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Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactors

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SUMMARY

A laboratory screening protocol was designed and conducted to test the efficacy of eight commercial bacterial cultures and two non-bacterial products in enhancing the biodegradation of weathered Alaska North Slope crude oil in closed flasks. Three lines of evidence were used to support the decision to progress to field testing in Prince William Sound: rapid onset and high rate of oxygen uptake, substantial growth of oil degraders, and significant degradation of the aliphatic and aromatic hydrocarbon fractions of the weathered Alaska North Slope crude oil. A product had to enhance biodegradation greater than that achieved with excess mineral nutrients. Experiments were conducted in closed respirometer flasks and shake flasks, using seawater from Prince William Sound and weathered crude oil from a contaminated beach. Analysis of the data resulted in selection of two of the ten products for field testing. Both were bacterial products. Findings suggested that the indigenous Alaskan microorganisms were primarily responsible for the biodegradation in the closed flasks and respirometer vessels.

INTRODUCTION

Bacterial degradation of petroleum hydrocarbons has been known and recognized for decades. The subject has been reviewed comprehensively in the literature [1,3,5,7]. Vestal et al. [13] reported that, although oil degraders comprise approximately 1% of the total heterotrophic population in unpolluted waters, the oil degrader population increases to as high as 10% in response to a spill. In 1989, research conducted by the U.S. Environmental Protection Agency (EPA) in Prince William Sound demonstrated that microbial communities on the contaminated beaches were highly competent in their ability to degrade the Alaska North Slope crude that was spilled from the Exxon Valdez [9]. The purpose of the study was to determine if application of water soluble and oleophilic nutrients could enhance the natural biodegradation rate.

After the EPA study showed that bioremediation of oil-polluted beaches was enhanced by the addition of fertilizer, the question then arose whether further enhancement was possible with the addition of microbial inocula prepared from oil degrading populations not indigenous to Alaska. Seeding or bioaugmentation experiments have been conducted in previous studies with mixed results [7]. In a recent study, Dott et al. [4] compared fuel oil degradation rates of activated sludge microorganisms with nine different commercial bacterial cultures in separate laboratory flasks. They found that the rate and extent of *n*-alkane and total hydrocarbon degradation by the diverse populations in activated sludge were significantly higher than any of the highly adapted commercially available cultures. Most success with biodegradation enhancement by allochthonous microbial cultures has been achieved when chemostats or fermentors were used to control conditions or reduce competition from indigenous microflora [14].

In February 1990, EPA issued a public solicitation for proposals to the bioremediation industry to test the efficacy of commercial microbial products for enhancing degradation of weathered Alaskan crude oil. The Agency commissioned the National Environmental Technology Applications Corporation (NETAC), a non-profit organization dedicated to the commercialization of environmental technologies, to convene a panel of experts to review the proposals and choose those that offered the most promise for success in the field. Forty proposals were submitted, and 11 products were selected for the first phase of a two-tiered testing protocol (only ten were tested because one company did not participate). The laboratory testing consisted of electrolytic respirometers

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set up to measure oxygen uptake over time and shake flasks to measure oil degradation and microbial growth. If one or more products were found effective, the second tier would take place, consisting of small field plots on an actual contaminated beach in Prince William Sound in the summer of 1990. This paper discusses the first phase of testing, the laboratory batch flask and respirometric evaluations.

The objective of the laboratory protocol was to determine if commercial bioremediation products can enhance the biodegradation of weathered crude oil to a degree significantly better than that achievable by simple fertilizer application. Testing was conducted in a controlled and closed environment designed to give quick results under ideal conditions. It was not meant to simulate the open environment of the oiled beaches of Prince William Sound, where conditions are in a constant state of flux with respect to tidal cycles and washout, temperature variation, climatic changes, freshwater/saltwater interactions, etc. The organisms inside the respirometer vessels were in continuous contact with the oil, seawater, and nutrients that were added initially. The seawater was not replenished every 12 h, as is the case in nature. The test was a screening procedure designed to determine if there was sufficient enhancement by the commercial additives to justify proceeding to the next tier of testing. To proceed to the field phase, three lines of evidence were used for decision-making: rapid onset and high rate of oxygen uptake, substantial growth of oil degraders, and significant degradation of the aliphatic and aromatic hydrocarbon fractions of the weathered Alaska North Slope crude oil.

The ten companies participating in the laboratory testing phase were (in alphabetical order): Alpha Environmental, Bioversal, Elf Aquitaine, ERI-Microbe Masters, Imbach, Microlife Technics, Polybac, Sybron, Waste Microbes, and Woodward Clyde. Of the ten products tested, eight were microbial and two were nonmicrobial formulations (i.e., fertilizers and/or dispersants). The products were randomly coded A through J and are presented that way in this paper. EPA and the venders established an agreement not to reveal the identity of the coding assigned to the individual participants in publications derived from the testing.

MATERIALS AND METHODS

Electrolytic respirometry

The studies were conducted using four automated continuous oxygen-uptake measuring Voith Model B-12 Sapromats. The instrument consists of a temperature-controlled water bath containing measuring units and a recorder for digital indication and direct plotting of oxygen uptake curves. The measuring units comprise 12 reaction vessels each with a carbon dioxide absorber mounted inside, 12 oxygen generators each connected to its own reaction vessel by tubing, and 12 pressure indicators connected electronically to the reaction vessels. The measuring units are interconnected by tubing, forming an air-sealed system, so that the atmospheric pressure fluctuations do not adversely affect the results.

Depletion of oxygen by microbiological activity creates a vacuum, which is sensed by the pressure indicator. The oxygen generator is triggered to produce just enough oxygen to counterbalance the negative pressure. The electrical current used to generate the oxygen is measured by the digital recorder, and the data are converted directly into mg/l oxygen uptake. CO_2 produced by microbial activity is absorbed by soda lime. The oxygen generators of the individual measuring units are electrolytic cells that supply the required amount of oxygen by electrolytic decomposition of copper sulfate/sulfuric acid solution.

A recorder/plotter constructs an oxygen uptake curve as a function of time and displays it on a computer screen while digitally saving the data on disc. For frequent recording and storage of oxygen uptake data, the Sapromat B-12 recorders are interfaced to an IBM-AT personal computer via the Metrabyte interface system. A software package, Labtech Notebook version 2.8 (Laboratory Technologies Corp., Wilmington, MA) allows the collection of data at 15-min intervals.

Experimental design

All commercial products were tested in duplicate at the concentration recommended by the manufacturer. Each 500-ml respirometer flask was charged with the following materials in the order listed: 250 mg weathered crude oil, 250 ml seawater from Prince William Sound, and commercial product. Seawater was prepared as follows: 25 g of oiled rocks from a contaminated beach in Prince William Sound (PWS) was placed in a 4-l flask to which was added 2 l of PWS seawater. The gravel was from a beach on Snug Harbor, an isolated cove on the southeast tip of Knight Island, where a moderate layer of oil from the Exxon Valdez had washed ashore earlier that year. PWS seawater was collected offshore from Snug Harbor. The mixture was shaken on a rotary shaker at 200 rpm for approximately 30 min to wash off a microbial inoculum from the rocks. The flask contents were allowed to settle, and the supernatant fluid was mixed with more seawater for use in the respirometer vessels. Table 1 summarizes the experimental design showing all control and experimental flasks.

Flasks F_1 and F_2 represented simple inorganic

TABLE 1

Experimental design of respirometric study showing contents of reaction vessels

Reaction Vessel	Nutrients	Weathered Oil	Commercial Product	Seawater	No. of flasks
Test flasks:					
T_{Pn}[a]	+	+	+	+	20
$F_{1,2}$[b]	+	+	−	+	2
Subtotal test flasks					22
Control flasks:					
C_{Pn}[c]	+	−	+	+	20
$C_{F1,2}$	+	−	−	+	2
C_1-inoculum[d]	−	−	−	+	2
C_2-no nutrients[d]	−	+	−	+	2
Subtotal control flasks					26
Total flasks					48

[a] T_{Pn} = duplicate commercial product flasks (n = 10).
[b] $F_{1,2}$ = fertilizer flasks (mineral N and P nutrients).
[c] C_{Pn}, $C_{F1,2}$ = no-oil controls for products and fertilizer, respectively.
[d] C_1, C_2 = inoculum and no-nutrient controls.

fertilizer application and contained (mg/l final concentration): KH_2PO_4, 6.33; K_2HPO_4, 16.19; Na_2HPO_4, 24.86; NH_4Cl, 38.5; $MgSO_4 \cdot 7H_2O$, 45; $CaCl_2$, 55; $FeCl_3 \cdot 6H_2O$, 2.5. Additional trace elements were included (μg/l final concentration): $MnSO_4 \cdot H_2O$, 60.4; H_3BO_3, 114.4; $ZnSO_4 \cdot 7H_2O$, 85.6; and $(NH_4)_6MO_7O_{24}$, 69.4. The above formulation was identical to a minimal salts medium developed previously [8], except for the concentrations of NH_4Cl and the phosphate salts, which were reduced to simulate more closely the concentrations being used in the field. The pH of the medium was 7.2. Addition of products did not alter the pH with the exception of product H, which reduced it to pH 2.5.

Respirometer flasks were incubated at 15 °C in the dark and continuously stirred at 300 rpm by magnetic stirrers. The first set of control flasks (C_{Pn}, $C_{F1,2}$) represented background oxygen uptake of the product and seawater without oil. Results from these flasks were subtracted from the appropriate test flasks to obtain the net oxygen uptake on weathered oil. The inoculum control represented the endogenous oxygen uptake of the organisms from the washed beach material and seawater alone. The no-nutrient control represented the oxygen uptake of the organisms from washed beach material and seawater on weathered oil without any external source of nutrient addition (i.e., background nutrient levels from Prince William Sound).

Flask experiments

Shake flasks duplicating the respirometer flasks were used to assess quantitative changes in oil composition by chromatographic separation of the individual components. Although it was possible to remove samples from the respirometer flasks, it was more prudent to sacrifice the shake flasks containing proportionately higher levels of oil and commercial products, to facilitate sampling for and precision/accuracy of the analytical chemistry. Table 2 summarizes the shake flask experimental design.

The test flasks corresponded exactly to the 22 test flasks listed in Table 1 but with the following modifications: flask size, 250 ml; seawater, 100 ml; weathered oil and commercial products, ten times the final concentrations used in respirometer flasks; and mineral nutrients, same final concentration used in the respirometer flasks. The higher concentration of weathered oil was used to improve the final sensitivity of the chemical analyses.

In addition to the 22 test flasks, 18 supplemental flasks were set up. Nine of these contained pre-sterilized products in non-sterile seawater and oil, which determined whether the enhancement was due to the microorganisms or to the nutrients or metabolites in the product. The other nine flasks contained pre-sterilized seawater and oil inoculated with non-sterile products to evaluate the effect of competition from naturally occurring organisms (one of the ten products did not receive these pre-sterilized treatments). Sterilization of materials was accomplished by autoclaving at 121 °C for 15 min.

Sampling

There were three sampling events for analytical chemistry and microbiology: at days 0, 11, and 20. These

TABLE 2

Experimental design of he shake flask study showing contents of reaction vessels

Reaction Vessel	Nutrients	Weathered Oil	Commercial Product	Seawater	No. of flasks
Test flasks:					
T_{Pn}[c]	+	+	+	+	20[a]
S_{Pn}[d]	+	+	sterile	+	9[a]
$T_{Pn}S_n$[e]	+	sterile	+	sterile	9[a]
$F_{1,2}$[f]	+	+	−	+	2[a]
Subtotal test flasks					40[a]
No. flasks sacrificed at days 11 and 20					80[a]
Control flasks:					
C_{Pn}[g]	+	−	+	+	10[b]
C_{F1}[g]	+	−	−	+	1[b]
C_1-inoculum[h]	−	−	−	+	1[b]
C_2-no nutrients[h]	−	+	−	+	2[a]
Subtotal control flasks					14
Total flasks					94

[a] = microbiological and chemical analysis.
[b] = microbiological analysis only.
[c] T_{Pn} = duplicate commercial products (n = 10), non-sterile seawater/oil/product system.
[d] S_{Pn} = sterile products in non-sterile seawater/oil, non-duplicated.
[e] $T_{Pn}S_b$ = non-sterile products in sterile seawater/oil, non-duplicated.
[f] $F_{1,2}$ = fertilizer (mineral N and P nutrients) in non-sterile seawater/oil/product system.
[g] C_{Pn}, C_{F1} = no-oil controls for products and fertilizer, respectively.
[h] C_1, C_2 = inoculum and no-nutrient controls.

events were determined by the shape of the oxygen uptake curves from the respirometry experiments. Each shake flask was sacrificed at the indicated sampling time by mixing the contents with methylene chloride and performing the extraction on the entire mixture. Before sacrificing a flask, a small aliquot was removed for determination of microbial density changes.

Analytical chemistry

Quantification of the hydrocarbon target analyses was performed by gas chromatography using a flame ionization detector (GC/FID). Quantification of polynuclear aromatic hydrocarbons (PAHs) and alkylated PAH homologues was performed by gas chromatography/mass spectrometry (GC/MS) in the selected ion monitoring mode.

Sample extraction

Contents of the entire shake flask were treated with methylene chloride and extracted according to EPA SW 846 Method 3510 (separatory funnel method)[12]. The methylene chloride/oil mixture was added to a 250-ml separatory funnel and 1 ml of a 50 ppm hydrocarbon surrogate standard (*n*-decylcyclohexane) and 1 ml of 1 ppm PAH surrogate standards (naphthalene-d8, acenaphthene-d10, and chrysene-d12) were added. The extraction was performed three times. The three extracts were poured through an anhydrous Na_2SO_4 column, mixed together, and concentrated to 1 ml in a Kuderna-Danish concentrator.

Sample clean-up

A 60/200-mesh silica gel column was prepared by activating the silica gel overnight at 210 °C in hexane and placing the silica gel slurry into a 10 mm ID × 25 cm long column. After eluting with hexane, 1 to 2 cm of anhydrous Na_2SO_4 was placed on top of the silica gel. The 1 ml sample extract was transferred onto the column. The column was eluted with 15 ml hexane to remove the aliphatics and 45 ml of a 1 : 1 mixture of hexane : benzene to remove the PAHs.

GC/FID

The GC instrument was a Hewlett-Packard 5880A equipped with a 0.75 mm ID × 30 m long DB-5 column with direct injection in the splitless mode. The operating parameters were: injector port temperature, 250 °C; detector temperature, 350 °C; temperature program: initial temperature, 50 °C; hold time, 5 min; program rate,

7 °C/min; final temperature, 300 °C; final hold time, 35 min; total run time, 75 min or less; injection volume, 0.002 ml; carrier gas, He at 5 ml/min; make-up gas, He at 20 ml/min; detector, air at 240 ml/min; and H_2 at 50 ml/min.

Calibration curve

A five-point calibration curve was prepared using 10, 50, 100, 200, and 250 ppm concentrations of the following compounds in methylene chloride: pristane, phytane, *n*-C7 through *n*-C26, *n*-C28, *n*-C30, *n*-C32, *n*-C34, and *n*-C36. Added to each standard was 0.050 mg of *n*-decylcyclohexane surrogate standard and 0.050 mg α-androstane internal standard per 1 ml of the calibration standard.

Sample analysis

Prior to analysis 0.050 mg α-androstane internal standard was added to each 1 ml of sample.

GC/MS

The instrument used was a Hewlett-Packard 5970A gas chromatograph with an MSD detector equipped with a 0.25 mm ID × 30 m long DB-5 column with direct injection in the splitless mode. The operating parameters were: injector port temperature 270 °C; detector temperature 350 °C; temperature program: initial temperature 50 °C; hold time 5 min; program rate 8 °C/min; final temperature 300 °C; final hold time 30 min; total run time 50 min; scan start time 8 min; splitless valve time 0.8 min; injection volume 0.002 ml; selected ion monitoring (SIM) mode 100 ms dwell time.

Calibration curve

A six-point calibration curve was prepared using 50, 100, 500, 1000, 2500, and 5000 ppb concentrations of the 16 PAHs shown below. To those standards 1000 ng each of naphthalene-d8, acenaphthene-d10, and chrysene-d12 surrogate standards were added per 1 ml of the standard. In addition, 4000 ng of nitrobenzene-d5, anthracene-d10, and perylene-d12 were added per 1 ml of the standard. The list of target PAHs used in the GC/MS analysis included acenaphthene, acenaphthylene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, chrysene, dibenzo(a,h)anthracene, dibenzothiophene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, anthracene, and pyrene.

Sample analysis

Prior to analysis 4000 ng of the internal standard was added to 1 ml of sample.

Quality assurance

For the GC/FID analyses, the percentage of recovery for *n*-decylcyclohexane surrogate had to fall within 60 to 140% to be accepted. Matrix spikes and matrix spike duplicates were analyzed every 20 samples or within a sample set, whichever was more frequent. The spike criteria were as follows: 1) matrix spike percentage of recovery of C15, C20, and C28 had to be within 60 to 140% to be accepted; and 2) only one compound could be below its required minimum percentage of recovery.

For the GC/MS analyses, the percentage of recovery of surrogates had to be as follows: 1) naphthalene-d8, 14 to 108%; 2) acenaphthene-d10, 20 to 130%; and 3) chrysene-d12, 10 to 118%. Matrix spikes and matrix spike duplicates were analyzed every 20 samples or with every sample set, whichever was more frequent. Matrix spike percentage of recovery of acenaphthene and pyrene had to be within 20 to 150% for acceptance.

Nutrient analysis

The nitrogen species NH_3-N, NO_2^--N, and NO_3^--N were determined by U.S. EPA Methods [11]. The NH_3-N method was No. 350.1 and the NO_2^-N/NO_3^-N method was No. 353.1.

Microbiological testing

Growth of oil degraders was measured by spread plates on oil agar. The oil agar was prepared by sterilizing Bushnell-Haas salts mixture [2] with 1.5% (w/v) agar in distilled water. The salt content of the mixture was 20 g NaCl/l to approximate observed salinities in surface waters of Prince William Sound. The pH of the medium was adjusted to 7.2 prior to autoclaving. After sterilization the agar was cooled to 50 °C in a water bath. The molten agar was poured into sterile stainless steel Waring blender jars, and 10 ml/l of fresh Alaska North Slope crude was added. The contents were blended for 30 s at low speed and then 60 s at high speed to homogenize the mixture. The oil agar was then dispensed into sterile Petri plates and allowed to harden and dry overnight. Plates were inoculated by spreading with glass hockey sticks 0.1-ml aliquots of samples that had undergone decimal dilutions in Bushnell-Haas broth. Plates were incubated at 15 °C for 21 days prior to counting. All determinations were performed in triplicate. Controls consisted of seawater only, seawater plus oil, and seawater plus nutrients.

Growth of total heterotrophs on marine agar spread plates was also monitored, but results are not presented in this paper. Oil degraders, as represented by colony formation on the oil agar plates above, numbered approxi-

mately 1% of the total heterotrophic count consistently among all plates.

RESULTS

Nutrient levels in each product

Product flasks requiring nutrient addition, as specified by the product manufacturer, received the same level of mineral nutrients as the fertilizer flasks. The NH_3-N concentrations measured in each product flask at day 0 are summarized in Table 3.

Total alkane reduction

The percent reductions of the resolved aliphatic constituents of the weathered oil (n-C_{12} through n-C_{34} plus the isoprenoid hydrocarbons pristane and phytane) were computed at days 11 and 20 for each product flask to compare with the results from the mineral nutrient flasks. The data from the non-sterile seawater/oil/product flasks and the corresponding flasks containing either pre-sterilized products in non-sterile seawater and oil or pre-sterilized seawater/oil with non-sterile products at days 11 and 20 are summarized in Fig. 1. The bar labelled "00" refers to the control seawater/oil flasks containing no nutrients i.e., seawater with inoculum and oil and no further amendments. At day 11 (Fig. 1a), better degradation was observed in every case when the commercial products were first sterilized, suggesting that the indigenous Alaskan populations were performing most, if not all, of the biodegradation. In contrast, less degradation occurred in every case except product I when the seawater and oil were first sterilized. This suggests that, when left alone, the product organisms were less able to degrade the alkane fraction than the indigenous organisms. In the non-sterile seawater/oil/product treatments, enhancement was observed for products E and G compared to mineral nutrients, suggesting that the products exhibiting the enhancement were providing metabolites or some other form of nutritional benefit that was lacking in the mineral nutrient flask. By day 20 (Fig. 1b), all products except products F and I caught up, giving greater than 85% reduction in the total alkane levels in the flasks. However, most of the flasks containing oil and seawater that had been previously sterilized still lagged significantly behind the non-sterile seawater/oil/product systems.

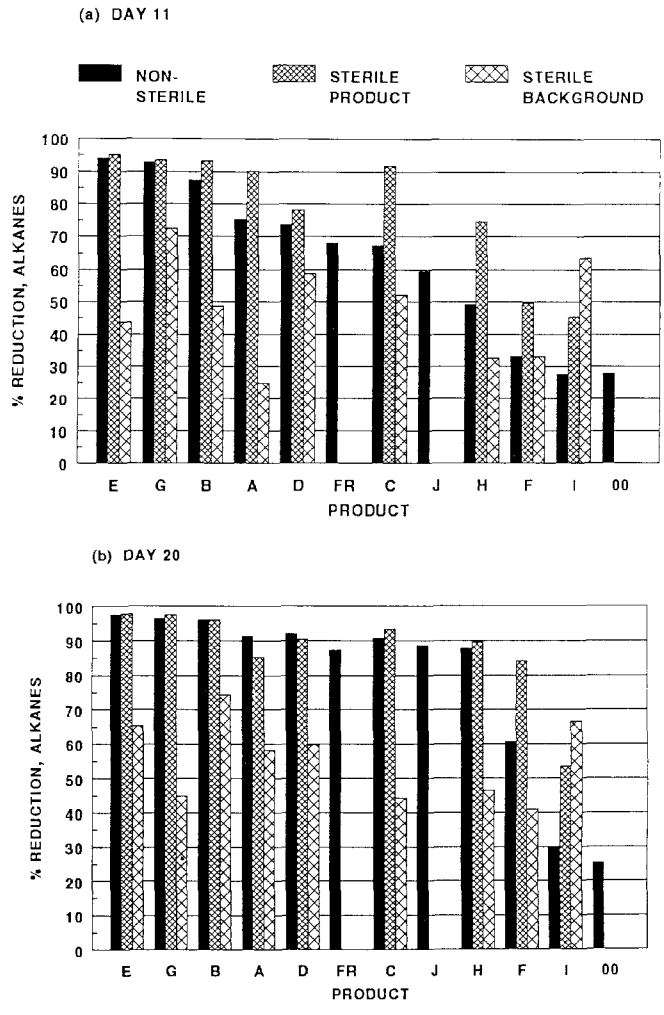

Fig. 1. Total alkane reduction in the product flasks. (a) day 11, (b) day 20.

TABLE 3

NH_3-N levels in each product flask at the start of the experiment

Product	NH_3-N mg/l	Nutrients added
A	8.0	Yes
B	2.1	No
C	1080.0	No
D	11.8	Yes
E	11.3	Yes
F	10.0	Yes
G	24.9	Yes
H	426.0	No
I	0.5	No
J	1.5	No
FR[a]	6.9	Yes

[a] FR = mineral fertilizer.

Statistical analysis of alkane degradation data

An overall analysis of variance was first performed to determine if there were significant differences in total alkane degradation among any of the treatments. After the F-test revealed the presence of significant differences with 10 (product) and 190 (error) degrees of freedom at the $P < 0.0001$ significance level, Tukey's Studentized

Range Test [10] was performed to compare paired results of products with mineral nutrient flasks. Table 4 summarizes the statistical differences observed. The products are arranged in descending order of significance. Only products E and G gave significantly higher removals ($P < 0.05$) than inorganic fertilizer after 11 days. Six of the other products gave results no different from mineral nutrients, while two actually gave significantly lower removals. The latter results suggest that the products may have been inhibitory to the biomass at the levels used in closed flasks.

Typical chromatographic profiles

Bar charts of typical 11-day chromatographic profiles of the individual alkane components for three of the products are shown in Fig. 2. Digitized representations of the data are shown rather than the chromatograms to facilitate quantitative viewing of changes in the individual aliphatic compounds. The three profiles shown were selected to represent products giving significantly better (product E), equivalent (product A), and lower (product I) alkane removals than mineral nutrients after 11 days. The solid bars in the figure are the non-sterile seawater/oil/product systems, i.e., as they would be used in the field. The shaded bars are the flasks containing non-sterile oil and seawater inoculated with pre-sterilized product, while the hatched bars are those containing pre-sterilized seawater and oil inoculated with non-sterile products. The no-nutrient controls are represented by solid lines with symbols, which are presented that way to facilitate comparisons.

In the case of product E, virtually all of the normal alkanes in both the non-sterile seawater/oil/product flasks and the flasks containing pre-sterilized product in non-sterile seawater and oil were at or below detectable limits. Even the branched chain aliphatics were substantially reduced. In the flasks containing pre-sterilized seawater and oil with non-sterile product, the normal alkanes C15 and above were unchanged, while some decreases occurred in the lower molecular weight fractions. In the case of product A, the concentrations of normal alkanes in the non-sterile seawater/oil/product flasks were higher than the corresponding product E flasks. Levels of the branched chain alkanes pristane and phytane were unchanged. In the flasks containing pre-sterilized product in non-sterile seawater and oil, most of the normal alkanes were nearly undetectable while pristane and phytane remained close to the starting levels. In the pre-sterilized seawater and oil flasks containing non-sterile products, levels of all constituents were similar to the corresponding product E flasks. Finally, in the case of product I, most of the alkanes were unchanged in all flasks, regardless of the nature of the pre-sterilization conditions. The exceptions were the normal alkanes C16 through C22, which averaged approximately 53% lower than starting levels.

The reason why the ordinate of the bottom third of Fig. 2 is twice that of the other two is that the *n*-alkanes shorter than C15 were substantially higher at day 11 than any other product or control flask. Inspection of the

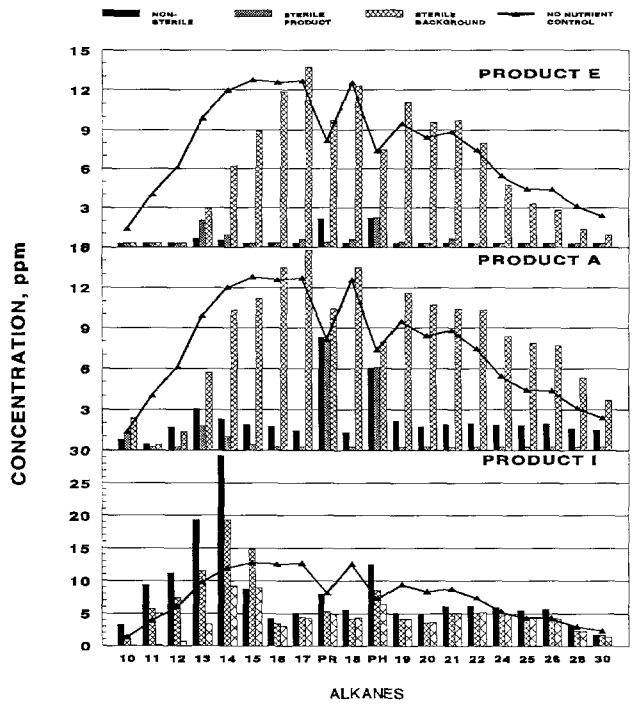

Fig. 2. Bar chart representation of the chromatographic profile of the alkane data at day 11 for products E, A, and I.

TABLE 4

Tukey's Studentized Range Test for detecting differences in mean percent removal of alkanes by products in 11 days

Product	% Removal	Significantly different from inorganic nutrients[a]
E	94.5	Yes
G	93.6	Yes
B	87.9	No
A	75.9	No
D	74.2	No
FR	68.4	–
C	67.8	No
J	59.9	No
H	49.5	No
F	33.3	Yes
I	27.9	Yes

[a] Minimum detection difference = 21.3% at 5% significance level.

digitized chromatographic data revealed that product I had three to four times the concentrations of those alkanes at day 0 than any other product flask. Also, product I had a distinct kerosene-like odor in its dry state. These observations suggest that the manufacturer of product I uses alkanes as a carrier for its product, presumably to assure that its culture is well induced prior to inoculation.

Total PAH reduction

A summary of the total PAH reduction data at days 11 and 20 is presented in Fig. 3. Differences are less clear among the products, although products C, F, H, and I gave total reductions considerably less than mineral nutrients. By day 20, PAH reduction by product C was somewhat closer to the others, while products H, F, and I lagged substantially. Excellent removal of aromatics was observed in all other flasks.

Typical mass spectral profiles

Bar charts of typical day-11 digitized mass spectral profiles of the individual PAHs for the same three products as in Fig. 2 are shown in Fig. 4. Again, digitized representations of the mass spectral chromatographic peaks are shown to facilitate viewing of individual changes in the PAHs. Mass spectral analyses of the fractionated extracts from the pre-sterilized product in non-sterile seawater/oil flasks or pre-sterilized seawater/oil flasks containing non-sterile product were not performed. Shown on the figure are day 0 and day 11 PAH levels for each of the non-sterile seawater/oil/product flasks. Differences in the PAH levels among the three product flasks are clearly evident. Higher concentrations of PAHs, especially in the substituted naphthalene group, occurred in the product A flasks and even higher in the

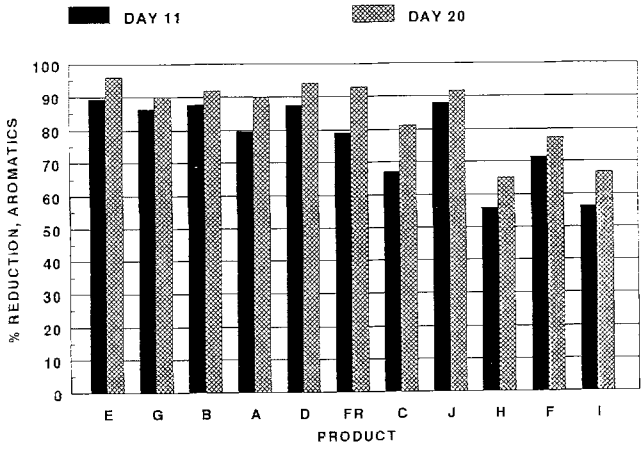

Fig. 3. Total reduction of aromatics in the product flasks at days 11 and 20.

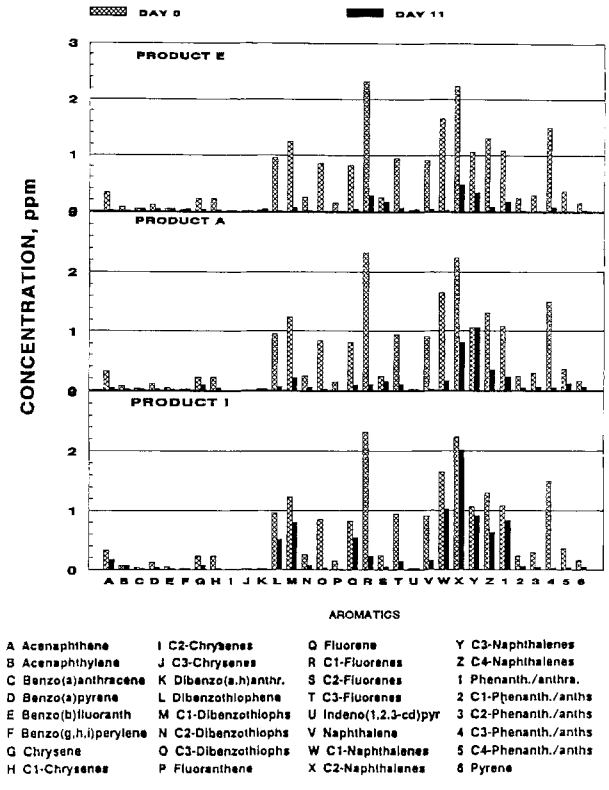

Fig. 4. Bar chart representation of the GC/MS profile of the aromatic data at day 11 for products E, A, and I.

product I flasks. The dibenzothiophene group was also more resistant to degradation in the product I flasks than the other two.

Respirometric results

Net oxygen uptake curves (oxygen uptake in product flasks with oil minus oxygen uptake in flasks without oil) for all ten products (curves with symbols) compared with the curve for mineral nutrients (curve with no symbols) are summarized in Fig. 5a,b; the two products giving significantly higher alkane degradation, E and G, also exhibited higher net oxygen consumption than mineral nutrients. The final plateau in total oxygen uptake was slightly less than 500 mg/l for both products E and G compared with about 340 mg/l for the mineral nutrient flasks. The acclimation lag periods for product E and G were approximately 2 and 4 days, respectively, compared with 5 days for mineral nutrients. Product A gave the highest maximum net uptake (630 mg/l compared with 340 for mineral nutrients) but the lag period was almost 10 days. Products B and D exhibited O_2 uptake characteristics no different from the nutrient control.

Products J and C gave higher overall net O_2 consumption than mineral nutrients (Fig. 5b), although product F exceeded the control after 27 days. The lag period

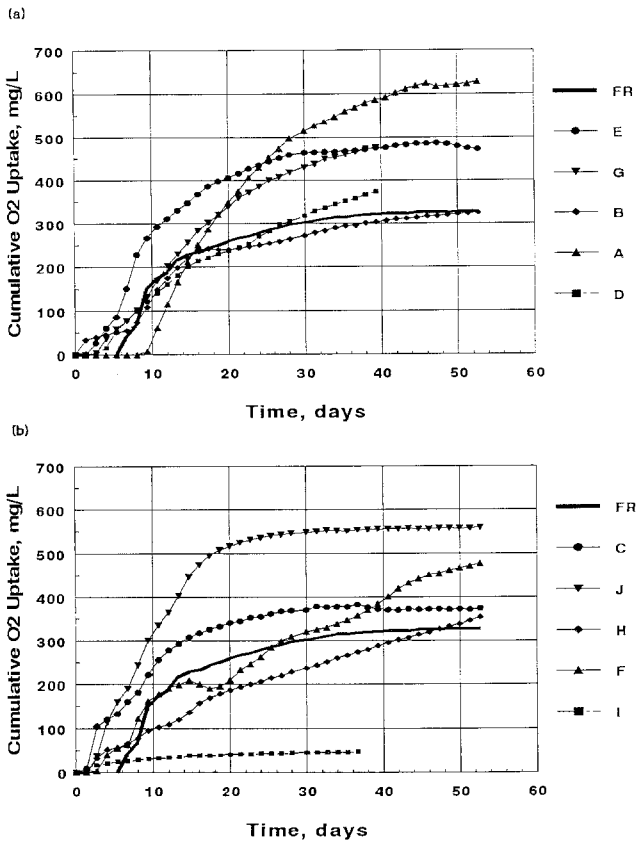

Fig. 5. Net oxygen uptake curves for products and mineral nutrients: (a) products E, G, B, A, and D; (b) products C, H, H, F, and I.

for products J and C was only 1 day. The shape of the product F curve was multi-phasic, suggesting the organisms were consuming different substrates at different rates and at different times (diauxie). Little net oxygen consumption was observed with product I.

Microbiological results

Virtually all changes in oil degrader densities occurred by day 11. The populations levelled off in all flasks thereafter. Consequently, the growth of oil degraders has been summarized for days 0 and 11 only, and the results from all flasks, including the pre-sterilized treatments, are presented in Figs. 6 and 7. Figure 6 depicts the yield of oil degrader for all products at day 11, and Fig. 7 shows the $\log_{10}$ change in oil degraders in 11 days. Data absent from the figures were caused by missed dilutions.

Products E and G, which gave the best alkane degradation of all the products (Table 4 and Fig. 1) and displayed net oxygen uptake characteristics superior to most (Fig. 5a), also exhibited excellent yield and growth of oil degraders in 11 days. Products C, J, and F yielded high levels of oil degraders and good oxygen uptake

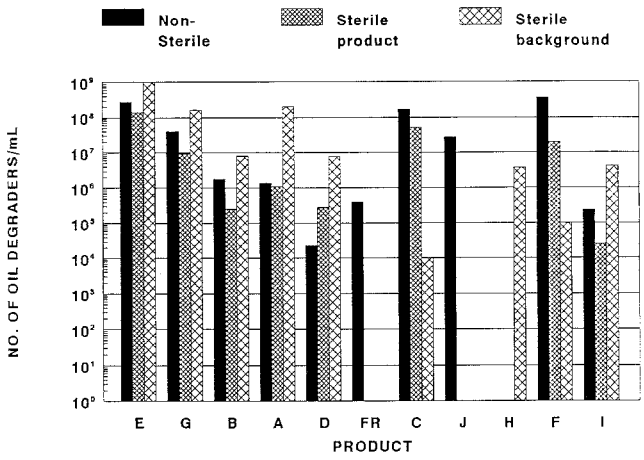

Fig. 6. Yield of oil degraders for all products at day 11.

curves, but alkane degradation was no better than by populations growing in simple mineral nutrients. Oil degrader populations actually declined in the product B and A flasks, and the increase in oil degraders in the flasks containing products D and I was minimal.

Flasks containing pre-sterilized seawater and oil inoculated with non-sterile products (large-hatched bars in Fig. 6) gave higher oil degrader counts in all cases except products C and F. The organisms in the latter two products were less able to grow on the weathered crude oil than the indigenous populations. If products C and F were first sterilized, however, the indigenous populations grew better than either the non-sterile seawater/oil/product systems or the pre-sterilized seawater/oil flasks. This suggests that either there was an antagonism between the indigenous populations and product organisms or the products contained one or more inhibitors that prevented indigenous bacteria from achieving their ultimate biodegradative potential.

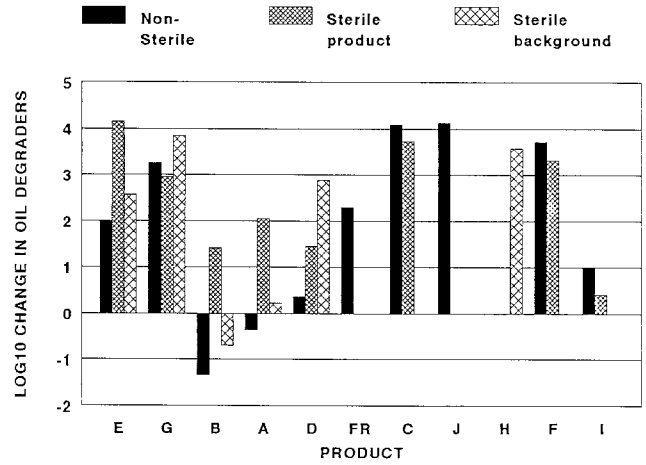

Fig. 7. Increase in oil degraders for all products in 11 days.

Flasks containing products B and A also gave better growth when the products were first sterilized (Fig. 7, small-hatched bars), again suggesting either a microbial antagonism or the presence of a heat-labile inhibitory substance. Product D gave the best growth when the indigenous populations were first sterilized. Products E, G, and J gave good final yields and density increases, but product J did not perform well with respect to oil constituent degradation.

DISCUSSION

The objective was to determine if weathered crude oil could be degraded faster when natural microbial populations were supplemented with exogenous oil degraders and excess nutrients than when the natural populations were supplemented only with excess nutrients. Oil degradation chemistry, oxygen uptake in respirometer flasks, and microbial density changes were used to decide which product(s) would proceed to field testing.

Of the ten products tested, the two that provided the most consistent results in all three tests were products E and G. Each gave higher oxygen uptake with more rapid onset, greater growth of oil degraders, and superior alkane degradation than mineral nutrients. Two of the products, C and J, showed good growth of oil degrader populations and gave excellent net oxygen uptake curves but were no better in oil degradation than indigenous populations supplied with simple mineral nutrients. Product F yielded the highest oil degrading population of all, yet its oxygen uptake curve was no better than the mineral nutrient curve until after day 27, and alkane degradation was relatively poor. Product A gave the best overall net oxygen consumption, but the change in oil degraders and the relative alkane degradative capability at day 11 were unsubstantial, perhaps because of the extended acclimation lag period as noted in the oxygen uptake curve for product A. Flasks containing products B, D, H, and I also produced only minor changes in oil degrading populations and unenhanced oil degradative capability.

The pre-sterilized product and pre-sterilized seawater/oil trials revealed that the indigenous Alaskan oil-degrading populations were performing most, if not all, of the biodegradative activity. The organisms present in products E and G did not appear to contribute significantly to such activity. This suggests that a co-metabolite or a nutrient or some other factor exists in these two products that stimulates indigenous microorganisms to degrade crude oil constituents at rates faster than with simple nutrient addition. Further work needs to be done to define the enhancement factor(s) in these products.

It is possible that autoclaving the oil might have altered its bioavailability or nature. It is unlikely, however, that this was the case when it is considered that the oil was highly weathered to begin with and that further "weathering" by autoclaving would have resulted in volatilization of the lighter ends of the crude oil material. Since most of the lighter ends were substantially gone at day 0, it is unlikely that the nature or availability of the oil was substantially altered by the heat treatment.

Correlations have not as yet been made between weathered crude oil degradation and oxygen uptake, nor have carbon balances been performed. Work is planned to measure carbonaceous metabolic end products, CO_2 production, the extent of biodegradation of selected crude oil fractions, and total biomass yield, and then to correlate this information with oxygen consumption data. If such correlations can be established, then use of oxygen consumption data for estimating biodegradation efficacy as part of a screening protocol will be made possible. The respirometric technique requires much less effort than conventional shake flask studies because data gathering is automated and computerized, and it is not necessary to collect samples manually during the course of an experiment. All that is required, assuming the proper correlations have been established, is the careful measurement of initial substrate (oil) and biomass levels followed by the measurements of the residual soluble products, biomass, and remaining undegraded substrate levels at the plateau of the uptake curve [6]. From the analysis of this information, treatment decisions can be facilitated.

CONCLUSIONS

Results from all three lines of evidence, i.e., respirometry, microbiology, and oil chemistry, supported the decision to field test only products E and G. It appears from the available evidence that the indigenous Alaskan microorganisms were primarily responsible for the biodegradation in the closed flasks and respirometer vessels, and that any enhancement provided by products E and G might have been due simply to metabolites, nutrients, or co-substrates present fortuitously in the products. Questions remain unanswered, and further research is being planned to increase our knowledge base regarding oil spill bioremediation enhancement using commercial inocula.

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