J Ind Microbiol Biotechnol (2005) 32: 171–185
DOI 10.1007/s10295-005-0218-1

ORIGINAL PAPER

**John R. Haines · Eric J. Kleiner · Kim A. McClellan
Karen M. Koran · Edith L. Holder · Dennis W. King
Albert D. Venosa**

Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems

Received: 19 July 2004 / Accepted: 2 March 2005 / Published online: 3 May 2005

Abstract Ten oil spill bioremediation products were tested in the laboratory for their ability to enhance biodegradation of weathered Alaskan North Slope crude oil in both freshwater and saltwater media. The products included nutrients to stimulate inoculated microorganisms, nutrients plus an oil-degrading inoculum, nutrients plus compounds intended to stimulate oil-degrading activity, or other compounds intended to enhance microbial activity. The product tests were undertaken to evaluate significant modifications in the existing official United States Environmental Protection Agency (EPA) protocol used for qualifying commercial bioremediation agents for use in oil spills. The EPA protocol was modified to include defined formulas for the exposure waters (freshwater, saltwater), a positive control using a known inoculum and nutrients, two negative controls (one sterile, the other inoculated but nutrient-limited), and simplified oil chemical analysis. Three analysts conducted the product test independently in each type of exposure water in round-robin fashion. Statistical tests were performed on analyst variability, reproducibility, and repeatability, and the performance of the various products was quantified in both exposure media. Analysis of variance showed that the analyst error at each time-point was highly significant (P values ranged from 0.0001 to 0.008, depending on water type and oil fraction). In the saltwater tests, six products demonstrated various degrees of biodegradative activity against the alkane fraction of the crude oil and three degraded the aromatic hydrocarbons by $>10\%$. In the freshwater tests, eight products caused $>20\%$ loss of alkane hydrocarbons, of which five degraded the alkanes by $>50\%$. Only four products were able to degrade polycyclic aromatic hydrocarbons (PAHs) by $>20\%$, one of which caused 88% removal. However, when the variability of the analysts was taken into consideration, only one of the ten products was found to yield significant percent removals of the PAH fraction and only in freshwater. Viable microorganism population analysis (most-probable-number method) was also performed on every sample by each operator to measure the changes in aromatic and alkane hydrocarbon-degrading organism numbers. In general, little evidence of significant growth of either alkane- or PAH-degraders occurred among any of the ten products in either the saltwater or freshwater testing.

Keywords Bioremediation · Biodegradation · Oil spill · Product test protocol · Freshwater · Saltwater

J. R. Haines (✉) · E. J. Kleiner · K. A. McClellan · A. D. Venosa
United States Environmental Protection Agency,
26 W.M.L. King Drive, Cincinnati, OH 45268, USA
E-mail: haines.john@epa.gov
Tel.: +1-513-5697446
Fax: +1-513-5697105

K. M. Koran · E. L. Holder
Department of Civil and Environmental Engineering,
University of Cincinnati, Cincinnati, OH 45268, USA

D. W. King
Statking Consulting, 759 Wessel Drive,
Suite 6, Fairfield, Ohio, USA

Introduction

Following the Exxon Valdez oil spill in March 1989, the United States Environmental Protection Agency (EPA) made the decision to conduct a full-scale research project to determine whether bioremediation is a viable technology for the cleanup of such a catastrophic spill. A large team of scientists was put together to design and conduct this field study [16]. At that time, product vendors began pressuring EPA and other governmental agencies to use their products for treating the spill. As a result of this mounting pressure, the EPA Office of Research and Development (ORD) was mandated in the fall of 1989 to develop and validate a protocol to test the claims of these product vendors on the effectiveness of their products in biodegrading crude oil spilled into the environment. In November 1989, EPA requested the National Environmental Technology Applications

Corporation (NETAC) to assemble a panel of scientific experts to develop screening criteria that would compare the efficacy and environmental safety of these products. In February 1990, EPA issued a public solicitation for proposals from the bioremediation industry to provide products for testing the feasibility of commercial bioremediation agents for enhancing the degradation of weathered North Slope crude oil. Meanwhile, ORD had proposed a laboratory screening protocol based on measuring the disappearance of crude oil in shake-flasks containing seawater, weathered North Slope crude oil, and commercial products [17]. The results of the first generation testing were presented in 1993 [18]. Subsequent work refined and adjusted the testing protocol [20].

A tiered approach was developed from this effort [13], consisting of: (1) a base tier used to identify the presence of chemical or biological agents that might be considered unacceptable (pathogens, carcinogens, hazardous substances), (2) tier 1, in which a vendor provides a description of the use of the product, its mode of action, and basic information on its toxicity, (3) tier 2, a laboratory batch-screening test that provides empirical evidence that a product is efficacious, (4) tier 3, a bench-top continuous-flow microcosm to simulate more closely environmental conditions, and (5) tier 4, consisting of actual field monitoring. Eventually, the final protocol consisted of the first three tiers; and tiers 3 and 4 were never adopted due to cost.

As more product tests were conducted throughout the 1990s, it became evident that refinements in the tier 2 test were needed. The analytical chemistry procedure, which was based on gas chromatography/mass spectrometry (GC/MS), was somewhat cumbersome; and the gravimetric and microbiological analyses became an unnecessary and burdensome step. Also, since the tier 2 test was written exclusively for saline environments, there was no equivalent test for freshwater environments. Because the test used natural seawater, it was found to give variable results due to the variability of seawater, both in composition and in oil-degrader numbers. Finally, a positive control was needed as an indication that the test was performing properly. Therefore, modifications were made in the tier 2 protocol. Such modifications included: (1) use of synthetic seawater and freshwater in separate tests, (2) incorporation of a standard microbial inoculum and nutrients in a positive control, (3) incorporation of two negative controls (sterile control, nutrient-limited control with inoculum), and (4) streamlining of the GC/MS procedure. The inoculum is used in the protocol not only as a positive control but also as an inoculum to test abiotic products such as fertilizers, enzymes, and other chemical enhancements.

The objective of the investigation reported in this paper was to perform the modified protocol in both synthetic saltwater and synthetic freshwater to determine whether it screens commercial products for efficacy in the biodegradation of weathered crude oil so that they may be listed on the National Contingency Plan (NCP) product schedule. Ten products were acquired from various product vendors who were listed on the NCP product schedule (http://www.epa.gov/oilspill/ncp/bagents.htm). Three analysts conducted the tier 2 testing in round-robin fashion independently of each other, first in synthetic seawater and then in synthetic freshwater. Results were analyzed statistically for reproducibility and repeatability and are presented in this paper. A decision rule was established for qualifying products that pass the protocol.

Materials and methods

Source of inocula

Mixed cultures of microorganisms capable of degrading weathered crude oil were derived from several sources. Our laboratory collected sediment samples from marine beaches and terrestrial locations contaminated with petroleum products. The marine beach samples originated from Alaska, Maine, Delaware, and Texas. The organisms for the freshwater tests were derived from riverbank sediments, junkyard soils, or soils from manufactured-gas plant sites. Samples of the sediments or soils were suspended in appropriate media with weathered crude oil and incubated on a shaker at 20°C for 28 days. After three successive transfers, the activity of each mixed culture against crude oil was assessed using the same measurement protocol as described below. Cultures demonstrating good activity were then grown in 10-L batches for preparation of stock cultures. The 10-L batch cultures were harvested by concentrating the organisms by centrifugation, washing twice with saline solution, and mixing with 10% glycerol as a cryoprotectant. The concentrated cultures were dispensed into 5-mL plastic tubes and placed into a –80°C freezer for storage. Periodically, frozen cultures were removed from storage and tested for oil-degrading ability. Cultures showing good activity from terrestrial and marine sources were chosen as the positive control cultures for this research.

Preparation of exposure water

A modified artificial seawater and freshwater were used as the exposure media for the bioremediation product test. Natural waters vary from location to location and season to season. Therefore, consistency among product vendors and regulatory agencies is very difficult to achieve using natural waters. The seawater used in this protocol is derived from a marine aquarium formula (GP2) [15] used in zoos. The GP2 contained (per liter): 21.03 g NaCl, 9.5 g $MgCl_2{\cdot}6H_2O$, 3.52 g Na_2SO_4, 1.32 g $CaCl_2{\cdot}2H_2O$, 0.61 g KCl, 0.088 g KBr, 0.034 g $Na\text{-}B_4O_7{\cdot}10H_2O$, 0.02 g $SrCl_2{\cdot}6H_2O$, 0.17 g $NaHCO_3$, 0.05 g $FeCl_3{\cdot}6H_2O$, 0.297 g $Na_5P_3O_{10}$, 2.89 g KNO_3.

The synthetic freshwater formula (SFW) is a modification of Bushnell–Haas (BH) medium (Difco Laboratories, Ann Arbor, Mich.) and contained (per liter): 0.2 g $MgSO_4$, 0.02 g $CaCl_2{\cdot}2H_2O$, 0.5 g NaCl, 2.89 g KNO_3, 1.32 g K_2HPO_4, 1.00 g KH_2PO_4, 0.05 g $FeCl_3{\cdot}6H_2O$. Trace minerals were not added as they have been found to be unnecessary for microbial growth. Batches of media sufficient for each product test were prepared and dispensed (100 mL) into 250-mL flasks prior to sterilization. The bicarbonate, phosphates, and minor components were prepared as concentrated solutions, filter-sterilized, and added to the flasks after the media had been steam-sterilized.

Most probable number analysis

The populations of oil-degrading microorganisms in each flask were estimated using a differential most-probable-number (MPN) method [20]. Microtiter 96-well plates were used in the analysis, resulting in an 8-tube, 11-dilution MPN. One row was filled with sterile medium as a sterility check control. Substrates used in the procedure were hexadecane for alkane degraders (0.7% w/v) and a combination of phenanthrene, anthracene, fluorene, and dibenzothiophene (10:1:1:1) for polycyclic aromatic hydrocarbon (PAH)-degraders (0.5% w/v, final concentration). Positive wells for alkane-degraders were scored by observing the pink/red color change from the addition of iodonitrotetrazolium violet (INTV) after a 2-week incubation period at 20°C. The PAH-degraders were scored after a 3-week incubation period by recording the brown/yellow color that develops naturally during PAH metabolism [5, 8, 9]. This color formation is thought to be due to the accumulation of products of meta-cleavage of aromatic rings [9, 14].

In this work, the MPN procedure was used as described [20] for saltwater and modified for fresh water by using SFW medium rather than GP2 [10]. For both alkane- and PAH-degrader enumeration, the MPN was calculated using a computerized enumeration program [11].

Residual hydrocarbon analysis

Following removal of 5 mL of sample from each flask for MPN analysis, a recovery surrogate solution consisting of D_{36}-heptadecane, D_{50}-tetracosane, D_{64}-dotriacontane, D_{10}-1-methylnaphthalene, D_{10}-phenanthrene, D_{10}-pyrene, and 5α-cholestane (androstane) in dichloromethane (DCM) was added to each reactor flask to obtain a final surrogate concentration of 4 ng μL^{-1} in the final extract. After addition of 50 mL of DCM to each shake-flask, the flasks were stirred for 10–15 min on a magnetic stirring plate. The DCM phase was passed through Na_2SO_4 to remove water. The DCM extracts were then exchanged into hexane under a stream of dry nitrogen. The solvent exchange was performed three times. The hexane extracts were prepared for GC/MS analysis [19] by adding an internal standard mixture of deuterated alkane and aromatic hydrocarbons to yield a final concentration of each standard of 10 ng μL^{-1}. Concentrations of hydrocarbons in the extract were quantified using a Hewlett Packard 6890 series GC with a 5973 MSD-MS as detector, operating in selected ion-monitoring (SIM) mode. The analytes included 28 alkanes ranging in carbon number from nC_{10} to nC_{35}, plus pristane, phytane, hopane, and 32 PAHs, consisting of 2-ring, 3-ring, and 4-ring PAHs (naphthalenes, phenanthrenes, dibenzothiophenes, fluorenes, naphthobenzothiophenes, pyrenes, chrysenes) and their alkylated homologues. The column was a DB5 column (30 m long, 0.25 mm ID, 0.2 μm liquid phase; Supelco, Bellefonte, Pa.). Alkane and PAH concentrations were summed to obtain the total alkane and total PAH concentrations in each flask. The percent remaining at each sampling event was determined relative to the concentrations of alkanes and PAHs in flasks sacrificed at time 0. The chemical analysis procedure given in the Federal Register [6] is more complex with more possibilities for error. The latter procedure differs from the one used in this research in that sample transfer to a separatory funnel is required as opposed to extraction in the incubation flask, a Kuderna-Danish concentrator is used to concentrate the analytes to quantifiable amounts, and the analytes listed include pyrogenic hydrocarbons, which were excluded from this research. In our experience, the extraction can be performed directly in the culture flask, solvent exchange can be performed in the sample storage vial, and the analytes can be changed without sacrificing the reliability of the method. Naphthobenzothiophenes and C_3 and C_4 chrysenes were added to the analysis and the 5-ring and 6-ring pyrogenic PAH compounds were eliminated. The pyrogenic PAH compounds, found in low concentrations in crude oil are of little importance in quantifying large changes in oil concentration. Hopane as a biomarker is useful in estimating physical losses of oil in open systems, but normalization to hopane is not necessary for closed systems where abiotic loss mechanisms are minimal.

Test procedure

Ten commercial bioremediation products were selected for this work. Commercial product manufacturers were advised that products were to be tested in both freshwater and saltwater systems. Table 1 lists the types of products and their assigned letter designations. Products are listed by letter to preserve confidentiality. Manufacturers of each product provided the product sample and instructions for use. The manufacturers specified whether the product required additional nutrients or inoculum. The appropriate additions were made to test-flasks at the beginning of each test. The manufacturers were: Acorn Biotechnical Corp., B&S Research, Bio-NutraTech, Elf Aquitaine, Enviro Zyme, Land and Sea Restoration LLC, Marine Systems, Oppenheimer Biotechnology, PetroRem, and Waste Microbes. Products

Table 1 Product type and letter designation

Letter designation	Product type
A	Bacteria/enzyme
B	Biological additive
C	Oleophilic fertilizer
D	Microorganisms with nutrients
E	Microorganisms with nutrients
F	Biological additive
G	Microorganisms with nutrients
H	Sorbent with microorganisms
I	Oleophilic fertilizer
J	Microorganisms

identified by letter in the results are not in alphabetical order by manufacturer. Tests were run independently by three different analysts to simulate a round-robin or inter-laboratory comparison type of process. All products and controls were run concurrently in a single medium by a single operator. One set was completed before another was started.

The experimental design for this study included one positive and two negative controls and ten product treatments, all in triplicate. Products that were powders or dry materials were weighed into small sterile test-tubes to provide the proper concentration for each flask. Liquid products were dispensed on the day the test was started. If a product supplied nutrients, the exposure water formula was modified by removing nitrogen. Products were supplemented with either nutrients or inoculum only at the specification of the manufacturer. Microbial products could have nutrients added. Fertilizer products could have inoculum added. Some required neither nutrients nor inoculum. Erlenmeyer flasks (250 mL) were filled with 100 mL of SFW or GP2, 0.5 g of weathered Alaska North Slope crude oil (ANS 521, previously heated to 272°C in vacuo to remove the light-end hydrocarbons), and product per the manufacturers' recommended concentrations. Each flask was inoculated with 1.0 mL of enriched culture (marine or freshwater) unless the product was a microbial product. Positive controls received 1.0 mL of culture, 0.5 g of ANS 521, and nutrients in 100 mL of exposure water. Negative control 1 (the sterile control) consisted of flasks with exposure water containing the nutrients specified and 0.5 g of ANS 521 but no inoculum. Negative control 2 consisted of 1 mL of inoculum, 0.5 g of ANS 521, and exposure water lacking nitrogen. Flasks were incubated at 20°C on an orbital shaker table rotating at 200 rpm. Triplicate shake flasks for each treatment were sacrificed on days 0, 7, 14, 21, and 28 of incubation and analyzed for microbial number by MPN and residual oil, as described above.

Statistical analysis

An overall analysis of variance (ANOVA) was conducted in each time-period. The ANOVA model included product, analyst, and product-by-analyst interaction. Then the least squares product from the ANOVA (adjusted for analyst and interaction) were compared with the negative control without inoculum. The seawater and freshwater analyses were conducted separately. The purpose of a round-robin multiple analyst evaluation of a method is to enable a regulatory group to formulate a precision statement about a method. The precision of a method is a function of two types of variability: the variances in reproducibility (R_1) and repeatability (R_2). The repeatability variance represents the within-laboratory variability, i.e., the variability of the method when carried out on identical samples in the same laboratory by the same analyst. The reproducibility variance represents the sum of within-laboratory and among-laboratory variability, i.e., the variability of the method when conducted on identical samples in two or more laboratories by two or more analysts. One goal of this study was to estimate the repeatability and reproducibility standard deviations and the corresponding repeatability and reproducibility coefficients for the laboratory method to be used to obtain biodegradability measurements from various commercial products by various analysts. To obtain the repeatability and reproducibility parameters, ASTM method E691 [2] was used and SAS served as the software program for making the calculations.

Results

Residual oil analysis

Figures 1, 2 summarize the alkane and aromatic hydrocarbon degradation in both SFW and GP2; and Fig. 3 shows the three controls. The data from all three analysts were compiled and averaged for the figures. The alkane data are plotted against the left axis and the aromatic data are plotted against the right axis. Of the ten products tested in SFW, nine demonstrated statistically significant degradation of the total alkane hydrocarbons analyzed at day 28, with P values ranging from 0.044 for product H to $P < 0.0002$ for all other products and the positive control. Fewer products demonstrated statistically significant degradation of aromatic hydrocarbons in SFW. Products C, E, H, I, and the positive control caused statistically significant degradation of aromatics ($P < 0.0001$) at day 28. Table 2 shows the percent loss of aliphatic and aromatic hydrocarbons in SFW and GP2.

In GP2 significant alkane degradation ($P < 0.033$–0.0001) occurred with six of the ten products after 28 days of incubation (A, C, E, G, H, I). Virtually complete biodegradation occurred in the positive control within 7 days. As shown in Figs. 1, 2, some of the products exhibited degradation earlier in the incubation period, but the most extensive degradation was observed at 28 days.

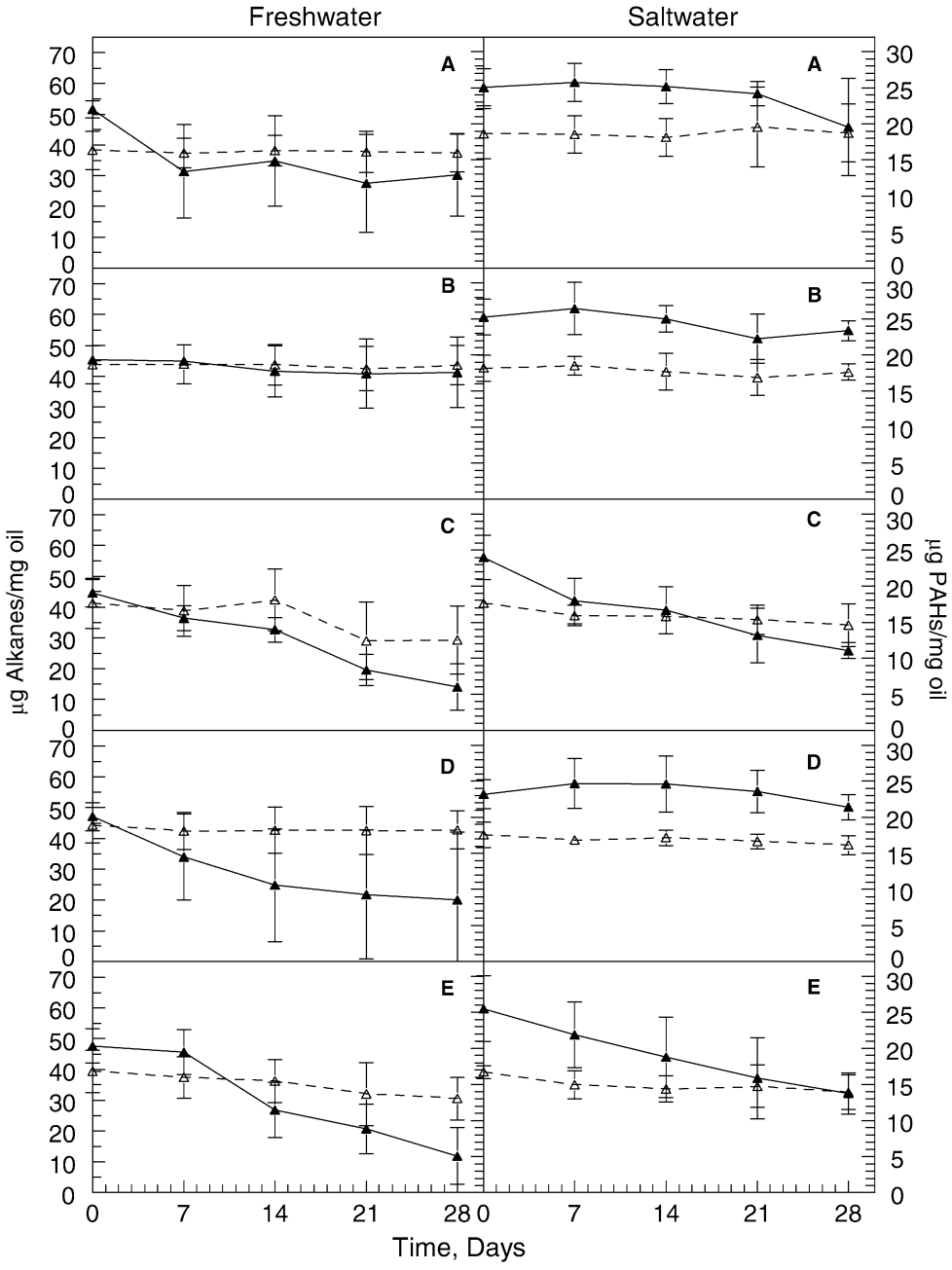

Fig. 1 Alkane (*solid lines*) and aromatic (*broken lines*) hydrocarbon degradation in flask test. *A–E* designate products

Degradation of aromatic hydrocarbons in GP2 was unimpressive. Only three products (C, E, I) caused statistically significant reduction of PAHs ($P < 0.0032$–0.0004), although in absolute terms these reductions were only about 15–17%. In contrast, approximately 86% reduction of PAHs occurred at day 28 in the positive control ($P = 0.0001$; Fig. 3). These results were surprising, because the products tested were originally intended for use in a saltwater environment. Yet, better degradation of PAHs occurred in SFW.

MPN analysis

Figures 4, 5, 6 summarize the alkane-degrader MPN data for the ten products in both exposure waters, and the three controls. The inoculum added to the abiotic products (C, F, I) and the control flasks contained approximately 10^4–10^6 cells mL^{-1} alkane-degrading organisms at time 0 in freshwater and ca. 10^7 cells mL^{-1} in the saltwater medium. Flasks with products A, B, D, E, G, H, and J were not inoculated because they contained their own microbial source. The ability of these cultures to grow on ANS521 in the two exposure waters was highly variable. Some grew better in saltwater than in freshwater, some did the opposite, and still others grew poorly or moderately in both. The positive control in SFW (Fig. 6) grew by two orders of magnitude by day 7 and then remained steady for the remainder of the incubation. The positive control culture derived from a marine source did not show the same growth pattern.

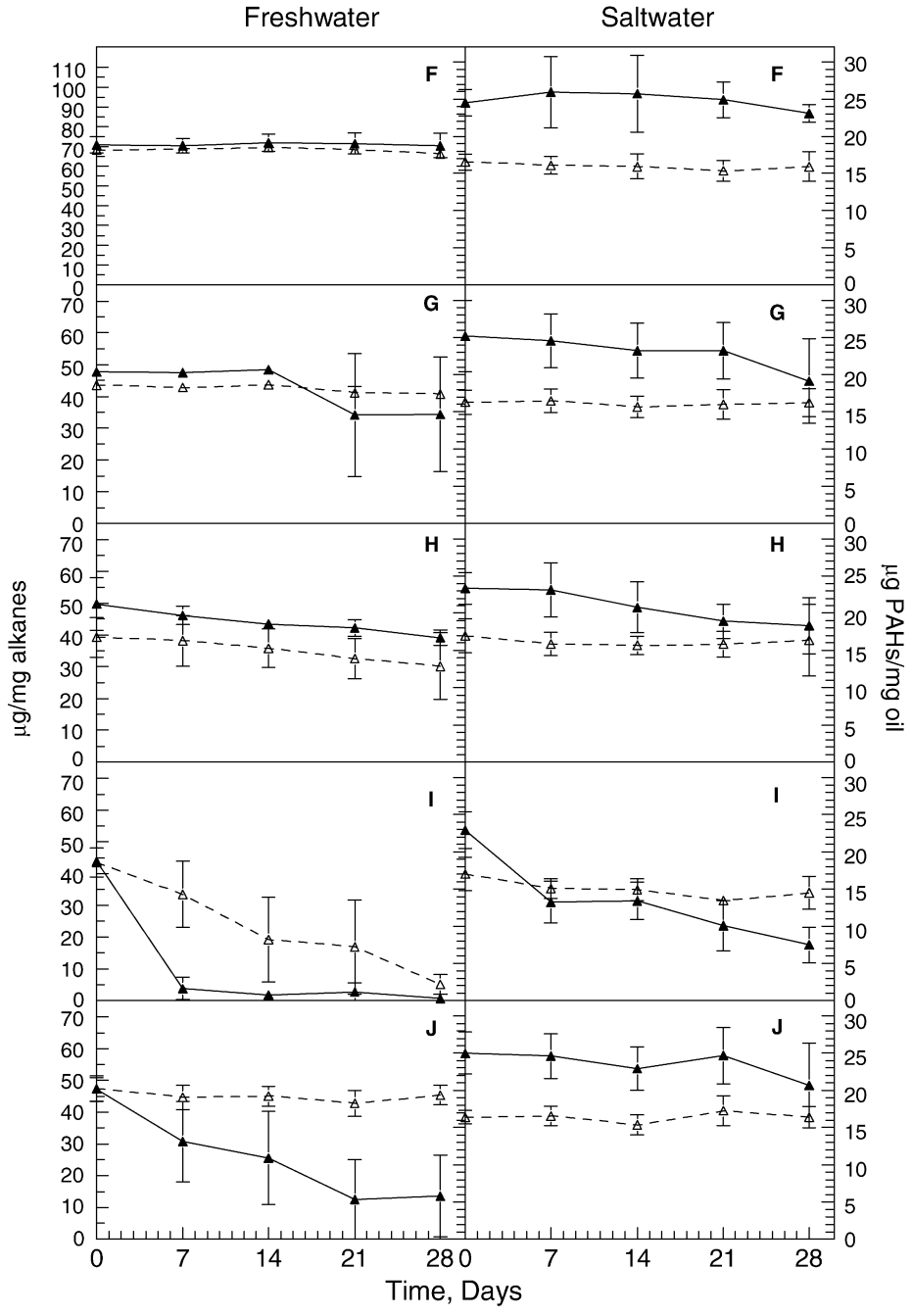

Fig. 2 Degradation of alkane (*solid lines*) and aromatic (*broken lines*) hydrocarbons in the flask test. *F–J* designate products

Figures 7, 8, 9 depict the MPN estimates for PAH-degrading organisms from both freshwater and seawater tests and the controls at each sampling event. The inoculum added to the abiotic products (C, F, I) and the control flasks contained approximately 10^3–10^4 cells mL^{-1} PAH-degrading organisms at time 0. Few of the products tested demonstrated significant growth of aromatic degraders in either water type. The MPNs in product C increased approximately two orders of magnitude in SFW; and product H increased approximately five orders of magnitude. Product F showed no change in MPN over the course of the test. Growth in GP2 was much less.

Reproducibility and repeatability

Table 3 summarizes the average R_1 and R_2 standard deviations derived from the ten-product test. The within-analyst error (R_2) should always be lower than the among-analyst error (R_1). In compiled data the R_2 standard deviation was slightly lower than the R_1 standard deviation, although R_1 and R_2 standard deviations were of similar magnitude. For individual products, this was not always the case (data not shown). Also, the variability at day 28 was higher than at day 0 when viewed as a percentage of the corresponding means. This is not surprising, since more variation is expected as

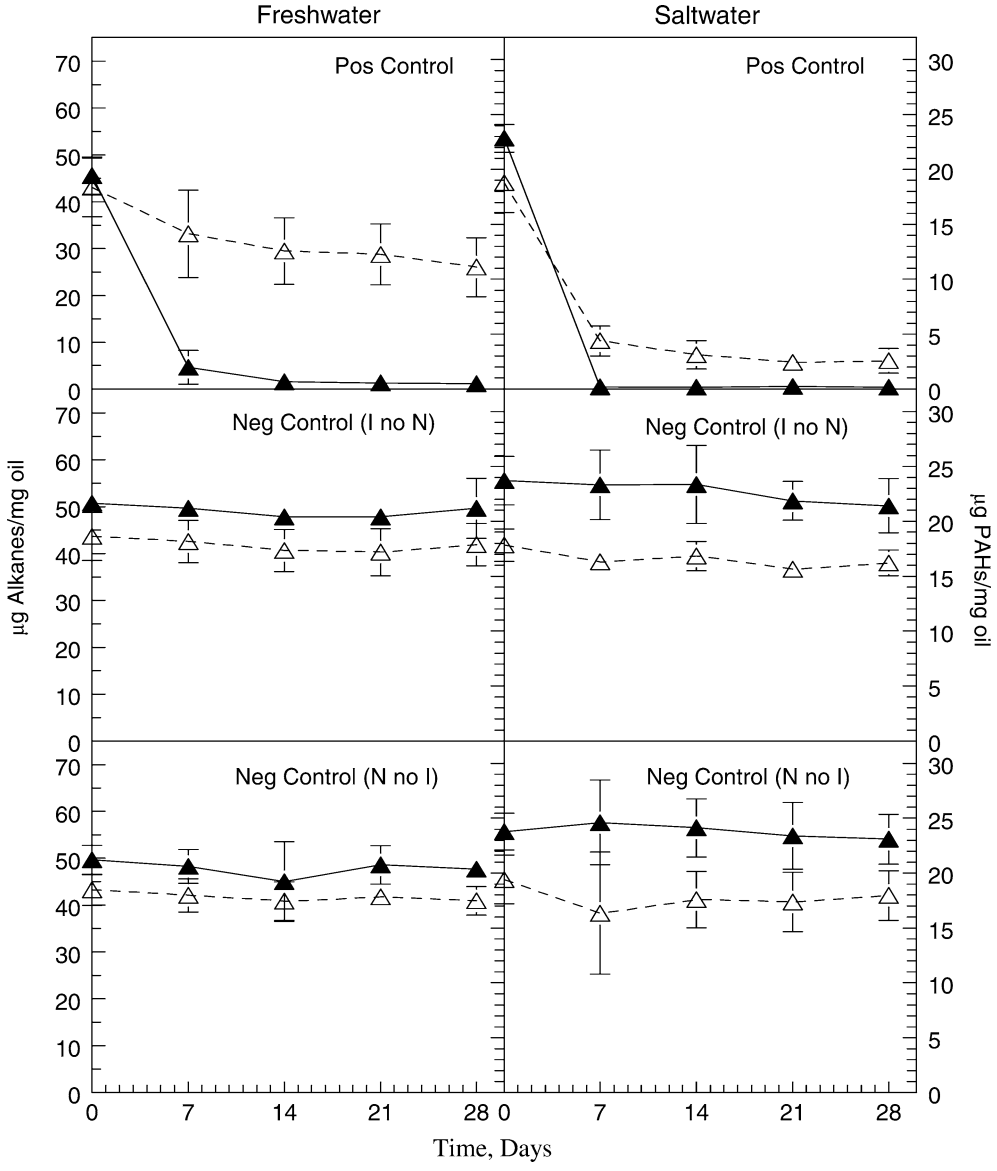

Fig. 3 Alkane (*solid lines*) and aromatic (*broken lines*) hydrocarbon degradation in flask-test controls. *I* Innoculum, *N* nitrogen, *Neg* negative, *Pos* positive

concentrations of hydrocarbons decline with time. In cases where the R_2 error was greater than or similar to the R_1 error, this signifies that it is somewhat difficult for an individual analyst to get precisely the same result for all three replicates. Biological variability is relatively high, especially as biodegradation takes place. For this reason, a pass/fail decision rule (discussed below) must account for the inherent variability expected from the protocol.

Discussion

This research was undertaken to determine whether the modified protocol was an adequate test of the ability of commercial products to demonstrate successful biodegradation of crude oil hydrocarbons. Two types of exposure water were used for these tests, synthetic saltwater and freshwater, and results indicated large differences between the two inocula originating from each source. This suggests that commercial products should be screened in both types of environments if the product vendor wishes his product to be used in both marine and freshwater spill responses. This agrees with the findings of Blenkinsopp et al. [4], who also used two different exposure waters for testing bioremediation products.

In the modified procedures, the exposure waters were chemically defined, a positive control was added, an inoculum was used for testing abiotic products, and residual oil analysis was simplified. Using synthetic waters eliminates the need to rely on natural waters to provide the oil degraders for testing abiotic products such as new fertilizers or other chemical stimulants. If an abiotic product is able to stimulate the inoculum to degrade the oil, this procedure should be able to accommodate that activity reproducibly. Chemical analysis was simplified by reducing the number of sample-transfers and the solvent-exchange procedure.

Table 2 Loss of hydrocarbon components over 28 days

Product or treatment	Freshwater		Seawater	
	Alkane loss (%)	Aromatic loss (%)	Alkane loss (%)	Aromatic loss (%)
Positive control	97.6	39.5	99.3	86.3
Negative control 1	4.0	5.4	2.9	7.5
Negative control 2	2.1	4.0	9.7	9.2
A	41.4	2.5	22.0	0.5 Increase
B	9.1	0.2	7.4	3.0
C	68.4	29.0	53.9	17.2
D	57.4	3.3	7.7	8.2
E	75.1	22.5	46.2	16.7
F	0.4	2.9	5.9	3.5
G	27.9	6.6	24.0	0.2
H	21.3	23.0	21.6	3.5
I	98.4	88.2	67.3	14.6
J	71.3	4.4	17.4	0.03 Increase

The number of analytes was changed to focus on the more prevalent components of crude oil. These modifications, which were consistent with the Canadian protocol [4, 7], were developed to improve the reproducibility and repeatability of the protocol. Results demonstrated that, of the three abiotic products tested in

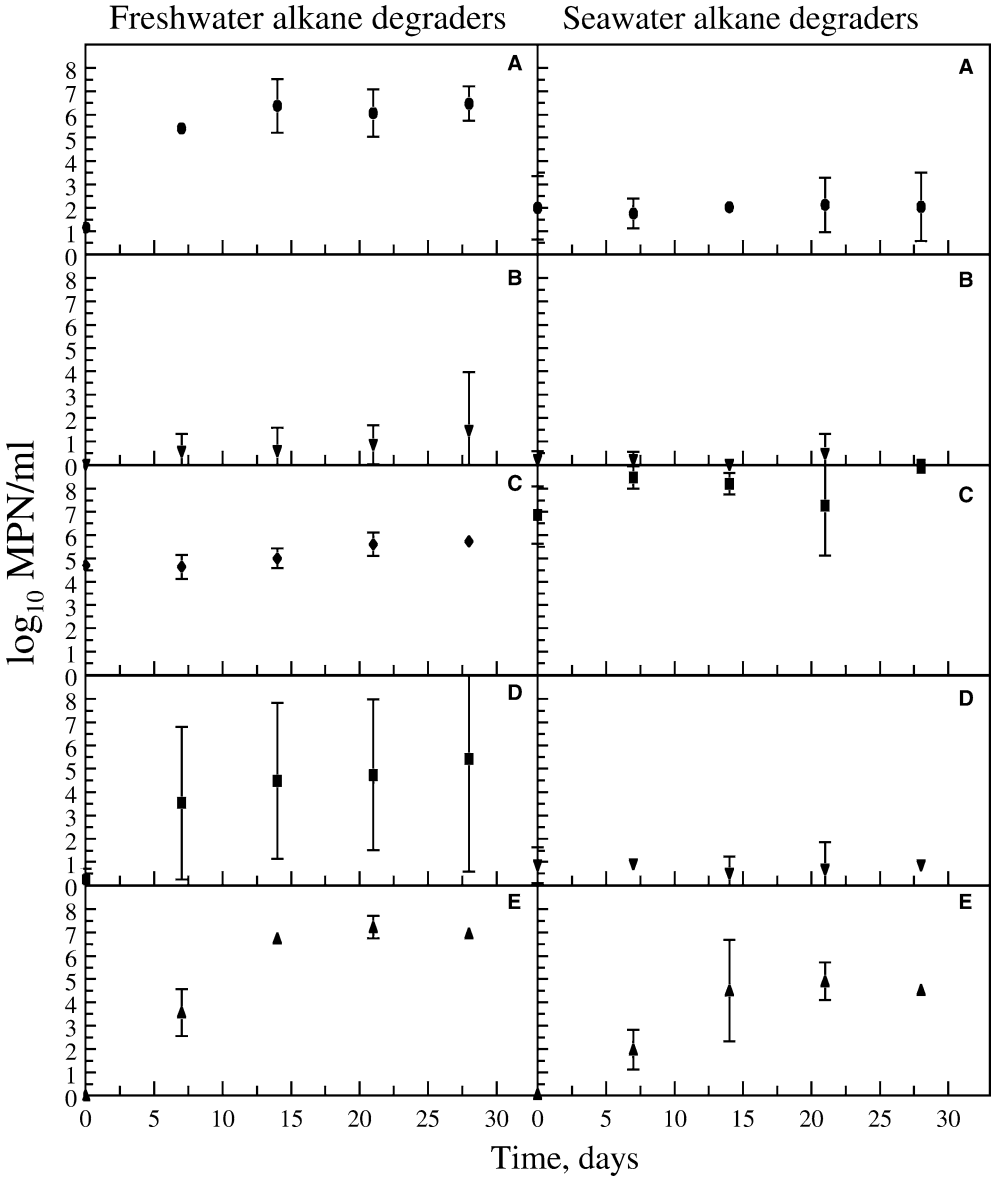

Fig. 4 MPN of alkane-degrading organisms. *A–E* designate products

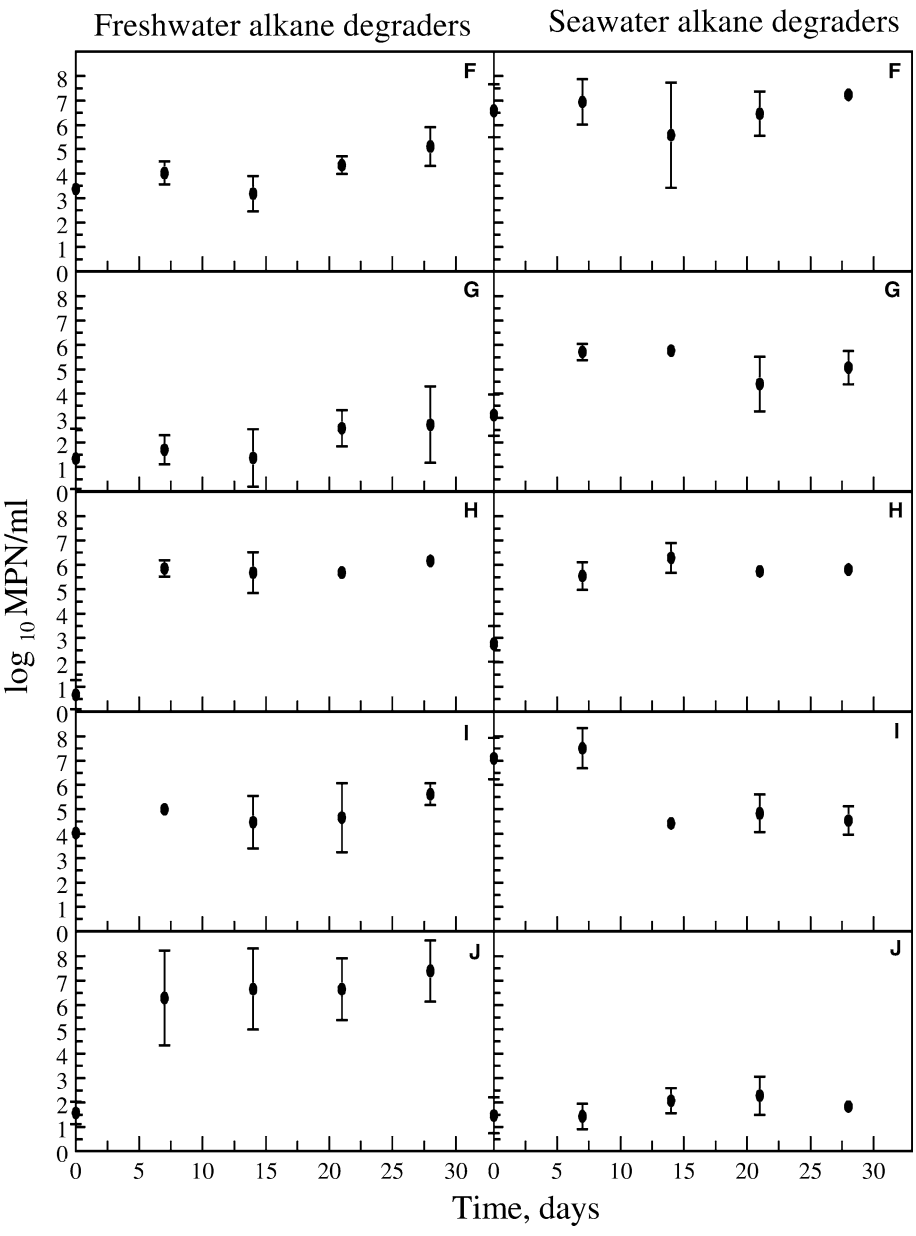

Fig. 5 MPN of alkane-degrading organisms. *F–J* designate products

both GP2 and SFW, two (products C, I) significantly degraded both the aliphatics and the aromatics in both exposure waters, whereas the other (product F) caused no biodegradation activity in either medium. Although statistically significant differences from the negative controls were observed for products C and I, the absolute degradation compared with the positive control was much lower in seawater. This was not true for product I in freshwater, which was able to achieve much better degradation of the PAH fraction than the positive control. These findings suggest that product C did not supply enough nitrogen and phosphorus to even approach the same level of degradation that the positive control did. Product I, however, did supply sufficient nutrients, but this stimulation occurred only in freshwater. Since the positive control cultures for freshwater and saltwater were from different sources, it is unclear why this product was unable to elicit the same stimulation for both cultures. One of the three abiotic products (product F) contained a substantial concentration of alkane hydrocarbons present in the product itself; and no degradation of either oil fraction was observed with that product.

An additional experiment with six of the ten products (A, B, C, D, H, I) was repeated later in GP2 with five times the product manufacturers' recommended dosage. Table 4 shows the results of the higher concentration tests. Products A, B, and D did not show any improvement in the degradation of hydrocarbons at a higher dose. The positive control elicited virtually identical results to the earlier tests performed. Products C, H, and I showed improved PAH degradation. The products were oleophilic fertilizers and one sorbent with organisms. This suggests that the nutrient products were

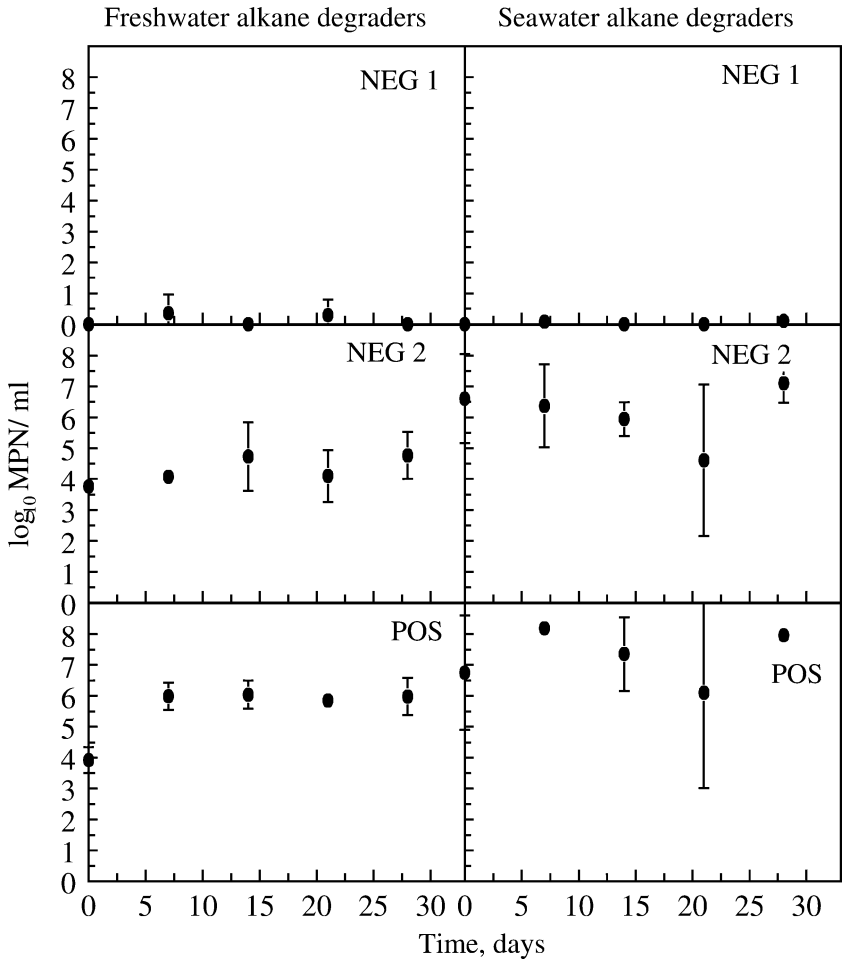

Fig. 6 MPN of alkane-degrading organisms in control flasks

nitrogen-limited and/or phosphorus-limited in the original tests. The manufacturers recommended a dose rate that may be inadequate for a batch-type test with no continuous input from the environment, such as would happen in an open beach setting.

With respect to the seven products containing microbial cultures, only product E was able to significantly biodegrade both oil fractions in both exposure waters. The other biotic products partially degraded the aliphatic fraction in one or other exposure water, but none of them degraded the PAH fraction significantly. This was despite the fact that they had the same nutrient concentration as the positive control. This suggests that the cultures in the biotic products were not effective in metabolizing the oil fractions within the 28-day period of the test. The positive control in saline GP2 was much more effective in metabolizing the PAH fraction than the SFW positive control. Average degradation in the latter case was 39.5%, whereas average degradation in GP2 was 86.3%. These results contrast with those of Blenkinsopp et al. [4], whose cultures showed that much better biodegradation occurred in synthetic freshwater than in marine water.

Laboratory-scale evaluations of bioremediation products have been performed in the past. However, the testing protocols have not been standardized, leading to an inability to compare results directly. Aldrett et al. [1] tested 13 products using a protocol similar to the EPA, and four of those products performed as well as or better than the nutrient controls, which achieved approximately 80% removal of alkanes and 35% removal of aromatic hydrocarbons. The four products reduced aliphatics of chain length less than C_{31} by more than 90%. Removal of C_{31}–C_{35} alkanes ranged from 31% to 90%. Pristane and phytane were completely removed by two products and were 73% and 89% removed by the other two products. Removal of two-ring aromatics ranged from 30% to 93%, three-ring aromatics from 13% to 77%, and four-ring aromatics from 11% to 28%. The authors observed unpredictable variability in the natural inoculum, which was a limitation to accurate and reproducible assessment of commercial products. Neralla and Weaver [12] tested ten commercial products in salt marsh microcosms over a 90-day period at two temperatures (10°C, 30°C). A 1-g amount of oil was added to a 1-cm layer of water covering salt marsh sediment and biodegradation was monitored with and without nutrient addition. After 90 days, only one product enhanced oil degradation without added nutrients at 10°C. With added nutrients, nine of ten products enhanced degradation at 10°C, compared with the fertilized control. At 30°C, seven of ten products enhanced

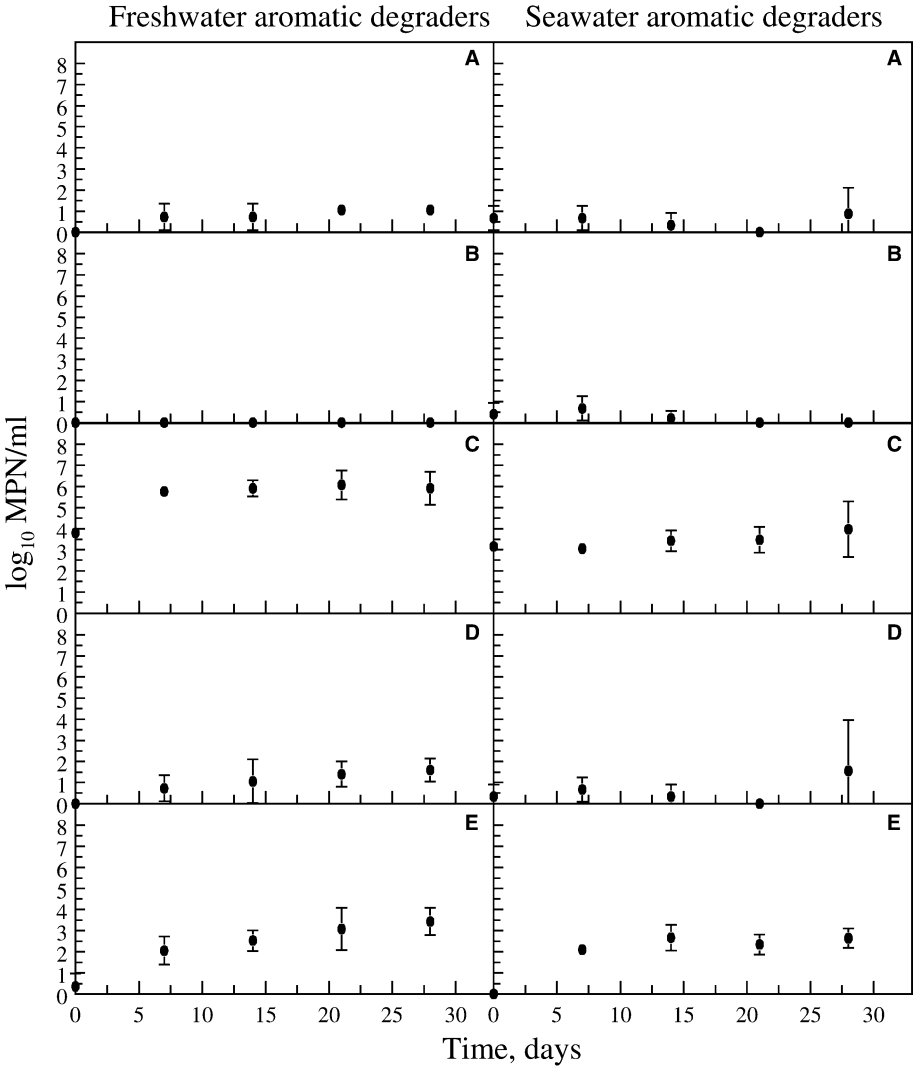

Fig. 7 MPN of PAH-degraders. *A–E* designate products

the biodegradation of total petroleum hydrocarbons (TPH) after 90 days without nutrients, whereas none of the products in the presence of nutrients was able to degrade TPH better than the nutrients alone. Focht et al. [7] developed a defined bacterial consortium of six bacteria for use as a standard inoculum for testing freshwater oil-spill bioremediation agents. This consortium was able to degrade both the alkane and aromatic fractions of crude oil reproducibly and predictably. Similar to the inoculum used in our study, it was not intended to be a super-concoction of oil degraders against which all commercial products would be compared. Rather, it was used as a quality control benchmark for product testing and as a reproducible inoculum for testing non-living biostimulation products.

Bachoon et al. [3] examined microbial community dynamics in salt marsh microcosms treated with oil and two bioremediation products. The products were a bacterial culture plus nutrients plus an activator solution and a dispersant. The treatments were oil plus sediment, oil plus nutrients plus sediment, and oil plus the two product treatments at the manufacturer's dosage. The results showed that nutrient formulas were most effective in stimulating population growth, as measured by DNA content and oil degradation. Oil degradation with the nutrient treatment was greater than 90% removal of alkanes and a significant reduction of aromatics, compared with the control or either product. The bacterial product with nutrients showed greater than 90% reduction of alkanes C_{19} and above. Pristane and phytane were reduced to about 76% of the control. Aromatics were not significantly reduced. Plate counts and MPN estimates of hydrocarbon-degrading populations were of limited utility.

In previous work conducted by our laboratory, Haines et al. [10] proposed a freshwater protocol for testing bioremediation products to include performance targets, a positive control, and a defined nutrient formula. Four products were tested and two were able to degrade $>90\%$ alkane and $>60\%$ aromatic hydrocarbons within 28 days. The Canadian freshwater protocol [4, 7] differs in the incubation period (14 days), inoculum size, and oil content. In our opinion, a longer incubation period, 28 days, allows more time for the growth of

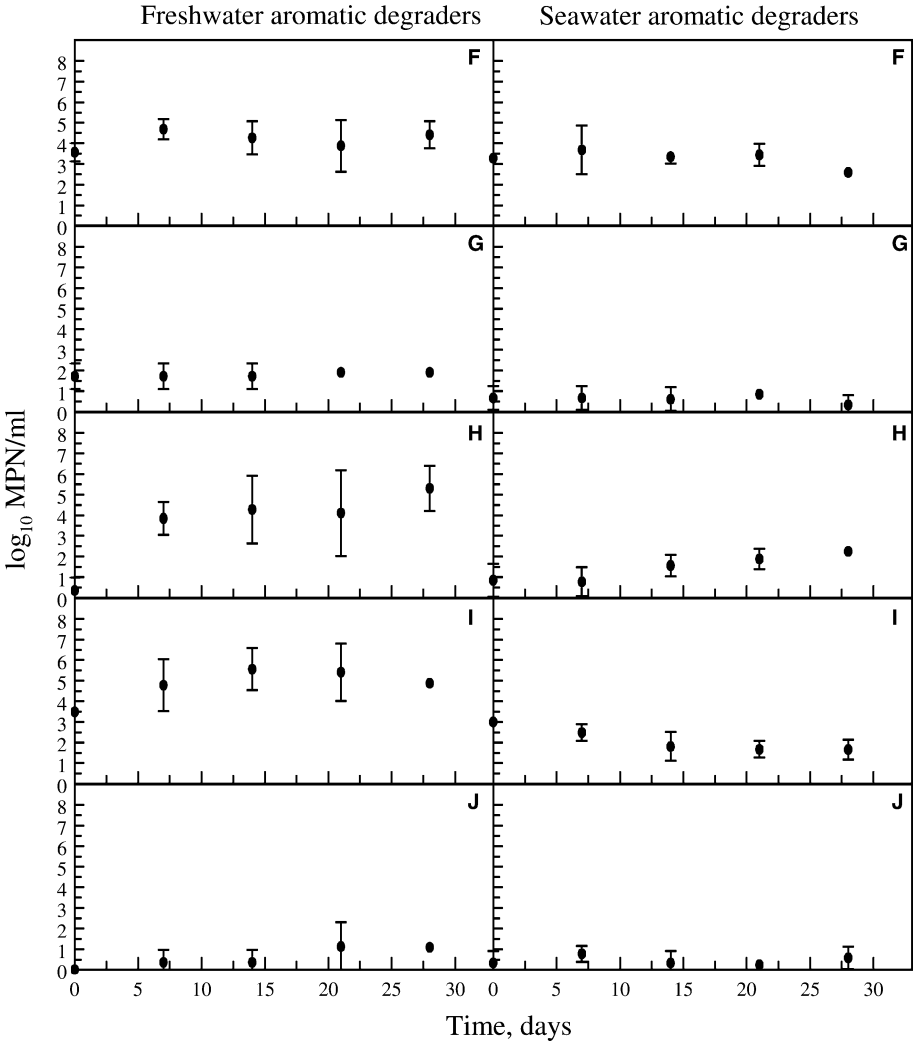

Fig. 8 MPN of PAH-degraders. *F–J* designate products

organisms that may be slow to metabolize some oil compounds, especially PAHs. A smaller inoculum in the order of 10^3–10^4 cells mL^{-1} rather than 10^6 cells mL^{-1} is somewhat more typical of what may be found in nature. In our research, the inoculum contained about 10^3–10^4 PAH-degraders mL^{-1} and about 10^6 alkane-degraders mL^{-1}. The response of an abiotic product without its own inoculum should reflect actual use conditions. In regards to the method of analyzing hydrocarbon degradation in the protocol, the Canadian protocol [4, 7] uses GC with flame ionization detection (FID) to measureme the aliphatic, aromatic, and total petroleum hydrocarbons and uses GC/MS-SIM for the target aliphatic and aromatic analytes. Our laboratory proposes using only GC/MS, because GC/MS is able to resolve and quantify the alkylated PAHs that GC-FID cannot. We also recommend only two sampling times, day 0 and day 28. The intermediate sampling events are not necessary to establish efficacy. The initial oil content in our protocol is 5 g L^{-1}. This is high enough to provide a good distinction between starting analyte concentrations and final analyte concentrations, especially with regard to aromatic hydrocarbons. The saltwater and freshwater exposure media are defined formulas, to enable interlaboratory comparison and to limit inoculum variability that would occur if natural water were used. Positive and negative controls must be included to ensure the procedures are working properly and that microbial contamination does not occur.

One of the objectives of this investigation was to establish quantitative decision rules that a product must be able to meet to qualify for use in an oil spill. Our freshwater positive control culture yielded 97.6% alkane and 39.5% aromatic hydrocarbon degradation in 28 days, while the saltwater culture yielded 99.3% alkane and 86.3% aromatic hydrocarbon degradation. Since the entire purpose of listing products on the NCP product schedule is to have a readily available source of effective bioremediation agents that responders can use to clean up an oil spill, it is reasonable to set a minimum target aliphatic and aromatic concentration level that a product must be able to meet in order to pass the test and therefore qualify for NCP listing. It is recognized that the higher-molecular-weight compounds in both oil fractions are more difficult and therefore slower to degrade than the lower-weight compounds. It is also rec-

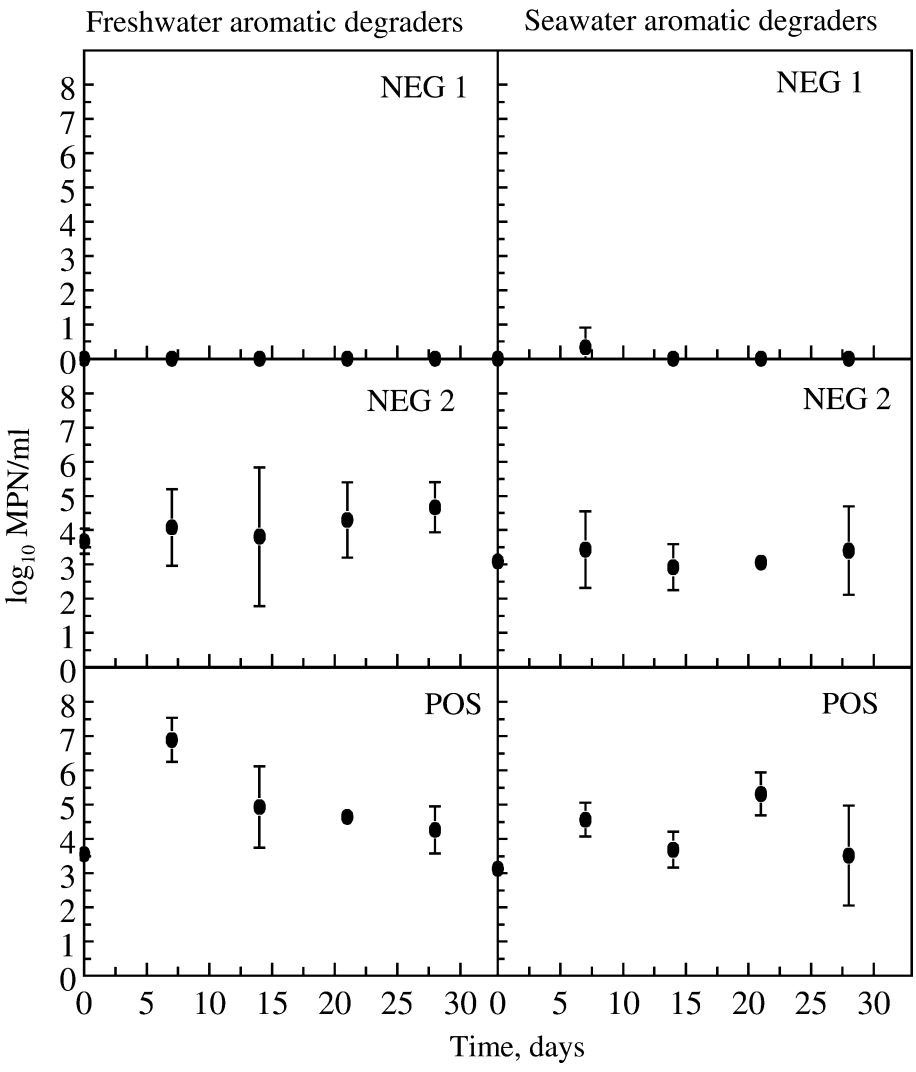

Fig. 9 MPN of PAH-degraders in control flasks

ognized that the 60+ target compounds measured by the GC/MS-SIM method represent only a minor fraction of the total hydrocarbons present in crude oil. Nonetheless, quantification of those compounds is the best we can do at the present time to infer that biodegradation is taking place. Most of the products tested in this research did not degrade the PAH fraction much at all, even though most products degraded the alkane fraction by a significant amount. Since PAHs are the most toxic compounds in oil, a product must show some evidence that it is able to degrade PAHs within the 28-day time-frame of the protocol. Since our two positive control cultures are known to degrade PAHs, and since the poorer performing culture was the freshwater inoculum, it is reasonable to set a minimum percent PAH reduction level at 39% after 28 days for both the freshwater and saltwater tests.

Recognizing that the data from this protocol can be highly variable, it is important to incorporate an accounting of the variability when establishing a target decision rule. We propose this be done as follows. First, calculate the mean and standard deviation of the total aliphatic and total aromatic concentrations from the three independent replicates at day 0 and day 28. Then, from those data, calculate the upper 90% confidence level (UCL_{90}) at day 28 using the following formula:

Table 3 Reproducibility and repeatability standard deviations

Water type	Oil fraction	Day 0			Day 28		
		Mean	R_1	R_2	Mean	R_1	R_2
Freshwater	Alkane	49,306	2,703	2,112	28,749	6,333	4,730
	PAH	18,147	1,505	796	14,934	1,605	900
Saltwater	Alkane	59,266	4,371	2,343	42,560	5,188	4,893
	PAH	17,517	1,237	1,027	15,157	1,644	1,456

Table 4 Hydrocarbon reduction with products at five-fold higher concentrations in seawater

Product or treatment	Alkane loss (%)	Aromatic loss (%)
Positive control	99.6	90.6
Negative control (innoculum, without nitrogen)	18.3	11.1
A	6.5	9.2
B	8.9	8.7
C	97.1	35.0
D	4.8	1.7
H	29.3	11.8
I	68.6	19.8

$$\mathrm{UCL}_{90} = \bar{x} + \left(\frac{t_{0.90,2\mathrm{df}} \times \sigma}{\sqrt{n}} \right) \quad (1)$$

where $\bar{x}$ is the total alkane or total PAH mean of three replicates on day 28, $t_{0.90,2\mathrm{df}}$ is the 90% 1-tailed t value with two degrees of freedom (1.886), σ is the standard deviation of the three replicates on day 28, and n is the number of replicates (three). Finally, calculate the percentage reduction of each oil fraction from day 0 to day 28, using the day 28 UCL_{90} value instead of the day 28 mean.

Using the above procedure, the freshwater positive control percent-reduction values for the aliphatic and aromatic fractions were calculated to be 96.6% and 23.6%, respectively, compared with 97.6% and 39.5% based simply on the means. For the saltwater tests, the positive control values for the aliphatic and aromatic fractions were 98.9% and 79.8%, respectively, compared with values of 99.3% and 86.3%, respectively, based on the means. Since the freshwater tests gave the poorer performance of the two tests, setting a target where the UCL_{90} has to meet a percent reduction of $>90\%$ for aliphatics and $>20\%$ for aromatics would be a reasonable requirement for passing the test. Using these criteria, only one of the ten products in these tests would pass the protocol in freshwater (product I) and none would pass in saltwater.

The above data are similar to the requirements established in Canada [4], which are 35% reduction for TPH, 30% for total aliphatics, and 10% for total aromatics. The 30% reduction in total saturates (including all resolvable and unresolvable but GC-detectable aliphatics) is equivalent to roughly 80% reduction in total GC/MS-resolvable target *n*-alkanes, according to the Canadian data; and the 10% reduction in total aromatics is equivalent to an approximately 50% reduction of the 5-PAH homologue group, consisting of naphthalene, fluorene, dibenzothiophene, phenanthrene, chrysene, and their alkylated homologues. Our PAH series includes two other four-ring PAHs in addition to those five PAH series considered by Blenkinsopp et al. [4]. Thus, the United States and Canadian protocols are quite similar. Before the United States EPA protocol is adopted, it must go through public comment and be approved by EPA headquarters. This will be pursued in 2005.

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