J Ind Microbiol Biotechnol (2003) 30: 107–113
DOI 10.1007/s10295-002-0018-9

ORIGINAL PAPER

J.R. Haines · K.M. Koran · E.L. Holder · A.D. Venosa

Protocol for laboratory testing of crude-oil bioremediation products in freshwater conditions

Received: 26 July 2002 / Accepted: 19 November 2002 / Published online: 16 January 2003
© Society for Industrial Microbiology 2003

Abstract In 1993, the Environmental Protection Agency, National Risk Management Research Laboratory (EPA, NRMRL), with the National Environmental Technology Application Center (NETAC), developed a protocol for evaluation of bioremediation products in marine environments [18]. The marine protocol was adapted for application in freshwater environments by using a chemically defined medium and an oil-degrading consortium as a positive control. Four products were tested using the modified protocol: two with nutrients and an oleophilic component; one with nutrients, sorbent, and organisms; and one microbial stimulant. A separate experiment evaluated the use of HEPES and MOPSO buffers as replacements for phosphate buffer. The oleophilic nutrient products yielded oil degradation similar to the positive control, with an average alkane removal of 97.1 ± 2.3% and an aromatic hydrocarbon removal of 64.8 ± 1.2%. The positive control, which received inoculum plus nutrients, demonstrated alkane degradation of 98.9 ± 0.1% and aromatic degradation of 52.9 ± 0.1%. The sorbent-based product with inoculum failed to demonstrate oil degradation, while the microbial stimulant showed less oil degradation than the positive control. Replacement of phosphate buffer with other buffers had no significant effect on one product's performance. Differences in product performance were easily distinguishable using the protocol, and performance targets for alkane and aromatic hydrocarbon degradation are suggested.

K.M. Koran · E.L. Holder
Department of Civil and Environmental Engineering,
University of Cincinnati, 741 Baldwin Hall,
Cincinnati, OH, 45221, USA

J.R. Haines (✉) · A.D. Venosa
National Risk Management Research Laboratory,
US Environmental Protection Agency,
26 Martin Luther King Drive, Cincinnati, OH, 45268, USA
E-mail: haines.john@epa.gov
Tel.: +1-513-569-7446
Fax: +1-513-569-7105

Keywords Bioremediation · Oil spill · Product test protocol · Freshwater

Introduction

The quantity of petroleum spilled into freshwater systems can equal or exceed that spilled into marine environments [20]. Between 1973 and 2000, the total reported spill volume in marine areas was 1.15×10^8 gallons of oil. In the same period the total volume of oil spilled in freshwater was 1.16×10^8 gallons. Most of the spills were less than 1,000 gallons. The majority of the spills occurred in rivers and canals. Because the majority of freshwater spills involve small quantities, the remediation problems may become more onerous than for marine spills. Remediation operations are often inefficient since these spills have high manpower and equipment needs relative to the size of the spills. In such cases, products that enhance biodegradation of spilled oil may be useful as sole treatment options or in conjunction with other remediation technologies.

Bioremediation as a method to ameliorate the impact of oil spills is a recently applied technology and has shown promise in different settings [2,9,10,19]. The most knowledge has been gained with regard to marine shorelines as a result of catastrophic tanker accidents. Freshwater spills have not received the same attention. The results of many studies have provided some guidance for the practice of bioremediation. Frequently, addition of nitrogen and phosphorus will enhance degradation of the oil [9,10,12,24]. However, large spills create a significant burden on the environment, and removal of oil by physical means prior to bioremedial treatment is recommended. Few statistically valid, peer reviewed studies have shown successful enhancement of oil degradation by adding inoculum to augment the resident microbial population [1,5,13,16,17]. Inoculation has had some success in soil treatment [5,16], but little success where active water movement occurs. Swannell

et al. [19] reviewed and summarized the published research involving oil-spill bioremediation. Study sites have included alpine soils [12,13], tropical soils [16], sandy beaches [14,24], freshwater wetlands and salt marshes [26]. Field-scale experiments are relatively rare due to cost and difficulty.

Laboratory-scale evaluations of bioremediation products have been carried out. However, the testing protocols have not been standardized, leading to an inability to compare results directly. Thirteen products were tested by Aldrett et al. [1], and four of them performed as well as or better than the nutrient controls, which achieved approximately 80% removal of alkanes and 35% removal of aromatic hydrocarbons. Another laboratory tested ten commercial products over 90 days at two temperatures (10 and 30 °C). After 90 days, only one product enhanced oil degradation without added nutrients. With added nutrients, nine of ten products enhanced degradation of petroleum at 10 °C [17]. Bachoon et al. [3] examined microbial-community dynamics in salt marsh microcosms treated with oil and bioremediation products. The products were a bacterial culture plus nutrients and a dispersant. The treatments were oil plus sediment, oil plus nutrients plus sediment, and the two product treatments. The results showed that plate counts and most-probable-number estimates of hydrocarbon-degrading populations were of limited utility. Products that were nutrient formulas were most effective in stimulation of population growth as measured by DNA content and oil degradation. The DNA analysis showed *Eubacteria* as dominant with *Archaea* increasing in all treatments except control. *Pseudomonas* increased only in oil treatments. Up to 40% of the DNA was uncharacterized.

In the aftermath of the 1989 EXXON VALDEZ oil spill in Alaska's Prince William Sound, many products were offered as possible bioremediation treatment options. At the time, there was no procedure for evaluating these products for their effectiveness in the spill response setting. Following enactment of the Oil Pollution Act of 1990, the Environmental Protection Agency, National Risk Management Research Laboratory (EPA, NRMRL), with the National Environmental Technology Application Center (NETAC), began development of a series of protocols for evaluation of bioremediation products. The initial protocol offered a standardized test designed to assess the performance of products intended for use in marine environments [18]. The marine laboratory-scale protocol employs natural seawater as an inoculum, a complex hydrocarbon analytical procedure, and has no positive control. A natural seawater inoculum may not provide an adequate array of microorganisms capable of degrading oil in a flask test. The results may depend too much on where and when the seawater was collected. Laboratories in different parts of the United States will have different results, due to the different characteristics of seawater near their locations. A positive control is needed in the procedure to ensure that the methods are working as they should. The hydrocarbon analysis procedure in the protocol can be simplified by changing sample-extraction and solvent-exchange procedures.

Other bioremediation product testing protocols have been developed in Canada and France [4,8,15]. The Canadian protocol includes defined media, defined inocula, and positive, negative, and sterile controls. Positive controls are defined as experimental tests that are known to produce significant oil degradation under the conditions of the test. The limitations of the protocol are that the inoculum concentration is very high, not reflective of environmental populations, and the incubation period is relatively short. The short incubation time could lead to poor results simply from lack of time for population development. The performance targets for the Canadian protocol are relatively low, with 35% of gas chromatography-total petroleum hydrocarbon (GC-TPH), 30% aliphatic and 10% aromatic loss being considered adequate. The French protocol is a field-scale protocol for determining the effectiveness of bioremediation in field plots. Laboratory testing is not addressed.

The following research was carried out to adapt the existing EPA/NETAC marine product protocol to freshwater conditions, to validate the revised protocol and to examine commercial products for effectiveness in the laboratory. These products are intended to enhance the biodegradation of petroleum hydrocarbons by supplementing nutrients, inoculating capable organisms, or mobilizing oil for greater accessibility. A product will eventually be required to submit effectiveness data using this protocol prior to being listed on the National Contingency Plan Product Schedule [6] for consideration in an oil-spill clean up action. Thus, the protocol must be usable with all classifications of products. For biostimulation products, which stimulate indigenous microbial populations through the addition of inorganic nutrients or starter compounds, the testing protocol must provide a standard inoculum to demonstrate that the nutrients supplied by the product are sufficient to support microbial degradation of oil. Bioaugmentation products, which add exogenous organisms, may require supplemental nutrients to ensure conditions are not limiting. In addition, the protocol must define good performance, be statistically sound and be repeatable by different laboratories.

Materials and methods

Most-probable-number analysis

The populations of oil degrading microorganisms in each flask were estimated using a differential most-probable-number (MPN) method. The MPN method of Wrenn and Venosa [28] produces estimates of numbers of alkane- and aromatic-hydrocarbon-degrading organisms. For both alkane and polynuclear aromatic hydrocarbon (PAH) degreder enumeration, the MPN was calculated using a computerized enumeration program [7].

Residual hydrocarbon analysis

Following removal of sample for MPN analysis, a recovery surrogate solution consisting of 5α-cholestane and D^{10}-phenanthrene was added to each reactor flask to obtain a final surrogate concentration of 4 ng/μl in the final extract. After addition of 50 ml dichloromethane (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_2SO4 to remove water. The DCM extracts were then exchanged into hexane under dry nitrogen for hydrocarbon analysis by gas chromatography-mass spectrometry (GC-MS) [25]. Concentrations of 28 alkanes and 32 PAHs (Table 1) were quantified using a Hewlett Packard 5890 Series II GC-MS with a 30 m×0.25 mm ID with 0.2-μm liquid phase DB5 column (Supelco, Supelco Park, Bellefonte, Penn.). Alkane and PAH concentrations were then summed and normalized to 17A(H), 21B(H)-hopane 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.

Culture development

A mixed consortium of microorganisms capable of degrading crude-oil components was developed in the laboratory. Soils and river sediments known to have a history of oil contamination were collected. Shake-flasks (250 ml) received 100 ml of Bushnell-Haas medium (BH) (Difco Laboratories, Ann Arbor, Mich.), 10 g wet weight of soil from each source, and 0.5 g of Alaskan North Slope oil. The oil was previously weathered by heating it in vacuo at 272 °C (521 °F) (ANS 521) to simulate oil that had weathered in the open air and to provide a standardized material for testing. This procedure removed alkanes lighter than C14 and aromatics lighter than C2 naphthalene. The flasks were incubated at 20 °C on an orbital shaker at 200 rpm for 28 days. At that time, 10 ml of liquid were transferred to fresh flasks with BH and oil. This enrichment procedure was repeated twice more and the flasks were then analyzed for bacterial populations and residual oil chemistry according to methods described above.

Table 1 Hydrocarbons analyzed to determine biodegradation of oil

Alkane hydrocarbons	Aromatic hydrocarbons
Decane	Naphthalene
Undecane	C1 naphthalene
Dodecane	C2 naphthalene
Tridecane	C3 naphthalene
Tetradecane	C4 naphthalene
Pentadecane	Phenanthrene
Hexadecane	Anthracene
Heptadecane	C1 phenanthrene
Pristane	C2 phenanthrene
Octadecane	C3 phenanthrene
Phytane	C4 phenanthrene
Nonadecane	Fluorene
Eicosane	C1 fluorene
Heneicosane	C2 fluorene
Docosane	C3 fluorene
Tricosane	Dibenzothiophene
Tetracosane	C1 dibenzothiophene
Pentacosane	C2 dibenzothiophene
Hexacosane	C3 dibenzothiophene
Heptacosane	Naphthobenzothiophene
Octacosane	C1 naphthobenzothiophene
Nonacosane	C2 naphthobenzothiophene
Triacontane	C3 naphthobenzothiophene
Untriacontane	Fluoranthene
Dotriacontane	Pyrene
Tritriacontane	C1 pyrene
Tetratriacontane	C2 pyrene
Pentatriacontane	Chrysene
	C1 chrysene
	C2 chrysene
	C3 chrysene
	C4 chrysene

Product test

Four commercial bioremediation products were selected for this work. Products labeled A and B were marketed as mineral nutrient additives, product C contained mineral nutrients and a sorbent carrier containing microbial spores, and product D was a bacterial stimulant. Product A was encased in an oleophilic coating, which enables attachment to an oil slick for greater bioavailability. Product B was an oleophilic liquid nutrient mixture with several organic components to keep the nutrients in suspension and promote attachment to the oil. In addition to being a nutrient supplement, product C was also a sorbent and reported to contain approximately 3.5×10^4 hydrocarbon-degrading organisms in spore form per gram of product. Product D was a liquid plant extract material with a surfactant intended to stimulate bacteria to a higher metabolic rate. This product was not specifically intended for use with crude oil, although the manufacturer suggested that it would be effective in stimulating bacterial activity.

The experimental design for this study included positive and negative controls and product treatments, each in triplicate. Sterile 250-ml Erlenmeyer flasks were filled with 100 ml freshwater media, 0.5 g ANS 521 and product per manufacturers' recommended dosages. The BH medium was prepared in the laboratory using the manufacturer's formula, but without KNO_3 for use with products known to supply nutrients. Each flask was inoculated with 1 ml of enriched culture unless the product claimed to contain an inoculum (product C only). Positive controls received culture and 0.5 g ANS 521 in 100 ml complete BH media. Negative controls consisted of flasks with BH medium and 0.5 g ANS 521 oil. Flasks were incubated on an orbital shaker table at 200 rpm and 20 °C. 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.

Respirometry

In addition to the flask experiment, a respirometry study was run concurrently to measure the oxygen (O_2) uptake and carbon dioxide (CO_2) production over the course of the experiment to provide supporting data for use in protocol development. The rate of microbial respiration is a direct measure of biological activity, and therefore directly correlates with the extent of hydrocarbon degradation within the system. The respirometry data were also useful in revealing the effect of bioremediation products on O_2 uptake and CO_2 production.

Triplicate reactors were set up for each treatment at the same concentrations used for the shake-flask experiment. Controls set up in addition to the product treatments included positive (nutrients, oil plus inoculum), negative (sterile, oil, and no nitrogen), and product with inoculum but no oil. The last control was to determine the O_2 uptake and CO_2 production due to product alone. Reactors were incubated at 20 °C in N-CON Model 512 respirometers (N-CON Instruments, Crawford, Ga.) with automated data recording. The total O_2 uptake within the reactor represents the amount of O_2 required by the microorganisms to metabolize the available substrate, oil plus product. The O_2 required to metabolize ANS 521 was estimated by subtracting the product O_2 demand from the total O_2 uptake at each time point (collected hourly) throughout the experiment.

Each reactor was equipped with a CO_2 trap containing 0.1 N KOH to collect evolved CO_2. The amount of CO_2 produced within the system was calculated at discrete time points from the pH of the

KOH solution. The KOH solution in the traps was replaced when the solution pH dropped below approximately 10.5, as indicated by a pH indicator, alizarin red. Curves were then fitted to the data to approximate the CO_2 produced in each reactor. The CO_2 curves were amended for CO_2 evolved due to the metabolism of the product without ANS 521.

Buffer tests

In addition to the other tests carried out with commercial bioremediation products, buffering compounds were evaluated. Most common biological media rely upon a phosphate buffer to control pH within incubation vessels. In some cases, it may be necessary to evaluate phosphorus dose effects on bioremediation product effectiveness. To that end, two non-phosphate buffers were tested for their effect on bioremediation product effectiveness in the protocol procedure. The buffers were HEPES {2-[4-(2-hydroxyethyl)-1-piperazine] ethanesulfonic acid, sodium salt} and MOPSO {3-(*N*-morpholino)-2-hydroxy-1-propanesulfonic acid} at 20 mM. In the test, the buffers were substituted for phosphate buffer in the BH solution. The product supplied the phosphorus needs of the organisms. The buffer test was done as previously described.

Results

MPN analysis

Figure 1 depicts the MPN estimates for alkane- and PAH-degrading organisms in flasks sacrificed at each sampling event. With the exception of product C which claimed to contain an inoculum, all other product and control flasks were inoculated with approximately $1{\times}10^3$ cells alkane-degrading organisms/ml and $1{\times}10^4$ cells PAH-degrading organisms/ml at $t = 0$. Flasks containing product C, which received no inoculum, revealed no significant number of PAH degraders (< 10 per ml) according to our method of MPN determination. The number of alkane-degrading organisms in product C treatments, when detected, was variable and one to three orders of magnitude lower than numbers found in other treatments. Some alkane degraders were detected in some flasks at days 21 and 28, indicating that longer incubation times may be required for this product.

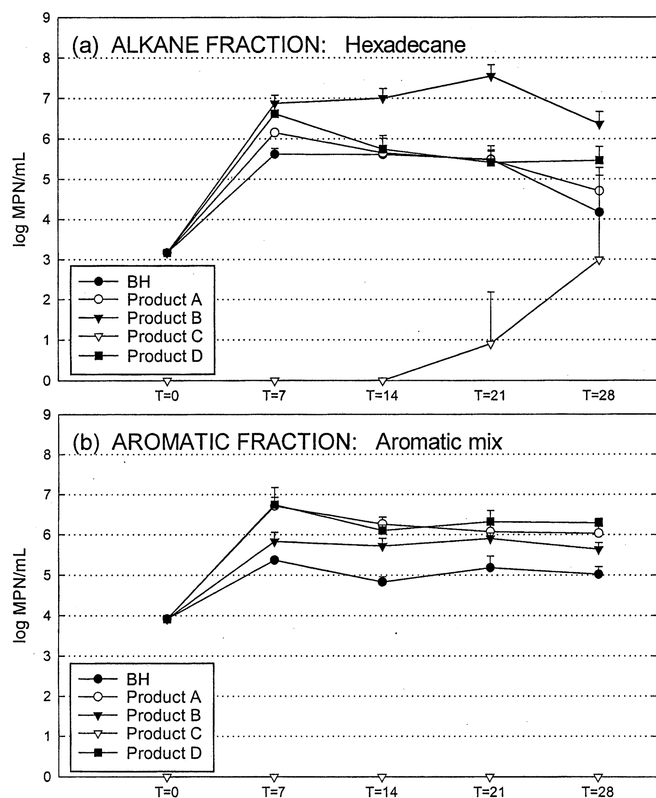

Fig. 1 Most-probable-number of (**a**) alkane- and (**b**) aromatic-hydrocarbon-degrading microorganisms incubated with bioremediation products or mineral nutrients and ANS 521 source oil

Residual oil analysis

Analysis of the residual hydrocarbon concentrations in the shake-flasks is given in Fig. 2. ANOVAs were carried out to determine whether product performance differed statistically from the control. Statistically different amounts of residual hydrocarbons are identified in Table 2. Flasks treated with product C had little removal of either class of hydrocarbons. Product D

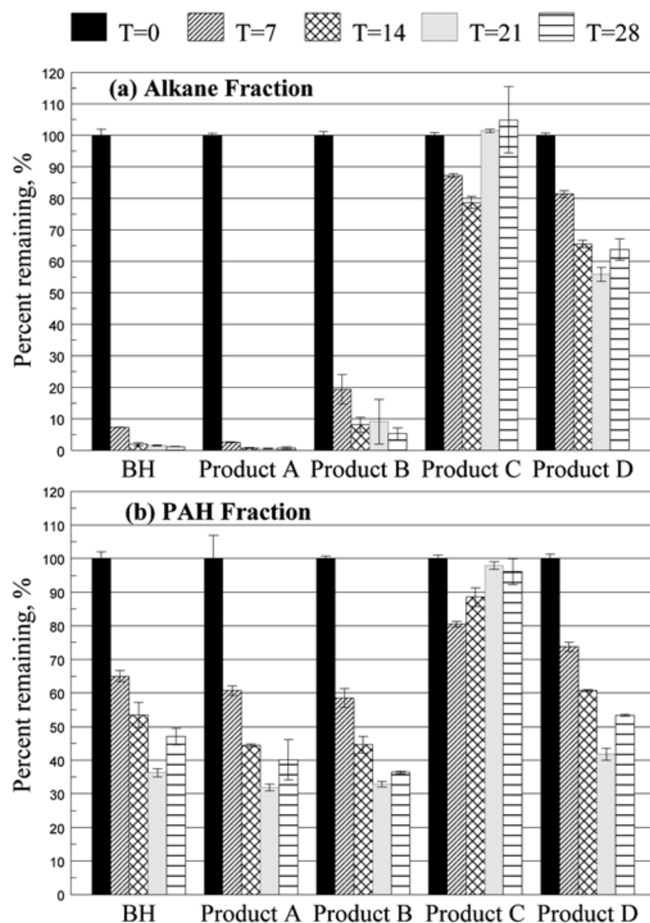

Fig. 2 Effect of bioremediation products on removal of (**a**) alkane and (**b**) aromatic hydrocarbons from ANS 521 crude oil

Table 2 Summary of day 28 results. *BH* Bushnell-Haas medium

	Alkane % remaining	SD	PAH % remaining	SD
BH vs	1.1	0.1	47.1	2.5
Product A	0.8[a]	0.3	40.2	6.0
Product B	5.2[a]	1.9	36.4[a]	0.4
Product C	101.5[a]	0.5	98.0[a]	1.1
Product D	63.7[a]	3.5	53.3[a]	0.3
	O_2 uptake (mmol)	SD	Corrected O_2 uptake (mmol)	SD
BH vs	84.3	2.6	84.3	2.6
Product A	92.2	7.5	87.6	7.5
Product B	88.4	3.5	46.8[a]	3.5
Product C	34.0[a]	2.5	10.4[a]	2.5
Product D	10.7[a]	6.3	5.6[a]	6.3
	CO_2 produced (mmol)	SD	Corrected CO_2 produced (mmol)	SD
BH vs	49.8	1.4	49.8	1.4
Product A	55.0	5.4	52.1	5.4
Product B	58.3[a]	2.0	22.3[a]	2.0
Product C	18.6[a]	1.3	4.5[a]	1.3
Product D	3.4[a]	0.5	1.2[a]	0.5

[a]Means differ significantly ($P < 0.05$) from the positive control BH; ANOVA α value = 0.05

treatments, which received the same inoculum as the control flasks and treatments A and B, demonstrated low to moderate removal efficiencies that were significantly lower than those of the control flasks.

Respirometry results

ANOVAs were carried out to determine whether oxygen uptake and CO_2 production differed between product treatments and controls. Table 2 shows that products B, C, and D consumed significantly less O_2 and produced significantly less CO_2 after correcting for metabolism of the product alone than either product A or the control.

Buffer test results

Substitution of HEPES and MOPSO for phosphate buffer in the incubation medium resulted in no statistical difference in hydrocarbon removal efficiencies among the three media tested with different buffers. Product A achieved 99.4% removal of total alkane hydrocarbons in each of the three media regardless of buffer used. PAH removal efficiencies were 61.0%, 61.6% and 58.4% for product A in modified BH, HEPES and MOPSO media, respectively. Product A treatments in modified BH yielded the highest respiration rate, with a total O_2 consumption of 90.4 ± 7.7 mmol/l after 28 days. HEPES and MOPSO treatments consumed 82.4 ± 4.7 mmol/l and 76.7 ± 7.2 mmol/l, respectively. Alkane removals observed for control and product A treatments were typical of removal efficiencies achieved in previous studies where nutrients were not limiting. Greater than 90% removal of total alkanes was achieved within 14 days of incubation, with greater than 98% removal achieved by day 28. Reduction of PAHs, however, was substantially lower. More extensive aromatic hydrocarbon degradation might be observed with longer incubation times.

Discussion

National Risk Management Research Laboratory undertook this research to modify the marine protocol for testing bioremediation products under freshwater conditions. In the modified protocol, the medium was chemically defined, positive controls were added, and residual oil analysis was simplified. The proposed Canadian freshwater protocol [4] differs in the incubation period, inoculum size, chemical analyses, oil content and suggested performance targets. A longer incubation period, 28 days, allows more time for growth of organisms that may be slow to metabolize some oil compounds, especially aromatic hydrocarbons. A smaller inoculum, on the order of 10^3–10^4 cells per ml rather than 10^6 per ml is more realistic in terms of what may be found in nature. The response of a product not carrying its own inoculum should reflect actual use conditions. The choice of chemical analyses to apply to determine residual oil content can be flexible. The Canadian protocol proposes gas chromatography-flame ionization (GC-FID) measurement of aliphatic, aromatic, and total petroleum hydrocarbons. Our laboratory uses GC-MS exclusively. Either GC-FID or GC-MS would be adequate for measurement of aliphatic and aromatic hydrocarbons. The protocol for testing freshwater bioremediation products should have sample times at least at 0 and 28 days. The starting oil content should be between 2 and 5 g/l. The oil content should be high enough to provide good distinction between starting analyte concentrations and final analyte concentrations especially with regard to aromatic hydrocarbons. Lower starting concentrations of oil may prevent adequate measurement of some of the lower concentration hydrocarbons. The medium should be a defined formula to enable interlaboratory comparison and to limit 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 has not occurred. Performance

targets must be high enough to ensure the ability to differentiate between good performance and poor performance. Our positive controls yielded >90% alkane and >35% aromatic hydrocarbon degradation in 28 days. Based on the findings of this study, reasonable performance goals for the protocol flask test would require greater than 75% reduction in alkanes and greater than 35% reduction in total PAHs within the 28-day incubation period.

Four commercial bioremediation products were tested using a freshwater Bushnell-Haas minimal salts medium for their ability to stimulate the removal of oil hydrocarbons. The BH control and product A treatment both effectively removed greater than 98% of total alkanes and 55–65% of total PAHs. Product B achieved comparable removal of PAHs, but was slightly less effective at treating alkanes. These results were confirmed by respirometry studies, which indicated similar respiration rates for BH and product A treatments. Reactors treated with product B revealed a significant O_2 demand due to the metabolism of the product itself, thus respiration due to the metabolism of oil components was less than for the control. Products C and D were least effective at stimulating the degradation of either class of hydrocarbons and exhibited minimal respiration due to metabolism of oil constituents.

The results of this study reveal that the protocol does effectively differentiate among products according to their performance. Thus, this protocol can be used as a tool to determine the potential of a bioremediation agent to enhance the biodegradation of crude-oil components in a freshwater system. According to the results of this study, buffer selection had no significant effect on protocol performance. Additional testing will be required to formulate a buffered medium that provides no phosphorus for use in the protocol. Further work will also be required to determine whether the performance goals suggested in this work are adequate to ensure that product performance is sufficient for the task of oil remediation. A broader array of products should be tested by multiple operators using both freshwater and marine methods to ensure better statistical reliability. A question to be answered is whether the positive control culture should perform as well as possible, if it should meet protocol performance goals, or if it should only show positive degradation of oil compounds. The positive control culture used in this work performed well in degradation of aliphatic hydrocarbons and moderately well in degradation of aromatic hydrocarbons. The positive control should probably perform as well as the targets specified in the protocol to have a consistent goal for product manufacturers to achieve. The protocol suggested here has improved upon the current seawater protocol in terms of defined media, positive control cultures, and simplified analytical chemistry. The performance targets are modest and should be easily achievable by manufacturers.

References

1. Aldrett S, Bonner JS, McDonald TJ, Mills MA, Autenrieth RL (1997) Degradation of crude oil enhanced by commercial microbial cultures. Proceedings of the 1997 International Oil Spill Conference, Ft. Lauderdale, Florida, April 7–10. American Petroleum Institute, Washington DC, pp 995–996
2. Atlas RM (1991) Microbial hydrocarbon degradation-bioremediation of oil spills. J Chem Tech Biotechnol 52:149–156
3. Bachoon DS, Araujo R, Molina M, RE Hodson (2001) Microbial community dynamics and evaluation of bioremediation strategies in oil-impacted salt marsh sediment microcosms. J Ind Microbiol Biotechnol 27:72–79
4. Blenkinsopp S, Sergy G, Wang Z, Finges MF, Foght J, Westlake DWS (1995) Oil spill bioremediation agents – Canadian efficacy test protocols. Proceedings of the 1995 International Oil Spill Conference, Feb 27–March 2, Long Beach, California. American Petroleum Institute, Washington DC, pp 91–96
5. Dott W, Fiedlieker D, Kampfer P, Schleibinger H, Strechel S (1989) Comparison of autochthonous bacteria and commercially available cultures with regard to their effectiveness in fuel oil degradation. J Ind Microbiol 4:365–374
6. Federal Register (1994) 40 CFR Chap. 1 (July 1, 1999 edn) Part 300, Appendix C. pp 234–245
7. Klee AJ (1993) A computer program for the determination of most probable number and its confidence limits. J Microbiol Methods 18:91–98
8. Lee K, Merlin FX, Swannell RPJ, Reilly T, Sveum P, Oudot J, Guillerme M, Ducreux J, Chaumery C (1995) A protocol for experimental assessments of bioremediation strategies on shorelines. Proceedings of the 1995 International Oil Spill Conference, Feb 27–March 2, Long Beach, California. American Petroleum Institute, Washington DC, pp 901–902
9. Lee K, Tremblay GH, Cobanli SE (1995) Bioremediation of oiled beach sediments: assessment of inorganic and organic fertilizers. Proceedings of the 1995 International Oil Spill Conference, Feb27–March 2, Long Beach, California. American Petroleum Institute, Washington DC, pp 107–113.
10. Lee K, Tremblay GH, Gauthier J, Cobanli SE, Griffin M (1997) Bioaugmentation and biostimulation: a paradox between laboratory and field results. Proceedings of the 1997 International Oil Spill Conference, April 7–10, Ft. Lauderdale, Florida. American Petroleum Institute, Washington DC, pp 697–706
11. Macnaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang Y-J, White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 65:3566–3574
12. Margesin R, Shinner F (1997a) Bioremediation of diesel-oil-contaminated alpine soils at low temperatures. Appl Microbiol Biotechnol 47:462–468
13. Margesin R, Shinner F (1997b) Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. Appl Environ Microbiol 63:2660–2664
14. Mearns AJ, Venosa AD, Lee K, Salazar M (1997) Field-testing bioremediation treating agents: lessons from an experimental oil spill. Proceedings of the 1997 International Oil Spill Conference, April 7–10, Ft. Lauderdale, Florida. American Petroleum Institute, Washington DC, pp 707–712
15. Merlin FX (1995) Devising an experimental protocol to evaluate the effectiveness of bioremediation procedures. Proceedings of the 2nd International Oil Spill Research and Development Forum, May 23–26. International Maritime Org, London, pp 37–44
16. Mishra S, Jyot J, Kuhad RC, Lal B (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl Environ Microbiol 67: 1675–1681

17. Neralla S, Weaver RW (1997) Inoculants and biodegradation of crude oil floating on marsh sediments. Bioremed J 1:89–96
18. National Environmental Technology Applications Corporation (1993) Oil spill bioremediation products testing protocol methods manual. University of Pittsburgh Applied Research Center, Pittsburgh, Pennsylvania
19. Swannell RPJ, Lee K, McDonagh M (1996) Field evaluation of marine oil spill bioremediation. Microbiol Rev 60:342–365
20. United States Coast Guard polluting incident compendium, cumulative data and graphics for oil spills, 1973–2000. United States Coast Guard, Washington DC
21. Venosa, AD, Haines JR, Nisamaneepong W, Govind R, Pradhan S, Siddique B (1990) Protocol for testing bioremediation products against weathered Alaskan crude oil. EPA 600/D-90/208. Risk Reduction Engineering Laboratory, USEPA, Cincinnati, Ohio
22. Venosa AD, Haines JR, Allen DM (1992) Efficacy of commercial inocula in enhancing biodegradation of weathered crude oil contaminating a Prince William Sound beach. J Ind Microbiol 10:1–11
23. Venosa AD, Kadkhodayan M, King DW, Wrenn BA, Haines JR, Herrington T, Strohmeier K, Suidan MT (1993) Testing the efficacy of oil spill bioremediation products. Proceedings of the 1993 International Oil Spill Conference, March 29–April1, Tampa, Florida. American Petroleum Institute, Washington DC, pp 487–494
24. Venosa AD, Suidan MT, Haines JR, Wrenn BA, Strohmeier KL, Eberhart BL, Kadkhodayan M, Holder E, King D, Anderson B (1995) Field bioremediation study: spilled crude oil on Fowler Beach, Delaware. In: Hinchee RE, Fredrickson J, Alleman BC (eds) Bioaugmentation for site remediation. Battelle, Columbus, Ohio, pp 49–56
25. Venosa AD, Haines JR, Eberhart BL (1997) Screening of bacterial products for their crude oil biodegradation effectiveness. In: Sheehan D (ed) Bioremediation protocols. Humana, Totowa, New Jersey, pp 47–58
26. Venosa AD, Suidan MT, Lee K, Cobanli SE, Garcia-Blanco S, Haines JR (2002) Bioremediation of oil-contaminated coastal freshwater and saltwater wetlands. Rhodes Conference, September 14–21, Rhodes Greece
27. Wood TM, Lehman RL, Bonner J (1997) Ecological impacts of a wetland oil spill and bioremediation experiments. Proceedings of the 1997 International Oil Spill Conference, April 7–10, Ft. Lauderdale, Florida, American Petroleum Institute, Washington DC, pp 415–421
28. Wrenn BA, Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can J Microbiol 42:252–258