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Screening of commercial inocula for efficacy in stimulating oil biodegradation in closed laboratory system*

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Abstract

In February, 1990, the U.S. Environmental Protection Agency issued a public solicitation for proposals to the bioremediation industry on testing 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 corporation 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 were selected for the first phase of a two-tiered testing protocol (only 10 were tested because one company did not participate). The laboratory testing consisted of electrolytic respirometers 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 respirometric evaluations.

Introduction

Bacterial degradation of petroleum hydrocarbons has been known and recognized for decades. The subject has been reviewed comprehensively in the literature [1–3]; the most recent one has appeared last year [4]. Vestal et al.

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[5] 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 in Prince William Sound, Alaska demonstrated that microbial communities on the contaminated beaches were highly competent in their ability to degrade the Prudhoe Bay crude that was spilled from the Exxon Valdez [6]. The purpose of the latter 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 experiments have been done in previous studies with mixed results [4]. In a recent study, Dott et al. [7] compared nine commercial mixed bacterial cultures to activated sludge microorganisms for their ability to degrade fuel oil in laboratory flasks. They found that fuel oil degradation by the naturally occurring bacteria in activated sludge did not depend on nor was it enhanced by the application of highly adapted commercially available cultures. Most success has been achieved when chemostats or fermenters are used to control conditions or reduce competition from indigenous microflora [8].

In February, 1990, the U.S. Environmental Protection Agency issued a public solicitation for proposals from the bioremediation industry on testing 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 corporation 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 were selected for the first phase of a two-tiered testing protocol (only 10 were tested because one company did not participate). The laboratory testing consisted of electrolytic respirometers 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 con-

ditions 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 added initially, and the seawater was not replenished every 12 hours as is the case in nature. The test was a screening procedure that was designed to determine if there was sufficient enhancement due to the commercial additives that would 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 fractions of the weathered Prudhoe Bay crude oil.

The 10 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.

Materials and methods

Electrolytic respirometry

The studies were conducted using four automated continuous oxygen-uptake measuring Voith Sapromats (Model B-12). The instrument consists of a temperature-controlled water bath containing measuring units; a recorder for digital indication and direct plotting of the oxygen uptake velocity curves; and a cooling unit for the conditioning and continuous recirculation of water bath volume. The recorder displays a digital readout of oxygen uptake and constructs a graph of the data for each measuring unit. The cooling unit constantly recirculates water to maintain a uniform temperature in the water bath. The measuring units are comprised of 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 current used to generate the oxygen is measured by the digital recorder, and the data are converted directly into mg/L oxygen uptake. The CO_2 produced by microbial activity is absorbed by soda lime. The nitrogen/oxygen ratio in the gas phase above the sample is maintained throughout the experiment, and there is no depletion of oxygen. 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 graph as a function of time and displays it on the computer screen while digitally saving the data on diskette. For frequent recording and storage of oxygen uptake data, the Sapromat B-12 recorders are interfaced to an IBM-AT personal computer via the Metrobyte interface system. A software package allows the collection of data at 15-minute intervals.

Experimental design

All commercial products were tested in duplicate at the concentration recommended by the manufacturer. Each experimental respirometer flask was charged with the following materials in the order listed: weathered crude oil, 250 mg; 250 ml seawater from Prince William Sound; and commercial product at the concentration specified by the manufacturer. Seawater was prepared as follows: 25 g of oiled rocks from a contaminated beach in Prince William Sound was placed in a 4-L flask to which was added 2 L of seawater. The mixture was shaken for approximately 30 min to wash off a microbial inoculum from the rocks. The flask contents were allowed to settle, and the supernatant was mixed with more seawater for use in the respirometer vessels. Table 1 presents the summarized experimental design showing all control and experimental flasks for the respirometric studies.

All respirometer flasks were incubated at 15° C in the dark and continuously

TABLE 1

Experimental design for respirometric studies

Reaction vessel[a]	Weathered oil	Commercial product	Seawater	Total
Test flasks[b]				
T_{Pn}	+	+	+	20
$F_{1,2}$	–	+	+	20
Control flasks				
C_{Pn}	–	+	+	20
$C_{F1,2}$	–	–	+	2
C_1-inoculum	–	–	+	2
C_2-no nutrients	+	–	+	2
Total				48

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

[b]Flasks F_1 and F_2 represented simple inorganic fertilizer application and contained the following ingredients (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. The following additional trace elements were included in the formulation (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.

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 the weathered oil. The inoculum control represented the endogenous oxygen uptake of the organisms from the washed beach material and the seawater alone. The no-nutrient control represented the oxygen uptake of the organisms from the 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

Shaker flasks duplicating the respirometer flasks were used to assess the 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 deemed more prudent not to disturb the respirometric runs but instead have the shake flasks with proportionately higher levels of oil, commercial products, etc., to facilitate sampling for and precision/accuracy of the analytical chemistry. Table 2 summarizes the shaker 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;

TABLE 2

Experimental design fore the shaker flask studies[a]

Reaction vessel	Weathered oil	Commercial product	Seawater	Total
Test flasks				
T_{Pn}	+	+	+	20[b]
S_{Pn}	+	sterile	+	9[b]
$T_{Pn}S_b$	sterile	+	sterile	9[b]
$F_{1,2}$	+	–	+	2[b]
Control flasks				
C_{Pn}	–	+	+	10[c]
C_{F1}	–	–	+	1[c]
C_1-inoculum	–	–	+	1[c]
C_2-no nutrients	–	–	+	2[b]
Total				54

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

weathered oil and commercial products, 10 times the final concentrations used in the 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. These reactors represented 9 sterile product controls, which determined whether the enhancement was due to the microorganisms or to the nutrients or metabolites in the product, and 9 sterile background controls (i.e., sterile oil and seawater, but non-sterile product) to evaluate the effect of competition from naturally occurring organisms (one of the 10 products did not receive these sterile 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: day 0, day 11, and day 20. These events were determined by the shape of the oxygen uptake curves from the respirometry experiments. Each shaker 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

The oil constituents were analyzed by measuring the aliphatic and aromatic fractions of the methylene chloride extracts. The extracts were concentrated and passed through a silica gel fractionation column to separate the alkanes and the aromatics. The column was first eluted with hexane to collect the alkane fraction and then a 1:1 mixture of hexane and benzene to collect the aromatic fraction. Any polar compounds remaining in the extract stayed bound to the silica gel column. Aliphatic fractions were measured by gas chromatography using a flame ionization detector. The aromatic fractions were characterized by gas chromatography/mass spectrometry (GC/MS).

Nutrient analysis

The nitrogen species NH_3-N, NO_2^--N, and NO_3^--N were determined by U.S. EPA Methods [9]. 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 (Bushnell-Haas medium supplemented with Prudhoe Bay crude oil as the carbon source). Plates were incubated at 15 °C for 21 days prior to counting.

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 ammonia-nitrogen concentrations measured in each product flask at day 0 are summarized in Table 3.

Statistical analysis of alkane degradation data

The percentage reductions of the resolved aliphatic constituents of the weathered oil (n-C_{12} through n-C_{34} plus the branched-chain compounds pristane and phytane) were computed at day 11 for each product flask and the results compared to the percentage reduction computed for the mineral nutrient flasks. Table 4 summarizes the statistical differences observed using Tukey's Studentized Range Test [10]. 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 toxic to the biomass at the levels used in the closed flasks.

Total alkane reduction

The total alkane degradation data from the product flasks and the corresponding sterile controls at days 11 and 20 are summarized in Fig. 1. The products are arranged on the x-axis in the order determined by the statistical analysis (this same ordering has been made on all figures). At day 11 (top half of Fig. 1), better degradation was observed in every case when the commercial products were first sterilized, suggesting that the indigenous Alaskan populations were doing most if not all of the bioremediation. In contrast, less degradation occurred in every case except Product I when the background (seawater and oil) was 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 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 (bottom half of Fig. 1), 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 were first sterilized still significantly lagged behind the non-sterile systems.

Total aromatics reduction

A summary of the total aromatics reduction data at day 11 and 20 is pre-

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.

TABLE 4

Tukey's [10] studentized range test for detecting differences in mean percent removal of alkanes by products after of 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	No
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.

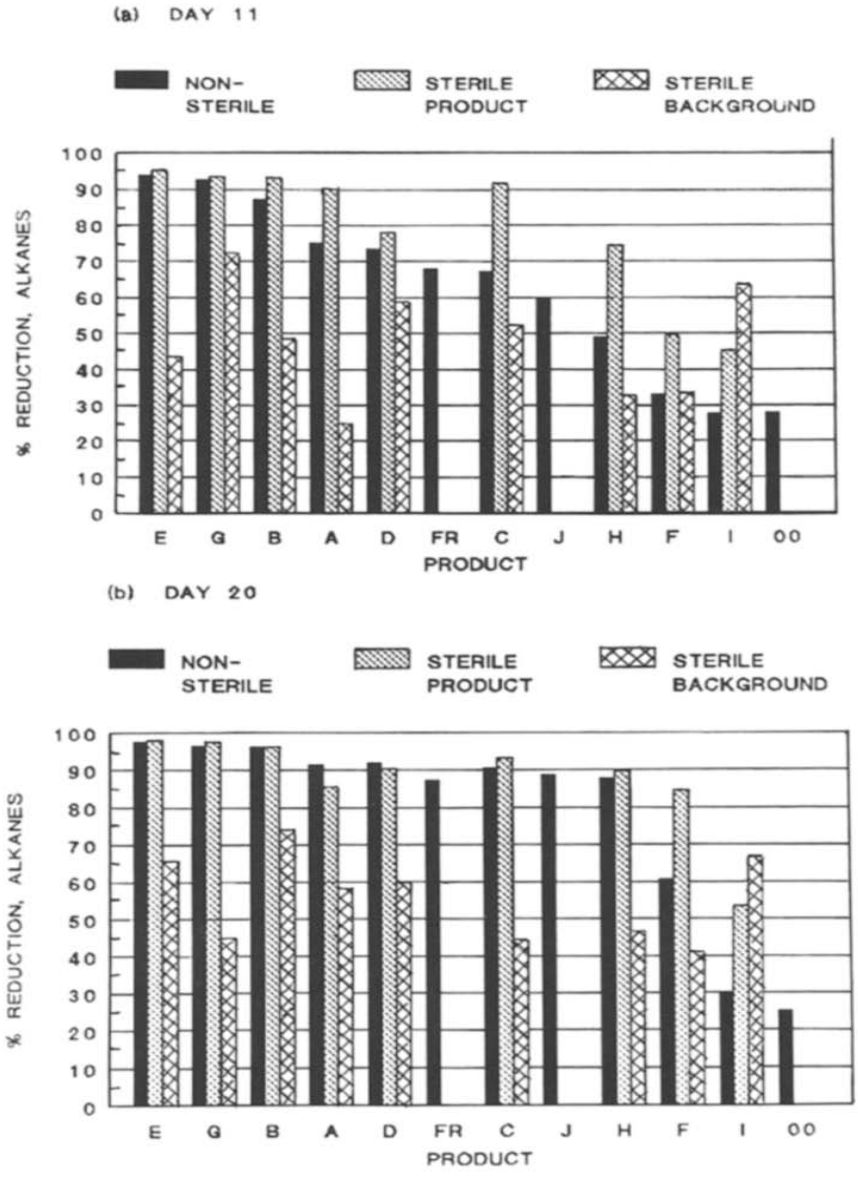

Fig. 1. Total alkane reduction in the product flasks at (a) day 11, and (b) day 20.

sented in Fig. 2. 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, aromatic reduction by Product C was somewhat closer to the others, while Products H, F, and I substantially lagged. Excellent removal of aromatics was observed in all other flasks.

Respirometric results

The net oxygen uptake curves (oxygen uptake in product flasks with oil

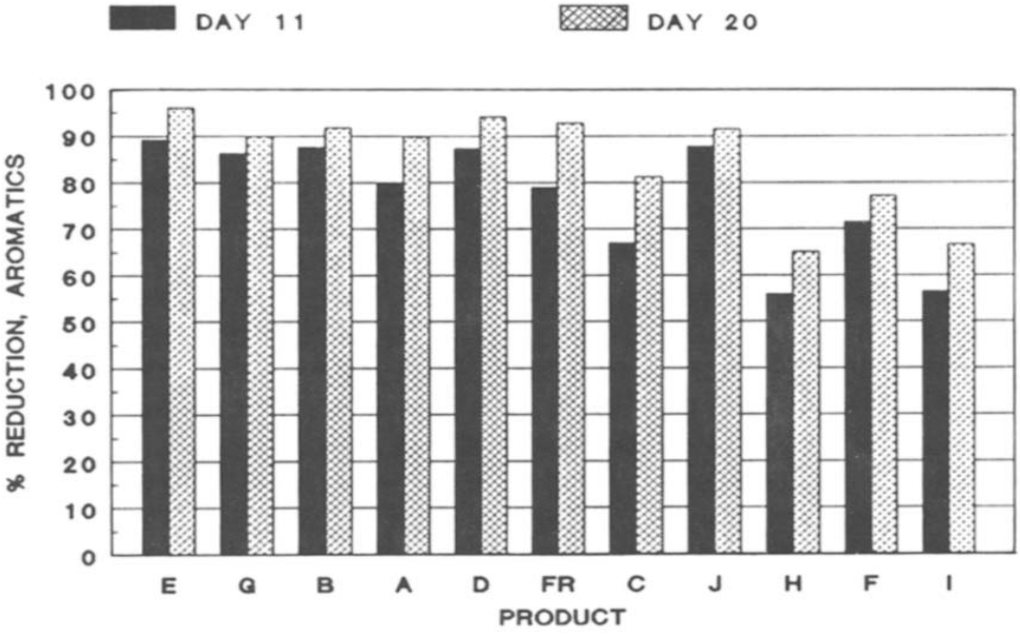

Fig. 2. Total aromatics reduction in the product flasks at days 11 and 20.

minus oxygen uptake in flasks without oil) for all 10 products (curves with symbols) compared to mineral nutrients (curve with no symbols) are summarized in Figs. 3a and 3b. In Fig. 3a 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 to about 340 mg/L for the mineral nutrient flasks. The acclimation lag period for products E and G were approximately 2 and 4 days, respectively, compared to 5 days with mineral nutrients. Product A gave the highest maximum net uptake (630 mg/L compared to 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.

In Fig. 3b only products J and C gave higher overall net O_2 consumption than mineral nutrients, although product F exceeded the control after 27 days. The lag period for both 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). Very 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

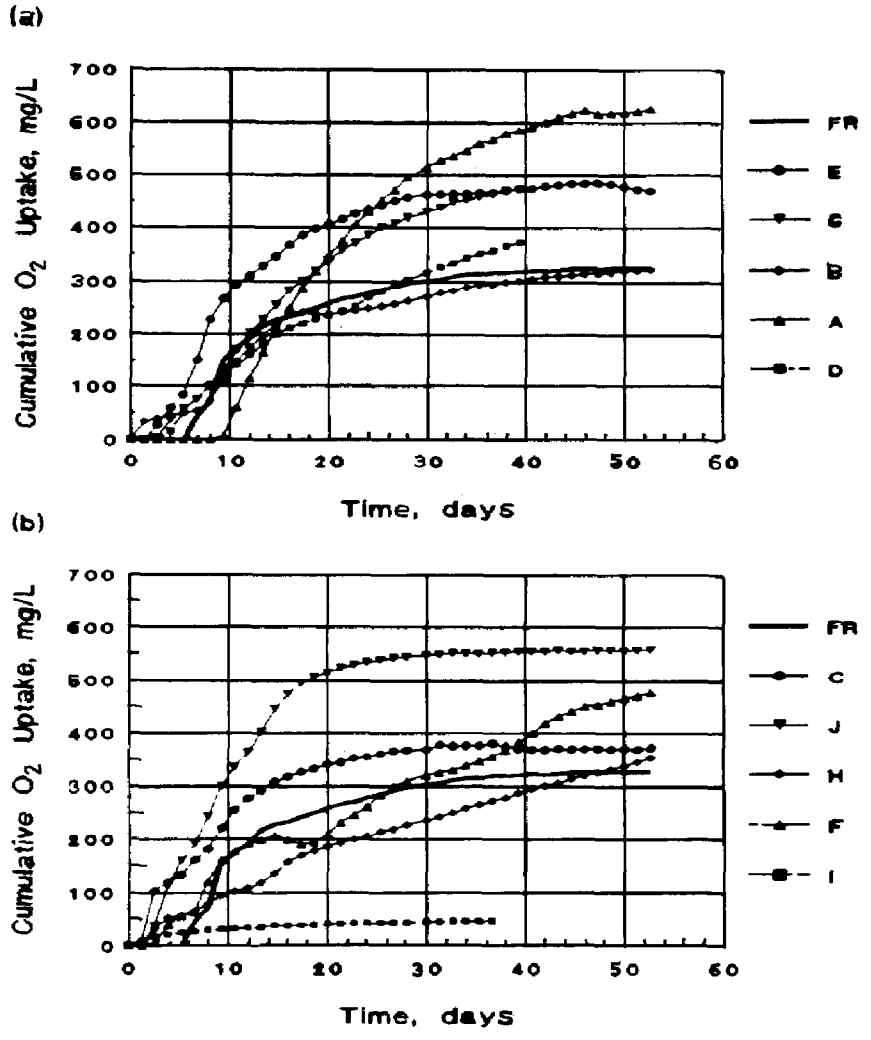

Fig. 3. net oxygen uptake curves for products and mineral nutrients: (a) products E G B A D, and (b) products C, J, H, F, I.

degraders has been summarized for days 0 and 11 only, and the results are presented in Fig. 4.

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. 3a), also exhibited excellent growth of oil degraders in 11 days. Products C, J, and F yielded high levels of oil degraders and good oxygen uptake curves, but alkane degradation was no better than the populations growing in simple mineral nutrients. Oil degrader populations actually declined in the product B flasks, and the increase in oil degraders in the flasks containing products A, D, and I was minimal.

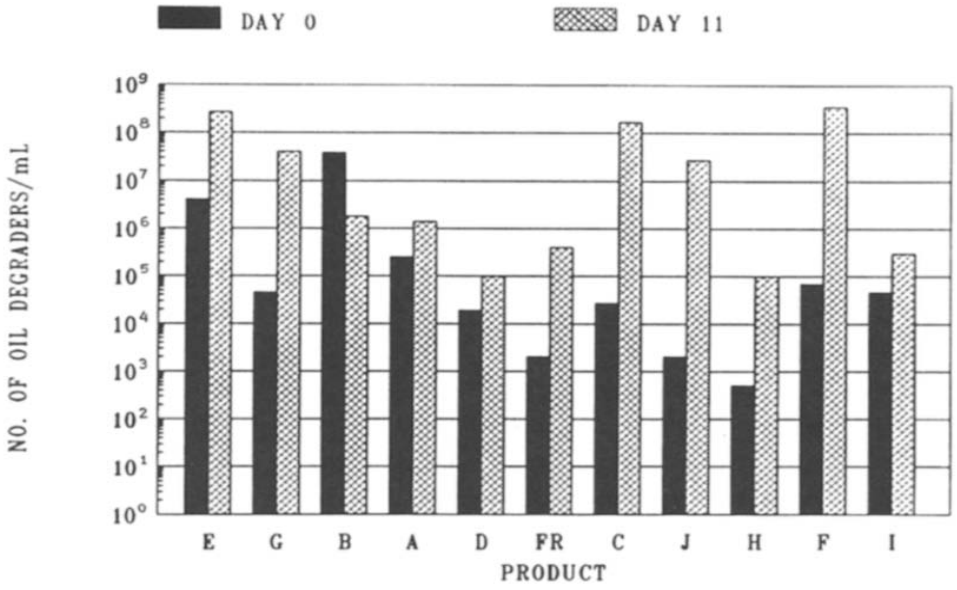

Fig. 4. Growth of oil degraders in the product flasks.

Discussion

The objective was to determine whether a commercial bioremediation product was able to effect weathered crude oil degradation better than natural Prince William Sound organisms when stimulated with simple mineral 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 all the products tested, the two that provided the most consistent results in all three tests were products E and G. Both gave higher oxygen uptake, greater growth in 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 than indigenous populations stimulated 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 increase in oil degraders and the relative alkane degradative capability were mediocre, as were the flasks containing products B, D, H, and I.

The sterile controls 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 unknown factor exists in these two products that stimulates the indige-

nous microorganisms to degrade the crude oil constituents at rates faster than is possible with simple nutrient addition. Further work needs to be done to define the enhancement factor in these products.

Correlations have not as yet been made between weathered crude oil degradation and oxygen uptake, nor have carbon balances been performed. Work is being planned to measure carbonaceous metabolic end-products, CO_2 production, and total biomass yield, and then to correlate this information with the 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 a biodegradation experiment. All that is required, assuming the proper correlations have been established, is the careful measurement of initial substrate and biomass values followed by the measurement of the residual soluble product value at the plateau of the uptake curve [11]. 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 all 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.

References

1 R.M. Atlas, Microbial degradation of petroleum hydrocarbons: an environmental perspective, Microbiol. Rev., 45 (1981) 180–209.
2 J.J. Cooney, The fate of petroleum pollutants in freshwater ecosystems, In: R.M. Atlas (Ed.), Petroleum Microbiology, Macmillan, New York, NY, 1984.
3 G.D. Floodgate, The fate of petroleum in marine ecosystems, In: R.M. Atlas (Ed.), Petroleum Microbiology, Macmillan, New York, NY, 1984.
4 J.G. Leahy and R.R. Colwell, Microbial degradation of hydrocarbons in the environment, Microbiol. Rev., 54 (1990) 305–315.
5 J.R. Vestal, J.J. Cooney, S. Crow and J. Berger, In: R.M. Atlas (Ed.), Petroleum Microbiology, Macmillan, New York, NY, 1984.

6 P.H. Pritchard, R. Araujo, J.R. Clark, L.D. Claxton, R.B. Coffin, C.F. Costa, J.A.J.A. Glaser, J.R. Haines, D.T. Heggem, F.V. Kremer, S.C. McCutcheon, J.E. Rogers and A.D. Venosa, Interim report: Oil Spill Bioremediation Project, U.S. Environmental Protection Agency, Office of Research and Development, Washington, DC, 1990; see also P.H. Pritchard, this issue, pp. 115–130.

7 W. Dott, D. Feidieker, P. Kampfer, H. Schleibinger and S. Strechel, Comparison of autochthonous bacteria and commercially available cultures with respect to their effectiveness in fuel oil degradation, J. Ind. Microbiol. 4 (1989) 365–374.

8 A.D. Wong and C.D. Goldsmith, The impact of a chemostat discharge containing oil degrading bacteria on the biological kinetics of a refinery activated sludge process, Water Sci. Technol. 20 (1988) 131–136.

9 U.S. Environmental Protection Agency, Methods for chemical analysis of water and wastes, EPA 600/4-79-020, U.S. Environmental Protection Agency, Washington, DC, 1979.

10 J.W. Tukey, The Problem of Multiple Comparisons, Princeton University, Princeton, NJ, 1953, 396 pp.

11 C.P. Grady, Jr., J.S. Dang, D.M. Harvey, A. Jobbagy and X.L. Wang, Determination of biodegradation kinetics through use of exectrolytic respirometry, Water Sci. Technol., 21 (1989) 957–968.