.

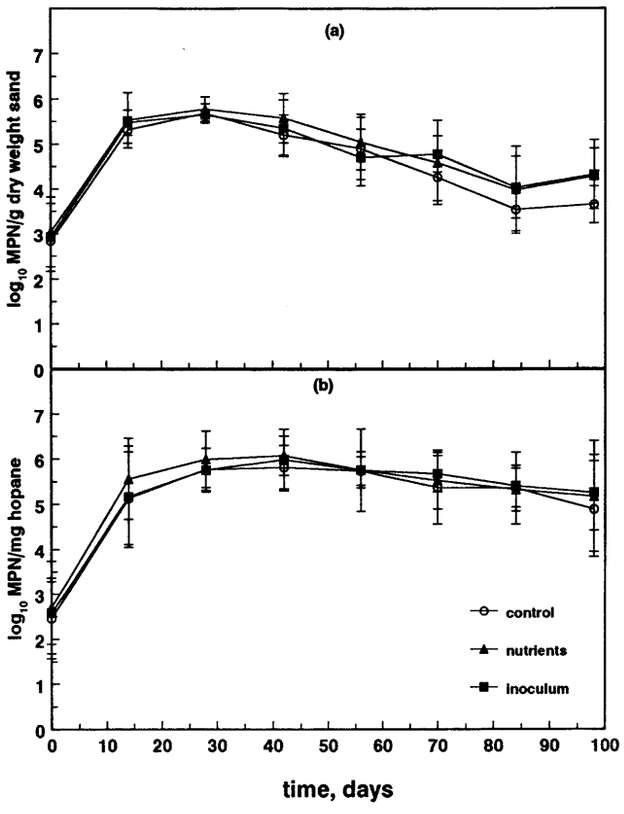

Figure 3 Most Probable Number estimates of alkane degraders per (a) g dry weight sand and (b) mg hopane. Error bars represent ±1 standard deviation unit.

that, in all three cases, the slopes of the best-fit lines were significantly different from 0. However, no statistically significant differences among the treatments were detected, although the alkane degraders in the control plots were always about 0.5 orders of magnitude lower than the nutrient-treated plots. The active bacterial population reached their maximum field capacity early and decreased slowly as the total amount of available hydrocarbon substrate declined.

In Figure 2b, where the alkane degraders are normalized to hopane, the numbers remained fairly constant over the 14-week experimental period. Linear regression of the data revealed that the slopes of the best-fit lines were not significantly different from zero. Again, no differences were evident among the three treatments. The reason for the apparent lack of significant microbial decline was that the actual decline in alkane degraders correlated with the decline in the hopane (and thus the oil) due to tide and wave action. The hopane half-life was about 28 days [23].

Changes in aromatic degraders are depicted in Figure 3a and b. Both panels show a three-order of magnitude increase within the first 2 weeks. Following that, the number of aromatic degraders g^{-1} sand progressively declined almost two orders of magnitude below their peak (Figure 3a), whereas the hopane-normalized MPNs declined to a far lesser extent (about a half order of magnitude). Linear regression analysis of the data from the highest MPN value revealed that, for both the sand-normalized and the hopane-normalized data, the slopes of all three best-fit lines were significantly different from 0 (the sand-normalized slopes were approximately 2 to 2.5-fold higher than the hopane-normalized slopes). Three explanations could account for the fact that the hopane-normalized MPNs for aromatic-degraders declined slightly while the MPNs for alkane-degraders remained the same: (1) the more readily degradable and more water-soluble PAH fraction decreased (either through washout, biodegradation, or a combination of both) enough to cause a drop in the total PAH-degrader population numbers; (2) the microorganisms responsible for metabolizing the PAHs might have been slightly more subject to physical washout; or (3) the death rate or maintenance requirements were higher for PAH-degraders, possibly due to the toxicity of the PAHs or their metabolic products. From the data shown we cannot distinguish among the three putative microbial loss mechanisms nor suggest which is the most important.

Fate of total target analytes

The total target alkanes are defined as the sum of all alkane analytes measured by GC/MS, ranging from *n*-C_{10} to *n*-C_{35} plus pristane and phytane. The total target aromatics are the sum of all groups of PAHs and sulfur heterocyclics analyzable by GC/MS and their alkyl-substituted homologues. Figure 4a summarizes the first-order decline in total

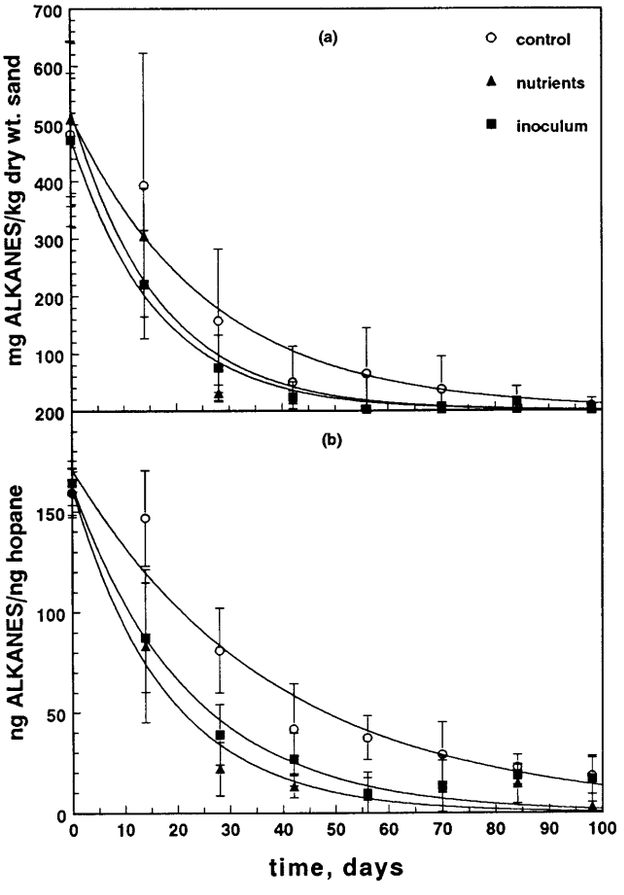

Figure 4 First-order decline in (a) mg total alkanes kg^{-1} dry weight sand and (b) ng total alkanes ng^{-1} hopane. Error bars represent ±1 standard deviation unit.

alkanes kg^{-1} dry weight sand, while Figure 4b depicts the same decline normalized to hopane. The RMANOVA revealed no statistically significant differences among the three treatments at any sampling event for the sand-normalized data, whereas highly significant treatment differences were detected in the hopane-normalized data at days 14, 28, and 56 with near significances ($P < 0.10$) indicated at days 42 and 98. Examining the results from the Protected LSD mean separation tests (summary data not shown), the differences at days 14, 28 and 56 were ascribed to the treated plots (nutrients and inoculum) giving lower means than the nonamended control plots. No differences between nutrient-treated plots and inoculum-treated plots were detected. The nonlinear regression analysis of the hopane-normalized data revealed that 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. No differences in the rate coefficients were detected in the sand-normalized data.

The same first-order declines in the aromatic fraction of the oil are shown in Figure 5a and b. The RMANOVA for the data presented in terms of mass per kg sand indicated no statistically significant treatment differences at any time,

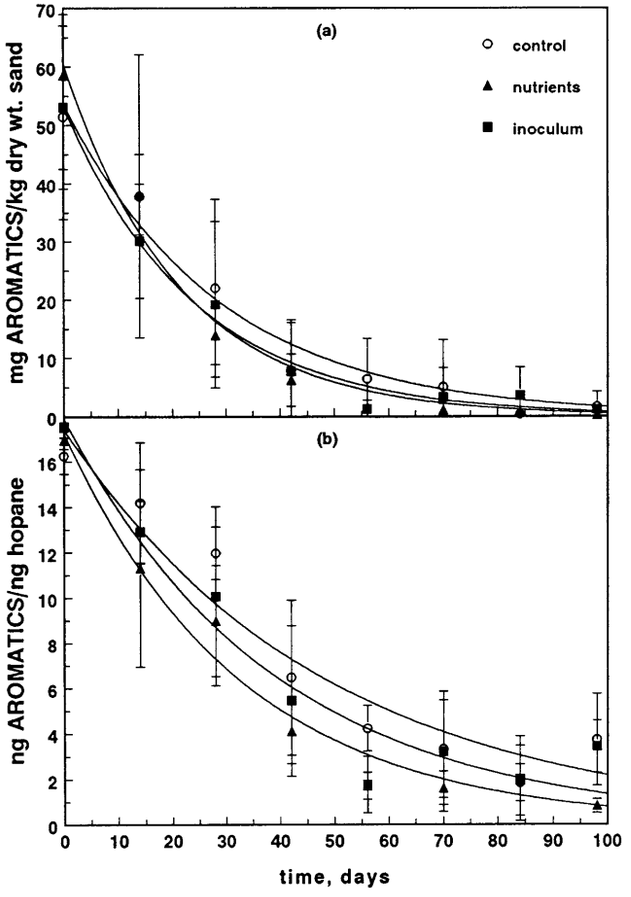

Figure 5 First-order decline in (a) mg total aromatics kg^{-1} dry weight sand and (b) ng total aromatics ng^{-1} hopane. Error bars represent ±1 standard deviation unit.

while significant treatment effects were observed at days 56 and 98 for the hopane-normalized data. These differences were due to the treated plots giving rise to lower means than the nonamended control plots, although at day 98, the nutrient plot means were significantly lower than both the control plots and the inoculum plots. No significant differences were detected between the hopane-normalized nutrient-treated plots and the inoculum-treated plots at any other sampling event. Results from the nonlinear regression analysis were the same as for the alkane data: rate coefficients for the hopane-normalized amended plots were significantly different from the unamended plots whereas no differences were detected among the sand-normalized treatments.

The most plausible explanation for the above observations is that the sand-normalized data were more variable than the hopane-normalized data, the high variability masking whatever differences might have existed among treatments. This can be seen graphically by plotting the coefficients of variation (CV) for the two normalization methods. The CVs were calculated by dividing the root mean square error (root MSE) at each sampling event by the means of all the data. The root MSE is the error that remains after removing the contributions of block and treatment effects to the variability of the response variable. The CV should vary randomly during the course of an experiment.

Figure 6a and b summarizes the CVs for the total alkanes and total aromatics, respectively, as a function of time for both the sand-normalized and the hopane-normalized data. The CV for the hopane-normalized data was relatively stable; however, the CV for the sand-normalized data increased systematically with time, suggesting that at each sampling event the variance and means were not independent, which indicates that the data do not follow a normal distribution. Thus, normalizing analytes to hopane produces not only field data that better satisfy the statistical assumptions necessary to use in the ANOVA but also lower variability that facilitates detection of treatment differences.

The first-order rate coefficients for the two sets of data are summarized in Table 1. These rate coefficients were computed by nonlinear regression analysis of all the data (ie 5 replicates/treatment × 8 sampling events). In all cases, the rate coefficients for the hopane-normalized data were lower than for the sand-normalized data because the latter include losses of hydrocarbons due to both physical and biodegradative processes, whereas the hopane-normalized losses were assumed to be due only to biodegradation. Note also the higher first-order rates for the treated plots compared to the control plots.

Individual analytes

A more graphic and definitive demonstration of the effect of hopane normalization on oil constituent biodegradation occurs when indivudal analytes representing the various analyzable fractions of crude oil are plotted as a function of hopane at separate sampling times. Figures 7–9 were constructed to illustrate this relationship.

Figure 7a, b, and c depicts phytane and *n*-C_{35} plotted as a function of hopane at three different sampling events (days 0, 56, and 98, respectively). These analytes were

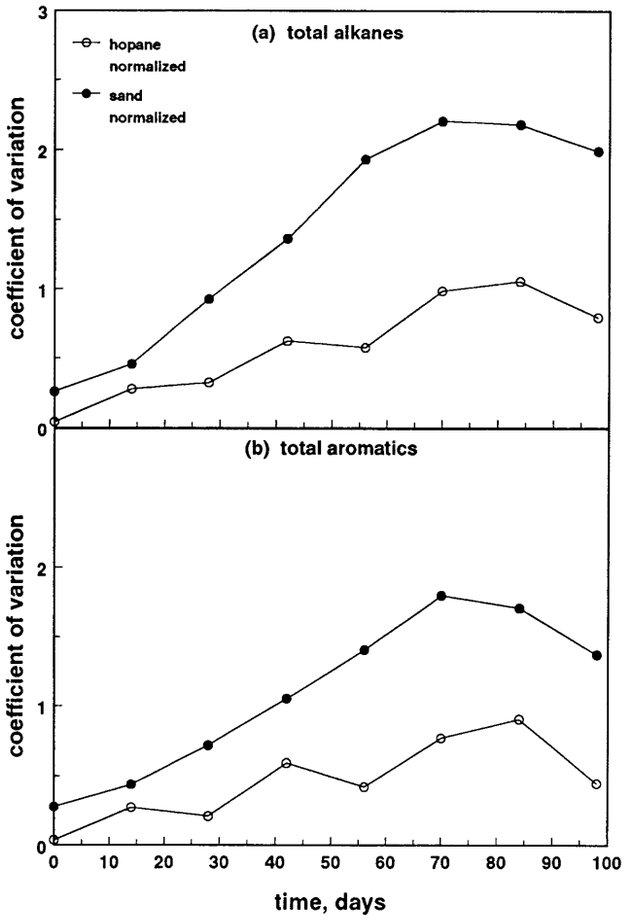

Figure 6 Change in coefficient of variation as a function of time for (a) total alkanes and (b) total aromatics.

Table 1 First-order rate coefficients (day^{-1}) for the sand-normalized and hopane-normalized data

Treatment	Alkanes		Aromatics	
	Sand	Hopane	Sand	Hopane
Control	–0.038	–0.026	–0.035	–0.021
Nutrients	–0.060	–0.056	–0.047	–0.031
Inoculum	–0.061	–0.045	–0.042	–0.026

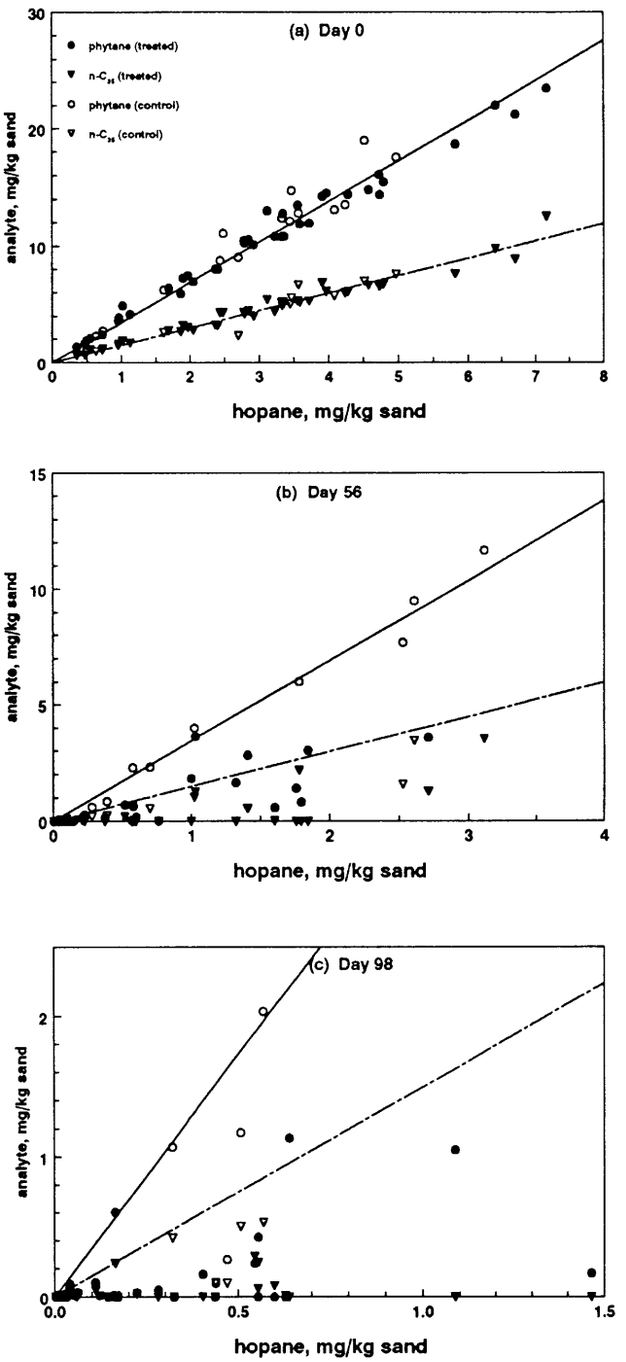

Figure 7 Correlation between phytane and *n*-C_{35} with hopane at (a) day 0, (b) day 56, and (c) day 98. The regression lines in (b) and (c) are repeated from (a) to give proper perspective to the changes that occurred with time.

selected because they biodegraded more slowly than the lower molecular weight normal alkanes. The figures depict both the physical variation in undegraded analytes as the hopane concentration varied due to physical effects of washout as well as the biodegradative losses that occurred over time. Open symbols represent the data from the unamended control plots, while closed symbols are the data from the nutrient- and inoculum-treated plots. At day 0 (Figure 7a), not much biodegradation had occurred, so all the measured data fit well on the linear regression curve. Hopane ranged in concentration from approximately 0.3 to over 7 mg kg^{-1} sand. At day 56 (Figure 7b), with the day-0 linear regression line repeated from Figure 7a, some of the data clearly diverged from the day-0 best fit line. Most of the data that still fit the day-0 relationship were from the control plots, while much of the data from plots that received nutrients and/or inoculum were clearly below the day-0 best fit line. Note that the maximum hopane concentration had dropped to less than 4 mg kg^{-1} sand. At day 98 (Figure 7c), most of the data fell below the day-0 best fit line, approaching the *x*-axis (undetectable concentrations).

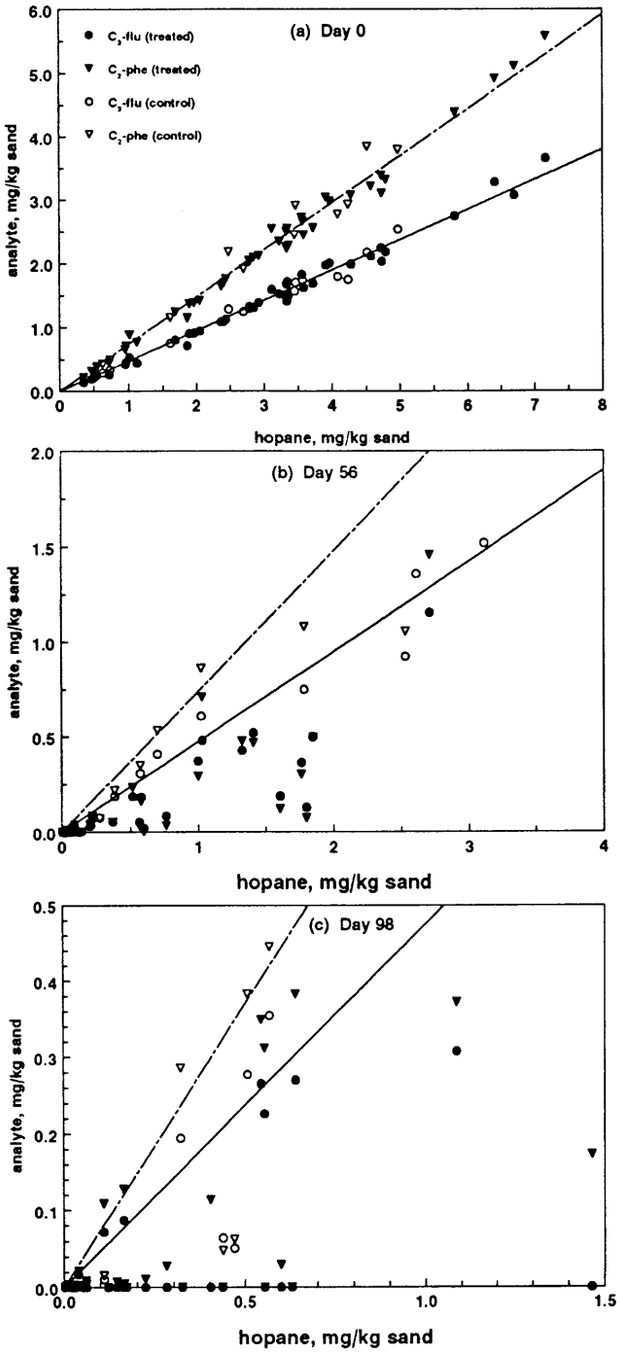

Figure 8 Correlation between C_3-fluorene and C_2-phenanthrene with hopane at (a) day 0, (b) day 56, and (c) day 98. The regression lines in (b) and (c) are repeated from (a) to give proper perspective to the changes that occurred with time.

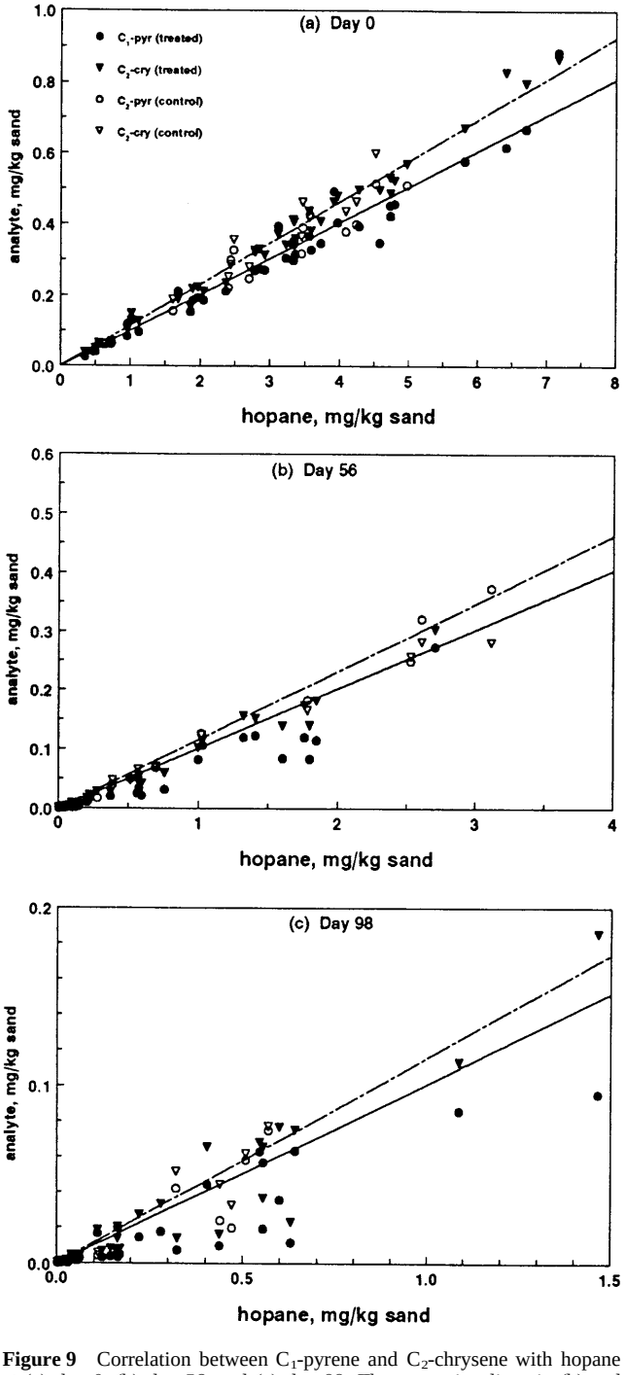

Figure 9 Correlation between C_1-pyrene and C_2-chrysene with hopane at (a) day 0, (b) day 56, and (c) day 98. The regression lines in (b) and (c) are repeated from (a) to give proper perspective to the changes that occurred with time.

The phytane data that still appear on the day-0 regression line were mostly from the control plots. Most of the data for *n*-C_{35} were below the day-0 regression line, although those that were nearer were from the control plots. Note that the maximum hopane concentration was only 1.5 mg kg^{-1} sand, reflecting the approximate 75–90% physical washout that had occurred by the end of the experiment.

Figures 8 and 9 depict results for two alkylated three-ring PAHs (C_3-fluorene and C_2-phenanthrene) and two alkylated four-ring PAHs (C_1-pyrene and C_2-chrysene), respectively. The data for these compounds behaved similarly to the data for phytane and *n*-C_{35} from Figure 7. As time went on, more data diverged from the day-0 best fit line and approached the *x*-axis, signifying that biodegradation took place. The data that diverged most slowly were typically from the control plots.

Discussion

The significance of the bioremediation findings from this study and the first-order rate coefficients computed from nonlinear regressions of the individual analytes with time have been discussed elsewhere [23]. The purpose of this paper was to present evidence that normalization of petroleum components measured by GC/MS to the nonbiodegradable biomarker C_{30}-17α(H),21β(H)-hopane mitigates much of the variability encountered when conducting field studies of oil spill bioremediation. Even normalizing microbial MPN estimates to hopane might help explain subtle differences in the various types of degrading populations actively metabolizing the biodegradable fractions. In the latter case, it should be pointed out that dividing the population numbers by hopane only serves to associate the putatively-active oil degraders with the undegraded oil and as such does not necessarily result in more accurate reflections of population changes. The reason for this is simply that oil degraders actively metabolizing the hydrocarbons sorbed to the sand particles may not reflect the entire population of oil degraders present in the surrounding environment, such as those that exist naturally in the sediment and in the seawater. Data from this study suggest but do not prove that either alkane degraders might adhere to the oiled sand better than PAH degraders or that loss of the more readily degradable PAH compounds might cause a greater loss in the PAH-degrader numbers over time. More explicit, independent supporting evidence needs to be obtained to enable a more definitive interpretation.

Another advantage of monitoring hopane when conducting oil spill bioremediation field studies is that it can be used to measure beach substrate distribution. Figure 1 demonstrated that early in the investigation the oiled sand was redistributed by wave and tidal action closer to the upper intertidal zone. As time progressed, however, the trend disappeared (data not shown). The reasons included not only physical washout of the oil (despite the presence of containment booms) but also mixing and dilution of oiled sand with unoiled sand, especially during storm events, which occurred several times during the experimental period.

Use of hopane as a biomarker for assessing biodegradation in the field is relatively new. Perhaps the most prominent example of its successful use was provided by Bragg *et al* [4] and Butler *et al* [7] in the case of the *Exxon Valdez* spill in 1989 and 1990. Hopane was used as a biomarker in these studies because the *n*-C_{17}/pristane and *n*-C_{18}/phytane ratios were inadequate for that purpose due to the substantial biodegradation rates of the branched alkanes [19]. When evaluating bioremediation of refined oil products such as diesel fuel, heating oil, etc, which do not contain hopane, use of alkylated multi-ringed PAHs (eg C_3-chrysene) can be used, at least for a moderate period of time [8]. For prolonged time periods, diterpanes [17] can be used successfully as field biomarkers in diesel fuel for bioremediation assessment [G Douglas, Arthur D Little Co, Boston, MA, USA, personal communication].

In general, biomarkers relieve some of the variability that results from the uneven distribution of the spilled oil or the physical factors that cause loss and redistribution of oil, such as washout from tidal and wave action. Highly variable data force the investigator to incorporate numerous replicates in an experimental design to enable detection of treatment differences. For example, for hopane-normalized total alkanes, the observed difference between nutrient-treated plots and control plots at day 56 was 2.75 standard deviation units. This calculation was made by dividing the differences in means at day 56 by the root MSE. This difference was detectable with five replicates of each treatment. The same calculation for the sand-normalized data at day 56 resulted in only a 1.25 standard deviation unit difference. For differences in means to have been statistically significant at the same level of confidence, the requisite number of replicate plots per treatment would have had to increase to 14, which is almost three times as many plots as were needed for the hopane-normalized data. When future oil spill bioremediation studies are conducted, biomarker normalization of oil chemistry data will substantially mitigate error variability, thereby enabling much more useful information to be generated with fewer replicate plots and thus at substantially reduced cost. Without use of an 'internal standard' such as hopane, bioremediation assessment will always be a complex and formidable task.

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