Biodegradation of Polycyclic Aromatic Hydrocarbons (PAH) from crude oil in sandy-beach microcosms

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ABSTRACT

Though the lower *n*-alkanes are considered the most degradable components of crude oil, our experiments with microcosms simulating oiled beaches showed substantial depletion of fluorene, phenanthrene, dibenzothiophene, and other PAH in control treatments consisting of raw seawater cycled through the microcosms over a 30-day period. PAH was not detectable in pooled test system effluents. To resolve the issue of wash out versus degradation, we ran oiled-beach microcosms with sterile synthetic seawater. Triplicate treatments were: sterile control, 10 ppm of a rhamnolipid biosurfactant added to the seawater, biweekly inoculation of the microcosms with two marine bacteria that produce biosurfactants but degrade only *n*-alkanes. The systems inoculated with the alkane-degrading microbes showed depletion of the *n*-alkanes, but essentially all of the aromatic analytes were recoverable from the oiled sand. We recovered all of the analytes (PAH or alkanes) from the other two treatments. The results support that lower molecular weight PAH were substantially depleted through biodegradation by microorganisms indigenous to natural seawater under aerobic conditions. In contrast, the *n*-alkane components were not significantly depleted under the same conditions.

Introduction

Measuring the success of bioremediation of oil spills is based on several parameters, among them the degradation of polycyclic aromatic hydrocarbons (PAH) in the crude oil. Though the lower *n*-alkanes are generally considered the most biodegradable compound class within crude oils [1, 9, 13], other studies point to exceptional conditions in which PAH degrade preferentially to *n*-alkanes. Jones and co-workers [8] showed that the biodegradation of alkylaromatic hydrocarbons was preferential to that of *n*-alkanes in crude oil when oil-contaminated sediments were aerobically incubated. Preferential biodegradation of the aromatic hydrocarbons was also observed by Connan and co-workers [2, 3] in asphalts of the South Aquitaine Basin in which the *n*-alkane had not been completely biodegraded.

Our experiments using flow-through microcosms that model biodegradation of crude oil on sandy beaches (Fig. 1) had consistently shown substantial depletion of phenanthrene, fluorene and other PAH, even in the "untreated" active controls, in which raw seawater was cycled through the microcosms over a 30 day period. The greater depletion of PAH

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relative to that of the *n*-alkanes was not enhanced by the addition of nutrients (inorganic N and P).

Since the pattern of depletion also followed that of their relative solubility, we initially attributed the phenomenon to dissolution of PAH into the tidal seawater, which was collected in the Santa Rosa Sound, Gulf Breeze, Florida, USA (SRS). However, the missing PAH was not detectable by gas chromatography/mass spectrometry (GC/MS) in the seawater effluent pooled from each microcosm. Subsequent experiments, which limited the oil-degrading microbial population in the microcosms, showed less disappearance of PAH, in a pattern that correlated with the degree to which bacteria were excluded from the systems.

In similar experiments, using microcosms preliminary to an intentional oil spill experiment on a mud-flat beach in the United Kingdom, Swannell and coworkers [15] found the PAH fraction was depleted from oiled sediment cores. Laboratory microcosms containing sediments from the Stert Flats test site near Bristol were treated with nutrient addition and subjected to tidal cycling. The nutrient-treated cores showed moderate *n*-alkane depletion, but levels of all PAH analytes were below the detection limit of GC/MS. In contrast to our microcosm experience, addition of N and P to the Stert Flats sediments stimulated PAH biodegradation from the crude oil over that occurring without added nutrients.

Since the depletion of the PAH analytes in all of these experiments correlated well with their relative solubility, the question remained as to whether the PAH had been removed from the microcosms by the washing action of seawater in the flow-through microcosms, perhaps aided by biosurfactants produced by microorganisms indigenous to the seawater. And we were unsure that PAH in the large volumes of effluent collected from the microcosms could be adequately quantified by GC/MS.

Most-probable number counts of PAH-degrading microorganisms in the SRS seawater revealed a consistently low number of microbes competent to degrade PAH (circa, 10-20 per liter). However, these populations could be substantially enriched when the seawater containing PAH or crude oil and nutrients was incubated with shaking at 20 °C.

During a final microcosm experiment, we successfully excluded all microorganisms that were culturable on one-tenth-strength tryptic soy agar from the microcosm system. In this experiment, we substituted sterile, synthetic seawater for the natural seawater. We found no loss of PAH from oil in the control chambers subjected to 30 days of tidal cycling. Two other treatments consisted of 10 ppm of a purified biosurfactant in the influent water, and a biweekly inoculation of the oiled sand with alkane-degrading, biosurfactant-producing marine bacteria. In all treatments the PAH remained intact within the oil until the conclusion of the experiment.

Thus, we concluded that depletion of PAH from our microcosms, including the natural seawater control treatments, was consistent with the preferential biodegradation of PAH by microbes indigenous to the SRS seawater under the aerobic conditions of our microcosms. We found no evidence that PAH in these oiled microcosms was being removed by dissolution or atmospheric losses. It is likely that the more soluble PAH compounds are similarly preferentially degraded by indigenous microbes in nature wherever aerobic conditions prevail.

Methods

All experiments described herein were done with triplicate microcosm chambers for each treatment. Specific treatments for each experiment are described in the text. The microcosm test systems used in this work (Fig. 1) were developed to model environmental parameters that affect biodegradation of oil on sandy inter-tidal beaches [10, 11]. All experiments described were done in a humidity-controlled environmental chamber at 20 °C. After tidal cycling for 1 week to allow colonization by indigenous microbial populations, a weathered crude oil (ANS521[1]) was added (1.9 ml; approx. 1.7 g) to water surface within the inner beaker containing the sand at high tide. The weight of oil added to each chamber was recorded to the nearest 0.1 mg to allow for mass-balance determinations. After an additional two days of tidal cycles, treatments were initiated at "T0," and continued for 28 days.

Effectiveness was assessed by comparing the total oil residue weight and the percentage of target hydrocarbon analytes remaining in the treatment test chambers at the conclusion of the experiment. The GC/MS results are expressed as the percentage of the designated analyte relative to its concentration in undegraded ANS521, which was used as its own standard. Since each component is present at differing concentrations in the ANS521 oil, a serial dilution of ANS521 generates a standard curve containing each component at concentration ranges that exist in the sample. A quantity of ANS521 equal to that delivered to each test chamber was diluted to the final volume of the sample extract and used as the 100% calibration solution.

At the conclusion of each experiment the oil residue was extracted from the microcosms with methylene chloride and treated by alumina chromatography to remove polar and asphaltenic components. The aromatic- and saturates- fraction was analyzed by GC/MS for selected *n*-alkanes from the series C15 through C35; the isoprenoids pristane and phytane (and the pristane/phytane ratio); hopane (C30, 17α, 21β); a range of aromatics including polycyclics, heterocyclics and alkyl-substituted compounds as follows: C2-, C3-, and C4-naphthalenes; dibenzothiophene; C1-, C2- and C3- dibenzothiophenes; phenanthrene; C1-, C2-, and C3- phenanthrenes; naphthobenzothiophene; C1-naphthobenzothiophene; chrysene; C1- and C2- chrysenes.

Fig. 1 is a schematic diagram of the microcosm/test system used in this work. Each chamber had an exterior 600 ml glass beaker containing a 250 ml fluorocarbon beaker filled with sand to which oil was added. The white, washed quartz sand was treated in a muffle furnace (550 °C, 4 h) to remove organic contaminants. Each chamber contained 50 g of coarse-grained (#18) sand over which was layered 200 g of medium-grained sand (#45). Holes drilled through the bottom of the inner beaker allowed water to flow between the beakers during tidal exchanges; a fluorocarbon screen in the bottom prevented loss of sand. The test chambers were clamped on an orbital shaker rotated at 70 rpm to simulate a gentle wave action. Timed peristaltic pumps controlled the flow of seawater over the sandy

[1] The Alaskan North Slope crude oil was treated by the "521" process according to the Draft International Standard ISO/DIS 8708 [7] method: the crude is heated to 374°F under atmospheric pressure, the system is then cooled and placed under partial vacuum (20 mm Hg) for the final distillation to an atmospheric equivalent of 521°F; the distillate is discarded and the residue, designated ANS521, was supplied to us by NETAC [12].

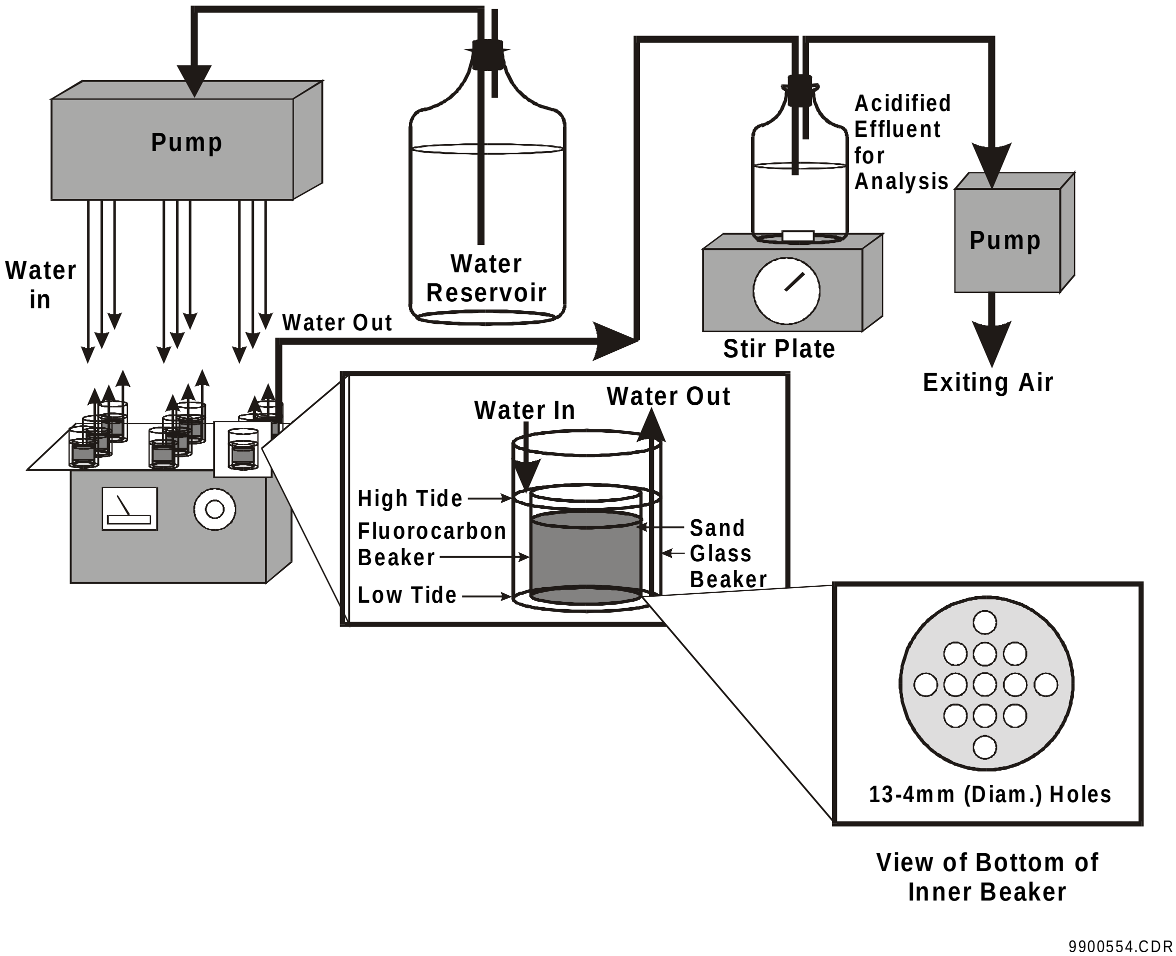

Fig. 1. Schematic diagram of microcosm/test system.

substratum effecting two tidal cycles of 200 ml per 24 hours. "Tidal" water was drained from the space between the glass and fluorocarbon beakers to ensure that it passed through the oiled sand as it exited the microcosm chambers. The effluent water was collected in a bottle and acidified to < pH 2.0 to stop microbial activity and stored for extraction later.

Surrogate bioremediation treatments

In this work, we employed two surrogate bioremediation treatments, one of which contained hydrocarbon-degrading marine bacteria in a nutrient solution delivered twice weekly to the surface of the oiled microcosms over the four week period of the experiments. The other treatment consisted of the nutrient solution without the microorganisms. Unless stated otherwise, the control microcosms were treated with unfiltered SRS seawater. The microbial inocula were prepared from pure cultures of the microorganisms described in Table 1, streaked to plates of one-tenth strength tryptic-soy agar containing 2% NaCl (TSA). After five days growth at 20 °C, the cells were aseptically washed from the surface of the TSA and suspended in a buffered mineral-salts medium containing no carbon substrate, but containing 400 ppm N as KNO_3 and 75 ppm P as $Na_5P_3O_{10}$. The optical density at 600 nm was adjusted to 1.0 (approx. 10^8 CFU/ml) and 5 ml applied to the microcosm surface. The nutrient-only treatment consisted of 5 ml of the same mineral salts plus nutrients solution without the microorganisms.

Table 1. Hydrocarbon-degrading microorganisms used in this work.

GOMEX2	**DF-1**	**AKPHEN6**
*Nocardia globerula**	*Rhodococcus fascians* *	*Pseudomonas saccharophila* *
hydrophilic cells	hydrophobic cells	hydrophilic cells
alkane-degrader	alkane-degrader	aromatic-degrader
emulsifies crude oil	emulsifies crude oil	does not emulsify oil

*Identified by fatty acid profiles MIDI™, Newark, DE, USA.

Results

The results of an experiment employing two surrogate bioremediation treatments, a microbial inoculum (alkane degrader, GOMEX2, and PAH degrader, AKPHEN6) and nutrients-only, applied twice weekly are shown in Table 2. It can be seen that the more

Table 2. Percentage of petroleum hydrocarbon analytes remaining in sandy beach microcosms after 28 days.

Targeted Analytes[1]	Seawater Control	Nutrients Only	Nutrients + Microbes
C16	56.0	23.9	15.7
C17	69.0	24.4	15.6
C18	75.2	24.9	16.3
C28	82.7	31.8	21.2
Pristane	76.3	68.4	54.3
Phytane	83.5	74.2	60.1
C2-Naphthalene	13.3	21.7	14.3
Fluorene	31.2	35.9	22.7
C1- Fluorene	64.2	62.1	56.2
C2- Fluorene	84.5	85.0	82.3
DBT	62.2	56.1	32.0
C1-DBT	84.0	78.8	66.1
C2-DBT	90.7	91.5	88.8
C3-DBT	92.9	95.5	93.3
Phenanthrene	61.8	51.1	28.2
C1- Phenanthrene	84.7	77.4	62.0
C2- Phenanthrene	93.4	91.2	87.6
C3- Phenanthrene	96.5	96.2	93.4
NBT	99.9	99.0	98.1
C1-NBT	100.2	99.2	96.5
Chrysene	92.4	100.2	88.5
C1-Chrysene	106.3	103.0	102.6
C2-Chrysene	102.8	104.5	102.9

[1]Target analytes included the *n*-alkane series of which the C16-28 are examples; DBT = dibenzothiophene; NBT = naphthobenzothiophene; C1, C2, C3 indicate the number of alkyl substitutions to polycyclic aromatic hydrocarbons.

soluble PAH were substantially depleted from the oil in the raw seawater control microcosms. The addition of the microbial inoculum was associated with somewhat greater depletion of PAH, however, the nutrients-only treatment did not enhance depletion of the PAH over that occurring in the control. Thus, the question arose as to whether the PAH were being biodegraded or physically lost from the system. When we extracted total effluents from several microcosms in which the PAH showed substantial depletion, we could detect no PAH in those effluents (data not shown).

In subsequent experiments we made attempts to exclude indigenous PAH-degrading microorganisms from the test systems (results in Table 3). We autoclaved the influent seawater used in the microcosms and took care to shield the microcosm chambers from ambient air, and we monitored culturable microbes (plated to TSA) in the effluent seawater. Sterile Control #1 showed culturable bacteria in the effluent within two weeks of setting up the experiment. Colonies on TSA from the effluent were sprayed with a diethyl ether solution of phenanthrene, and many developed cleared zones in the phenanthrene haze within seven to ten days, indicating degradation or solubilization of this PAH. The effluent from Sterile Control #2 remained free of culturable microorganisms for most of the four-week period. Other treatments were a non-sterile seawater control, and a non-sterile seawater treatment that was inoculated as described in Methods with N and P nutrients.

It can be seen that the Sterile Control #2, in which we effected a more stringent microbial control, suffered less depletion of the PAH from the residual oil. Whereas the nutrient addition did not enhance PAH depletion over that of the non-sterile control, nutrients did substantially enhance the degradation of alkanes from the oil. An exhaustive extraction and analysis of several of the effluents from microcosms that showed substantial depletion of PAH failed to recover more than trace amounts of any of the missing PAH analytes (data not shown).

In an effort to control the aseptic conditions of a microcosm experiment, we resorted to using autoclaved synthetic seawater (GP2) [14]. To each microcosm chamber we applied cheesecloth-covered cotton batting about 3 cm thick through which the hoses that conducted seawater into and out of the microcosm passed, and all contact with the microcosms used aseptic precautions. By these means, we maintained microbiological control of the microcosms over the entire test period such that no culturable microorganisms were found in the effluent. The experiment included triplicates of: (1) a sterile control, consisting solely of the GP2 influent water; (2) GP2 containing 10 ppm of the rhamnolipid biosurfactant EM; and (3) systems that were inoculated twice weekly with the alkane-degrading bacteria GOMEX2 and DF1 (Table 1) that also emulsify crude oil. The biosurfactant EM, a rhamnolipid surfactant produced by *Pseudomonas aeruginosa* (a gift from Santi Banerjee of Petrogen Inc. Arlington Heights, IL, USA) lowered the surface tension of GP2 to around 45 dynes/cm.

In this experiment we found that the PAH were not depleted by any of the three treatments (Table 4). Although microcosms inoculated with the alkane-degrading microbes showed marked depletion of n-alkanes and an 8% loss of oil residue weight, the microbes did not apparently produce biosurfactants that resulted in dispersion or dissolution of the PAH fraction. Moreover, the biosurfactant EM, (supplied at a concentration that reduced

Table 3. Percentage of petroleum hydrocarbon analytes remaining in sandy beach microcosms after 28 days.[1]

Targeted Analytes	Sterile Control #1	Sterile Control #2	Non-Sterile Control	Nutrient Non-Sterile
C16	78.3	84.4	63.1	31.5
C17	82.3	84.5	72.0	32.7
C18	83.5	84.2	76.1	33.1
C28	80.6	83.3	80.1	33.7
Pristane	80.9	81.9	74.3	71.9
Phytane	87.0	86.7	81.0	77.9
C2-Naphthalenes	51.3	74.8	22.9	25.8
Fluorene	74.0	84.4	39.8	39.6
C1 Fluorene	86.9	89.5	65.5	64.3
C2-Fluorene	89.3	90.3	81.3	82.8
DBT	88.5	92.6	60.8	55.1
C1-DBT	92.2	94.0	80.1	75.1
C2-DBT	92.9	94.6	87.3	88.1
C3-DBT	94.6	95.3	90.7	92.7
Phenanthrene	88.4	91.7	56.1	50.7
C1- Phenanthrene	93.2	94.7	80.6	73.6
C2- Phenanthrene	92.7	93.7	88.1	86.5
C3- Phenanthrene	94.9	96.8	91.5	93.5
NBT	95.3	95.3	93.0	92.4
C1-NBT	94.5	95.2	92.3	94.7
Chrysene	98.3	100.1	93.6	99.1
C1-Chrysene	94.1	96.0	93.4	95.5
C2-Chrysene	90.2	94.51	88.4	99.8

[1]Abbreviations as in Table 2. Sterile Control #1 and Sterile Control #2 are results of independent experiments; the Non-Sterile Control and Nutrient treatment were run concurrently. All values are averages of triplicate test system results.

the surface tension of the GP2 washwater to below that of natural SRS water, likewise did not effect a depletion of PAH from the microcosm chambers subjected to that treatment.

Discussion

The question we addressed was whether the depletion of PAH from oil in untreated controls in our beach microcosms was due to preferential biodegradation or washout of the PAH from the microcosms. We did a series of experiments to distinguish between these two potential mechanisms. Because PAH are more soluble than the alkanes, one might expect that a reduction in surface tension, effected by naturally occurring biosurfactants in the influent wash water, might provide a mechanism of dissolution of the PAH in water. Alternatively, PAH may have been washed out of the microcosms along with microparticles of oil in which they were dissolved. However, none of the missing PAH were analytically detectable in the microcosm effluent.

Our attempts to control the PAH-degrading microbial population within the microcosms demonstrated that the phenomenon correlated with the degree of microbial presence in the influent seawater. The addition of nitrogen and phosphorus nutrients to raw seawater

Table 4. Percentage of petroleum hydrocarbon analytes remaining in sandy beach microcosms after 28 days.[1]

Targeted Analytes	Sterile Control	EM Biosurfactant	Alkane Degraders
C16	96.0	95.2	15.4
C17	94.4	94.4	15.5
C18	93.4	92.7	15.2
C28	89.1	87.4	20.8
Pristane	93.2	92.4	60.8
Total Oil	98.4	99.4	92.1
C2 Naphthalenes	100.4	95.5	99.2
Fluorene	97.0	93.4	93.8
C1 Fluorene	94.7	94.5	92.9
C2 Fluorene	96.4	95.3	93.3
DBT	95.8	93.8	94.1
C1 DBT	95.4	95.8	93.7
C2 DBT	94.6	95.4	94.1
C3 DBT	92.4	95.6	94.1
Phenanthrene	96.6	95.6	94.5
C1-Phenanthrene	96.8	97.2	95.3
C2- Phenanthrene	94.3	95.7	93.9
C3- Phenanthrene	93.4	95.1	95.4
NBT	98.0	98.1	95.3
C1-NBT	93.9	97.4	93.9
Chrysene	93.4	93.1	89.5
C1-Chrysene	93.0	95.8	93.6
C2-Chrysene	94.6	100.8	92.1

[1]Abbreviations as in Table 2. All values are averages of triplicate test system results.

stimulated the indigenous alkane-degrading microbes, with a resultant substantial depletion of the *n*-alkane fraction, but did not substantially increase the degradation of PAH. We were unable to detect PAH or any other recognizable oil component in any of the selected effluents.

Because surfactants enhance solubilization and dissolution of PAH, thereby increasing the effective concentration of such compounds in an aqueous phase [4, 16], we investigated the influence of a purified biosurfactant and that of biosurfactants produced *in situ* in response to oil by introduced microorganisms. PAH-degrading microorganisms were excluded by using sterile artificial seawater and physical barriers to contaminating microorganisms.

EM at 10 ppm in the artificial seawater reduced its surface tension to about 45 dynes/cm. Because the critical micelle concentration of our preparation of EM was about 20 ppm, the surfactant was probably unable to form micelles that might facilitate washout of PAH into the aqueous effluent. Surface tension of distilled water is 73 dynes/cm and that of natural SRS seawater is typically 69 dynes/cm. We were surprised that the treatment supplying the bacteria DF1 and GOMEX2, emulsify crude oil in liquid culture, did not decrease surface tension of the effluent seawater, even though the oil was depleted of alkanes but not of PAH, as expected.

Our GC/MS results indicate that essentially all of the aromatic analytes were recovered from the oil residues in the microcosms in all three treatments. Thus, in the absence of PAH-degrading microbes, the PAH-depletion phenomenon was not replicated, arguing for biodegradation rather than washout of that compound class.

To determine the potential of the SRS seawater to harbor PAH-degrading microorganisms we used a most-probable-number counting method in which saturating concentrations of several PAH (phenanthrene, dibenzothiophene, fluorene) were added to nutrient-supplemented fresh SRS seawater. Endpoints included turbidity and color change from degradation intermediates or from redox indicator dyes. Although we found low numbers of PAH degrading bacteria (ca. 10/l), these populations could be modestly increased by adding inorganic P and N to seawater containing PAH as the sole C-substrate (200 fold increase).

Thus, even in the relatively pristine SRS, a potential for PAH biodegradation exists. The PAH fraction of crude oils in microcosm experiments such as ours may be substantially degraded by introduction of competent bacterial populations that colonize the oil on the sand matrix, effecting an enrichment over the 30-day period of these experiments.

Others have reported preferential biodegradation of PAH over biodegradation of saturates under aerobic conditions by indigenous microorganisms [2, 3, 5, 6]. Without nutrient supplementation, Fedorak and Westlake [6] found that the aromatic fraction of Prudhoe Bay Crude oil was more readily biodegraded by indigenous microorganisms than was the saturate fraction. More recently Fayad and Overton [5] reported degradation patterns by indigenous microorganisms in laboratory aquarium experiments on Arabian Crude oil similar to those we observe with respect to the PAH and alkane fractions. Fayad and Overton [5] also reported that the addition of nutrients slowed PAH biodegradation but stimulated degradation of saturates. We also find that nutrients stimulated depletion of the alkane fraction by indigenous microorganisms (Table 2). However, in our systems PAH biodegradation by indigenous microorganisms was not substantially affected by addition of nutrients. The patterns of oil biodegradation in all of the above-cited work are intriguingly similar.

Our experiments with aseptic systems and biosurfactants lead us to conclude that under the conditions of these microcosm studies, PAH were biodegraded, rather than simply washed out of the system, even though no significant losses of alkanes were observed. The aerobic and nutrient-limited conditions prevailing in our natural seawater controls appeared to favor the indigenous microorganisms that degraded PAH over those degrading alkanes. We believe that the pattern of *n*-alkane and PAH biodegradation observed in these microcosms is likely typical of that occurring in spill scenarios on sands and loose sediments where aerobic and low-nutrient conditions prevail.

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