

Field Evaluations of Marine Oil Spill Bioremediation

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INTRODUCTION

While accidental releases may contribute to only a small percentage of the oil released into the marine environment (95, 97), large accidental oil spills receive much attention and evoke considerable public concern (25, 66). Fortunately, such incidents occur rarely, although they can result in significant contamination of ocean and shoreline environments. For example, the *Amoco Cadiz* discharged 0.2 megatonnes of crude oil into the waters along the Brittany coast in 1978; the *Exxon Valdez* discharged 0.04 megatonnes into the Prince William Sound in 1989; the *Haven* caught fire and sank off the coast of Italy in 1991 with 0.14 megatonnes on board; and the *Braer* released approximately 0.08 megatonnes into the coastal waters of the Shetland Islands in 1993. Both the *Amoco Cadiz* and *Exxon Valdez* incidents contaminated a substantial length of coastline. Oil from the *Braer* spill posed a considerable threat to the coastal salmon farming industry, but fortunately the oil

has not persisted on the Shetland shoreline (60). Deliberate releases of oil can also cause considerable contamination. For example, during the Gulf War in 1991, 0.82 megatonnes of oil was released in Kuwait, threatening the desalination plants and coastal ecosystems of the Gulf (75).

These incidents have prompted the development and refinement of techniques for dealing with oil pollution both at sea and on shorelines. These include physical and chemical methods, which are fairly well established, and biological methods, which have been the subject of much debate and some research effort in recent years. A number of different technologies may fall into the category of biological methods; these include the use of straw or plant material as an absorbent for oil (109), biosurfactants to clean oiled surfaces (9), biological polymers to coat surfaces to prevent oil adhesion, and the addition of materials to encourage microbiological biodegradation of oil (111). It is the last procedure, known as bioremediation, which has received the most attention, notably after the *Exxon Valdez* incident (16, 81, 102).

As an oil spill countermeasure within the marine environment, bioremediation has been defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (111). Biodeg-

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radiation is known to be the principal natural process for the removal of the nonvolatile fraction of oil from the environment (76). Despite some favorable assessments of the technology (111), there is little consensus among the organizations charged with responding to oil spills as to its effectiveness. As the observed results of laboratory product tests may not be attained within the natural environment, where complex physical, chemical, and biological interactions occur (26), the most convincing demonstrations of bioremediation as an oil spill countermeasure are those which have been carried out under realistic field conditions.

The results of field investigations of various bioremediation techniques are reviewed in detail in this paper. Using this information, we have suggested operational guidelines for the use of bioremediation in response to a spill incident and have proposed research to refine the process. This document does not review the results of laboratory and mesocosm studies, because they have been the subject of several recent reviews (4, 5, 46, 48, 76).

In contrast to laboratory investigations, relatively few trials have been performed to test the effectiveness of bioremediation on oil spills in the field. This is because such trials are both difficult and expensive to conduct. Either experiments have to be carried out after a real spill incident or permission has to be obtained to release oil experimentally into the environment.

The first approach, based on "spills of opportunity," suffers from unpredictability. Scientists often have to work in inhospitable terrain, with little time for detailed preparation and with no control over the distribution of oil. Field trials at sea are virtually impossible to control without containing the slick in some way. When booms are used, they affect the normal behavior of the slick, compromising the validity of the data obtained. Experiments on contaminated shorelines following spill incidents are easier, but again the scientists have little choice in the type of beach, the concentration or type of oil, or the degree of weathering and emulsification of the oil. In short, the scientists must deal with conditions as they are presented and try to design carefully controlled experiments. This has been done with some success after recent spill incidents.

In contrast, controlled field trials allow the scientist to select the location of the experimental plots or enclosures, and the type, concentration, and degree of weathering of the oil. Such field studies have been limited by excessive cost and the difficulty in obtaining permits from regulatory agencies. Nevertheless, several research organizations have conducted controlled experimental oil spill field trials.

FIELD TRIALS

Field trials of bioremediation have been conducted both at sea and on beaches. However, owing to the complexity of working in open-water environments, beach trials predominate. For convenience, experiments at sea and on shorelines will be discussed separately. Each field trial is unique, as individual research groups have been constrained by different factors, such as the volume of oil permitted to be spilled and the availability of time and resources. Unfortunately, since few efforts have been made to standardize the experimental design and procedures used in field trials, only general conclusions can be drawn from the data.

Open-Water Studies

Most experiments to evaluate bioremediation efficacy have been carried out in laboratory mesocosm systems or small in situ enclosures. To counteract the effects of dilution in open-

water systems, most of the studies have focused on the development and evaluation of oleophilic formulations that maintain nutrients at the oil-water interface, where oil biodegradation activity occurs.

Tagger et al. (106) noted that added microorganisms disappeared rapidly from 10-m³ enclosures containing oiled seawater and that no increase in hydrocarbon-degrading potential was found. In the absence of added microorganisms under otherwise identical conditions, the natural microbial population adapted to hydrocarbon degradation after approximately 4 days. Atlas and Budosh (6) conducted experiments in floating Plexiglas cylinders in saline ponds at Barrow, Alaska. After 21 days of incubation of the cylinders in the saline pond, addition of oleophilic fertilizers (octyl phosphate and paraffinized urea) and an oil-degrading pseudomonad caused 65% removal of the Prudhoe Bay crude oil, whereas 51% was degraded in the presence of oleophilic fertilizers alone. In the poisoned control, 25% of the oil was lost by abiotic weathering. Addition of microorganisms alone caused only a 27% loss.

Horowitz and Atlas (40) carried out experiments in an open flowthrough system flushed continuously with seawater. The authors isolated a *Flavobacterium* sp. and a *Pseudomonas* sp. with hydrocarbon-degrading capabilities from Alaskan estuarine waters. Cultures of these organisms were grown on a mixture of oil and acetate before being freeze-dried. A fraction of these lyophilized cultures were also coated with octadecane to render them oleophilic. The experimental systems were treated with oleophilic nutrients and seeded with the prepared microbial inocula. The uncoated bacteria were found to be washed rapidly from the oil in the experimental system and did not enhance degradation, whereas the oleophilic strains enhanced the degradation of oil when added. It would have been interesting to determine the effect of killed oleophilic strains on oil biodegradation. This additional experiment would show whether the activity of the seeded organisms, rather than the influence of the additional nutrients provided with the inocula, stimulated the oil degradation.

Goldstein et al. (33) also noted that seeding had variable success in stimulating the breakdown of organic contaminants in nature. This was attributed to the following: (i) the concentration of the contaminant may be too low to support the growth of the inoculant, (ii) the concentration of the contaminant may be toxic to the inoculant, (iii) the added microorganisms may be susceptible to naturally occurring toxins or predators in the environment, and/or (iv) the inoculant may be unable to move through the environment to the contaminant. All of these difficulties could be encountered when seeding is used to treat oil spills, except that sufficient oil is normally present to support the added microorganisms.

Oliveiri et al. (71) tested the efficacy of a paraffin-supported MgNH₄PO₄ nutrient formulation to enhance the biodegradation rates of oil. Four 25-m² areas were delimited 300 m from shore within the Bay of Ortona, Italy, by a series of booms which penetrated 30 cm below the sea surface. A network of 5-cm mesh was laid on the water surface to prevent the crude oil drifting and sticking to the walls of the boom. A relatively small amount (0.25 kg) of topped Safir crude oil was applied to each boom. Two booms were treated with 2% (wt/wt) paraffin-supported fertilizer, and the other two booms were treated with paraffin only as controls. After 21 days, the nets and booms were recovered and washed with solvent and the oil content was determined. Approximately 60% of the added oil was recovered from the control plots, whereas 46% was recovered from the fertilized plots. In terms of chemical composition, the saturated fraction of the residual oil was degraded more extensively than the aromatic fraction. Thus, there is

TABLE 1. Composition of Inipol EAP 22 and Customblen^a

Ingredients	Chemical formula	Purpose
Inipol EAP 22 ^b		
Oleic acid	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$	Hydrophobic phase
Tri(laureth-4)-phosphate	$[\text{C}_{12}\text{H}_{25}(\text{OC}_2\text{H}_4)_3\text{O}]_3\text{PO}$	Phosphorus source and surfactant
2-Butoxyethanol	$\text{HO}-\text{C}_2\text{H}_4-\text{O}-\text{C}_4\text{H}_9$	Surfactant and emulsion stabilizer
Urea	$\text{NH}_2-\text{CO}-\text{NH}_2$	Nitrogen source
Water	H_2O	Solvent
Customblen ^c		
Ammonium nitrate	NH_4NO_3	Nitrogen source
Calcium phosphate	$\text{Ca}_3(\text{PO}_4)_2$	Phosphorus source
Ammonium phosphate	$(\text{NH}_4)_3\text{PO}_4$	Source of both nitrogen and phosphorus

^a Modified from reference 19.^b Elemental composition, 7.4% N and 0.7% P.^c Elemental composition, 28.0% N and 3.5% P.

some evidence that the addition of oleophilic nutrient stimulates oil biodegradation in open water. However, statistical verification of the study results was limited by the small number of experimental replicates and the short duration of the experiment.

Sirvins and Angles (96) briefly described a sea trial carried out in Antarctica with the oleophilic fertilizer Inipol EAP 22 (Elf Aquitaine, Artix, France) to treat Arabian light crude oil (Table 1). The results appeared to show that nutrient enrichment encouraged degradation, but too few data were provided to appraise the work critically. In response to these preliminary results, Sveum and Ladousse (100) carried out a field trial with Inipol EAP 22 in a lagoon system on the coast of Kings Bay, Spitsbergen, Norway. Statfjord crude oil (amount unspecified) was confined in large booms (3,250 m² for the fertilized slick and 800 m² for the control). Oil biodegradation was assessed by monitoring changes in the ratio of branched-chain (pristane and phytane) to straight-chain alkanes, assuming preferential metabolism of the straight-chain alkanes relative to their corresponding isoprenoids (2). The authors noted no difference in the biodegradation rate between the control slick and the treated slick over an 80 day period. They attributed the failure of bioremediation to the high photooxidation of the oil on the sea surface as a result of 24-h exposure to the Arctic sun. Evidence for a higher photooxidation rate of oil in the Arctic was supported by observations of a substantial increase in the oxygen content of the crude oil (0.1 to 10% [wt/wt] in 55 days) during the experiment, particularly in the oil amended with Inipol EAP 22. An alternative explanation, consistent with the data, is that the nutrients within Inipol EAP 22 dispersed fairly rapidly from the slick, making any stimulatory effect transient. The authors found no differences in the nitrogen content of the slicks 10 days after application and only small differences after 4 to 5 days. They concluded that Inipol EAP 22 was not effective for the treatment of oil slicks on open water in the Arctic.

Even though evidence has suggested that iron levels may limit oil biodegradation on the open sea under certain conditions (27), we have found no reference to the use of micronutrient additions (e.g., oleophilic sources of iron) in experimental field trials.

Shoreline Studies

In comparison with open-water experiments, many more bioremediation field trials have been carried out on shorelines. These experiments varied considerably with respect to geographic area, oil type and concentration, study site dimensions, position of oil on the shore, sediment composition, and the

analytical methods used to estimate biodegradation. The trials can be classified into three groups: (i) application of inorganic fertilizers, (ii) application of organic fertilizers, and (iii) application of specific microbes (seeding or bioaugmentation).

Application of inorganic fertilizers. One of the first field trials was carried out in Spitsbergen, Norway, in 1976 (90, 92). An oil spill was simulated on a seashore by spreading 10 liters of unweathered Forcados crude per m² on to two 10-m² test sites. One oiled site was treated with 0.1 kg of an unspecified commercially available fertilizer per m² at an application rate of 1.2% (wt/vol) N. The fertilizer stimulated oil biodegradation as measured by the *n*-C₁₇/pristane and *n*-C₁₈/phytane ratios and by the respiration rate of indigenous microorganisms (90). A threefold increase in microbial respiration rate was sustained until the summer of 1979. By the end of 1983, however, the oil on the unfertilized control plot biodegraded to a degree very similar to that observed on the fertilized plot (92). This experiment demonstrated the feasibility of bioremediation to accelerate the rate but not the extent of biodegradation.

The Baffin Island Oil Spill Project sponsored a multidisciplinary field study between 1980 and 1983 in Canada's eastern Arctic at Cape Hatt, on the northern end of Baffin Island. A 45-m³ volume (45,000 litres) of a sweet medium-gravity crude oil was released in a typical coastal arctic environment for purposes of scientific investigation (93). The experimental spills were monitored to quantitatively assess and compare the short- and long-term fate and effects of chemically dispersed oil and a beached oil slick, as well as the effectiveness of shoreline cleanup techniques including in situ burning, dispersant application, solidification, mixing, and bioremediation (28, 29, 74, 91).

Sendstad et al. (91) carried out the initial Baffin Island Oil Spill Project bioremediation experiments within the supratidal zone. Four experimental plots within the supratidal zone were each treated with a 50% oil-water emulsion of lightly weathered Venezuela Lago Medco crude oil at an application rate of 20 liters/m². One plot was left untreated as a control, and three were treated with a commercial agricultural fertilizer (Norsk Hydro, fullgjødsele C). At the start of the study, one plot was treated with 6.4 g of N/m² and the other two were treated with 10 times this amount; one was also tilled regularly.

At the start of the experiment, the highest available nutrient concentrations were observed in the tilled plot, which had received the largest amount of fertilizer. Tilling caused the oil and nutrient to penetrate more deeply into the subsurface (10 cm depth). Of the untilled plots, the one treated with 64 g of N/m² had less nitrogen retained within the surface layer

(depth, 0 to 5 cm) than did the plot which received 1/10 of this application. Surface sediments within the tilled plot contained only 1.5 times the nitrogen content of the plot treated with 1/10 the nutrient concentration. Hence, this experiment demonstrated that retention of nutrients within the sediments was not proportional to the amount added. Nevertheless, addition of nutrients resulted in an increase in the number of oil-degrading bacteria (after 17 days) and respiratory carbon dioxide production (after 23 to 24 days) in comparison with the untreated controls. Although little difference in oil concentration was noted between the nutrient-treated and control plots after 25 days, the results suggest that bioremediation may have some potential, even in an Arctic environment, where summer temperatures ranged from 5.7 to 10.7°C.

A 2-year subsequent follow-up study at the same field site (Bay 102), which became covered by sand and gravel because of unanticipated high tides and heavy wave activity, showed that the largest bacterial populations were sustained within the plot which received the greatest amount of fertilizer (28). Furthermore, under the conditions existing in the backshore of this high-energy beach, burial of the plots under 15 to 20 cm of sand did not seem to have been inhibitory to the development of substantial populations of bacteria in the oiled sediments. Nutrient-mediated enhancement of natural oil biodegradation rates was confirmed, after 2 years of exposure, by significant observed changes in the alkane/isoprenoid ratio in the residual oil. The combination of fertilization and mechanical mixing of oil and fertilizer into the sediment seemed to offer the best conditions which supported the highest rates of biodegradation, possibly because of improved oxygen and nutrient availability.

Experiments were also conducted within the Baffin Island Oil Spill Project to study the effectiveness of nutrient enhancement on the biodegradation of oil stranded within a low-energy beach environment (28, 29). Two small experimental plots (1 by 2 m) were established approximately 10 m up from the swash line. One plot was covered by 10 kg of the Lago Medio crude oil per m², while the other was treated with an oil-water emulsion (1:1). Because of the very low absorptive properties of the sediments, only about 10 kg of the oil-emulsion per m² could be applied. Each plot was divided in half, and one half was fertilized with 100 g of Norsk Hydro fullgjødse C per m². The scientific integrity of the results obtained is in question because of the absence of experimental plot replication and rigorous statistical analysis and the lack of a separation area between the fertilized and unfertilized parts of each plot (to reduce the danger of cross-contamination). Nevertheless, on the basis of the limited evidence, a stimulatory effect of fertilization on oil biodegradation was observed in both biological (oil-degrading bacterial numbers, respiration rates) and chemical (gas chromatography [GC] analysis) parameters.

Lee and Levy (49) studied the degradation of Scotian Shelf Condensate (SSC) and Hibernia Crude Oil (HCO) on a sandy beach site in Nova Scotia, Canada. Considering the importance of maintaining optimal nutrient concentrations within the sediments for effective bioremediation (10), this study used periodic additions of an inorganic agricultural fertilizer mixture (N/P/K ratio, 10:1:0), composed of "prilled ammonium nitrate" and "granular super-phosphate." To minimize the physical loss of oiled sediments caused by tidal action, the experiments were conducted with in situ sediment enclosures (Nitex bags; 264-µm mesh). Each treatment was replicated, and the bags were buried in the beach parallel to the shoreline, in random order, 1.7 m apart. The inorganic fertilizer mixture (4.8%, wt/vol) was applied 2 weeks after the addition of the oil

to the sand beach (1.14 liters/m²) and subsequently after each sampling period.

Detailed time series data collected by Lee and Levy (49) demonstrated clearly that bioremediation by periodic additions of inorganic fertilizers (following each routine sampling event) increased the removal rate of contaminant oil from beaches. The addition of the inorganic fertilizer substantially stimulated the rate of disappearance of the oils. The C₁₇/pristane ratios in the plots treated with the SSC or the HCO showed a steady decline over the 8- to 12-month duration of the experiment. On the basis of the concentration of nonadecane (n-C₁₉), 23% of the SSC oil remained on the unfertilized plot after 178 days, in comparison with undetectable levels on the plot treated with agricultural fertilizer. Similar results were obtained with the HCO oil. The addition of agricultural fertilizers also increased the rate of hexadecane mineralization in sand treated with each oil. Fertilizer application also encouraged the number of HCO-degrading bacteria (as measured by the most-probable-number technique). Furthermore, the authors noted that although the total numbers of heterotrophic bacteria were not affected by winter temperatures, the numbers of oil degraders declined in all the oil-treated plots during the winter months.

Lee and Levy (51) studied the fate of a waxy crude oil (Terra Nova) on both a sandy beach and a salt marsh environment on the coast of Nova Scotia, Canada. Waxy crude oils are viscous and fairly resistant to biodegradation owing to their high content of long-chain aliphatic compounds (10). The salt marsh environment consisted of a fine sediment with the cordgrass *Spartina alterniflora*, fibrous plant matter, and silt. The oxygen penetration depth was approximately 1 m in the sand beach and 0.1 m in the salt marsh under study. Terra Nova crude oil was added at two concentrations, 3% and 0.3% (vol/vol), to the sand retained in Nitex bags. Bioremediation was conducted on some of the oiled plots with the same 10:1:0 (N/P/K ratio) inorganic agricultural fertilizer formulation described by Lee and Levy (49). Two concentrations of the fertilizer were used: 0.34 and 1.36 g/liter of sediment. In this experiment, the oil was added to the sand 8 days before the fertilizer to allow weathering of the crude oil and adaptation of the indigenous microflora. The biodegradation of oil was studied by monitoring the aliphatic hydrocarbon content of the oil and quantifying the C₁₇/pristane ratio.

On the sandy beach, at the 0.3% oil concentration, oil biodegradation proceeded rapidly in both the fertilized plot and the unfertilized control. Therefore, the authors concluded that the natural nutrient concentration was not limiting the biodegradation of oil at the lower concentration and hence a bioremediation strategy was not required. However, at the higher oil concentrations (3%) on the sandy beach, natural biodegradation rates appeared to be nutrient limited, since the addition of nutrients promoted changes in oil composition consistent with that of enhanced biodegradation, as indicated by a dramatic decline in the C₁₇/pristane ratio.

Detailed GC-mass spectroscopy (MS) analysis of samples from this experiment (conducted after the publication of the paper) have shown that the concentrations of most nonpolar and aromatic compounds in the oiled beach sediments decreased over the duration of the experiment, regardless of treatment. However, the removal rates and responses to agricultural fertilizer treatment varied, reflecting the physical and chemical characteristics of the individual oil components. Removal of oil, through direct loss of oiled substrate, was determined by monitoring changes in a number of conservative oil biomarker compounds that are geochemically stable and relatively nonvolatile (boiling point, <300°C), exhibit very low

TABLE 2. Comparison of turnover times for alkanes, alkylcyclohexanes, and PAH by treatment with agricultural fertilizer

Compound	Boiling point (°C)	Turnover time (days)		Ratio of turnover times ^a
		No fertilizer	Fertilizer added	
<i>n</i> -C ₁₂	216	731	192	3.81
Naphthalene	218	247	221	1.12
<i>n</i> -C ₁₃	235	455	188	2.42
2-Methylnaphthalene	241	193	218	0.89
1-Methylnaphthalene	244	209	212	0.99
<i>n</i> -C ₁₄	253	395	186	2.12
2-Ethylnaphthalene	258	480	220	2.18
1-Ethylnaphthalene	259	559	166	3.37
<i>n</i> -C ₁₅	271	361	189	1.91
C ₃ -Naphthalene	263	381	205	1.86
Acenaphthylene	279	533	187	2.85
<i>n</i> -C ₁₆	287	354	195	1.82
<i>n</i> -C ₁₀ cyclohexane	294	540	219	2.47
<i>n</i> -C ₁₇	301	353	209	1.69
<i>n</i> -C ₁₈	316	367	206	1.78
<i>n</i> -C ₁₉	330	370	212	1.75
Phenanthrene	340	330	189	1.75
Anthracene	340	239	222	1.08
<i>n</i> -C ₂₀	345	401	218	1.84
<i>n</i> -C ₁₄ cyclohexane	355	645	352	1.83
<i>n</i> -C ₂₂	369	400	230	1.74
<i>n</i> -C ₂₄	391	512	261	1.96
<i>n</i> -C ₂₆	411	491	247	1.99
<i>n</i> -C ₂₈	432	726	243	2.99
<i>n</i> -C ₃₀	450	1,165	268	4.35
<i>n</i> -C ₃₂	467	1,261	293	4.30
<i>n</i> -C ₃₄	473	1,606	366	4.39

^a Ratio of turnover time without fertilizer to turnover time with fertilizer.

water solubility, and are resistant to biodegradation. Changes in concentration (per gram of oiled sediment) with time for these stable compounds are indicative of whole-oil loss. The trends for the time series plots for the isoprenoids (pristane and phytane), hopanes (17 α ,21 β -hopane), steranes (sterane S4: 24-ethyl-13 β ,17 α -diacholestane plus 14 β ,17 β -cholestane), and four-ring polycyclic aromatic hydrocarbons (benz[a]anthracene plus chrysene) all indicated bulk oil losses of between 10 and 15%. For all of the selected biomarkers, there was no significant difference between the nutrient-treated and untreated plots, suggesting that these chemical compounds were resistant to the microbial population stimulated by fertilizer additions over the 190 days of the bioremediation experiment.

Other than physical loss of oiled substrate, the principal removal mechanisms for the individual oil components include change of state (evaporation, dissolution), chemical degradation (oxidation, photolysis), and biodegradation processes (77). GC-MS analysis showed that the removal of many compounds is accelerated by nutrient treatment. Typically, turnover time decreased with fertilizer treatment for a range of selected compounds, the exceptions being 1-methylnaphthalene and 2-methylnaphthalene (Table 2).

The trends in the removal rates for *n*-alkanes are presented in Fig. 1. The data show that the disappearance of the low-molecular-weight alkanes is stimulated by fertilizer addition. It is generally thought that the *n*-alkanes of shorter chain length are more easily used as an energy source than are longer-chain alkanes (83). Recently, Setti et al. (94) have suggested that the degradation of alkanes within a heavy oil by a pseudomonad could be divided into three categories: liquid alkanes (C₁₂ to

C₁₆) are most rapidly degraded, followed by C₁₇ to C₂₈ alkanes and high-molecular-weight alkanes above C₂₈. However, the substantial increase in the rate of loss of the higher-molecular-weight alkanes (>C₂₆) is interesting (Table 2, Fig. 1). It is possible that another mechanism besides direct biodegradation, such as enhanced physicochemical removal caused indirectly by nutrient addition, is involved. Certainly, Bragg et al. (17) suggested that more oil was lost from a fertilized Alaskan beach than could be accounted for by enhanced biodegradation alone, and they postulated that enhanced physical oil loss was responsible. This phenomenon warrants further study.

Periodic additions of 1.36 g of nutrients per liter of sediment in the sand beach environment did not enhance the degradation any more than the lower nutrient concentration (periodic application of 0.34 g of nutrients per liter), suggesting that this application rate (subsequently calculated as 1.2 g of nutrient per m² per day) provided sufficient nitrogen and phosphorus concentrations to maintain optimal oil biodegradation rates during the experiment (51). This hypothesis was to some extent also supported by observations of higher hexadecane mineralization rates in the fertilized plots treated with higher concentrations of oil. Again, the numbers of hydrocarbon-degrading bacteria increased in response to oil addition but addition of nutrients did not result in a further increase. The authors also confirmed observations from their previous study (49), which indicated that the number of hydrocarbon degraders declined during the winter months.

On the salt marsh sediment, the results were quite different (51). The addition of nutrient to sediments containing the lower (0.3%, vol/vol) oil concentration resulted in enhanced rates of biodegradation. This suggests that in salt marshes, the rate of petroleum biodegradation is limited by nutrient availability even at low oil levels. At the higher level of oil addition (3%, vol/vol), little oil degradation was found even after fertilizer addition. The authors concluded that at these concentrations, the oil penetrated to the anoxic layer of the sediment and anoxia restricted hydrocarbon degradation.

The results of this study show clearly that the success of bioremediation will depend on the nature of the contaminated shoreline. On the sandy beach, the microorganisms are apparently carbon limited and respond to oil addition by proliferating. At low concentrations of oil, toxicity is reduced and the levels of nitrogen and phosphorus are sufficient to result in rapid oil biodegradation. Under these conditions, the authors recommend no treatment, because bioremediation would not result in any additional benefit. At higher oil levels, the sand microbial community eventually becomes nutrient limited, and bioremediation, by the addition of nutrients (inorganic ammo-

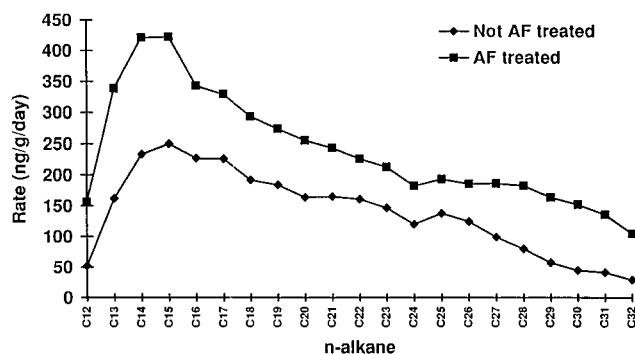


FIG. 1. Removal rate of *n*-alkanes from oiled sandy plots in Long Cove, Nova Scotia, Canada. AF, agricultural fertilizer.

nium, nitrate, and phosphate), stimulates biodegradation, causing enhanced rates of oil removal from the beach.

In salt marshes, the microbial community is not apparently starved of carbon but is limited by other nutrients such as nitrogen and phosphorus. Therefore, to encourage the degradation of even low concentrations of oil, sources of nutrients must be applied. Oil degradation was inhibited in the salt marsh sediments at higher concentrations, probably because of the penetration of oil into the anoxic layers of the sediment. In such cases, the addition of oxygen in some form may also be required as a part of the bioremediation strategy.

This work also substantiates previous work (49) that regular additions of inorganic nutrient are effective at sustaining enhanced biodegradation rates of oil. At present, the field work has not provided a method for determining when nutrients need to be reapplied to maintain optimal biodegradation rates. Lee and Levy (51) merely applied the nutrient after each sampling period. A more precise approach may be required for use of bioremediation in the field, perhaps based on regular measurements of the dissolved nutrient concentrations within the interstitial waters of the beach (16).

Application of organic fertilizers. In response to the *Amoco Cadiz* spill on the Brittany coast, Elf Aquitaine (France) developed an oleophilic microemulsion containing a solution of urea in brine, encapsulated in oleic acid and lauryl phosphate, called Inipol EAP 22 (96, 110). Inipol EAP 22 has two effects on petroleum: it apparently helps prevent the formation of water-in-oil emulsions by reducing the oil viscosity and interfacial tension, and it encourages oil biodegradation by supplying nutrients (45). Initial experiments with four different crude oils were conducted in four 18-m³ tanks filled with seawater (110). In each experiment, a stimulation of oil biodegradation was noted after 7 days in temperate conditions. Even at low temperatures of 3 to 8°C, some degree of enhancement was noted, and the optimal dose of the nutrient appeared to be 10% (wt/wt) of the oil, a much larger amount than that required for inorganic nutrients. Later studies used 5% (wt/wt) Inipol EAP 22 (101).

Halmö (35) conducted a field trial in 1983 in the supratidal zone. Three plots (4 by 2 m) were covered with oil emulsion (1:1 [wt/wt] mixture of weathered Statfjord crude oil and seawater) applied in even layers of 20 liters/m². One plot was treated with the Inipol EAP 22 at 10% (wt/wt) fertilizer-oil, the second was treated with a water-soluble commercial grade nitrogenous fertilizer at 4.5% (wt/wt), and the third remained untreated as a control. The oil/nitrogen ratio was the same for both the treated plots. Each test plot was divided geometrically into 200 subplots that were sampled at random with a soil corer over a 12-month period.

In response to the oil addition, the total number of microorganisms increased and remained large for the duration of the experiment. While the statistical methods were not identified, Halmö (35) reported that fertilization increased the degradation of the aliphatic fraction significantly. In the first 4 months, 45 to 85% of the saturated aliphatics were removed. The long-chain (>C₂₀) aliphatics were the most recalcitrant. After a year, no aliphatics could be detected in the fertilized plots whereas 10 to 25% remained in the untreated control, and the ratio of normal to branched hydrocarbons (C₁₇/pristane and C₁₈/phytane ratios) showed clear differences between the treatments and the controls. There was no significant difference between the rates of hydrocarbon removal from each of the fertilized plots. Hence, this field trial demonstrated that a single application of water-soluble or oleophilic sources of nitrogen could enhance the degradation of oil emulsions when coating the supratidal zone to an initial depth of 20 mm.

Further field experiments were conducted with Inipol EAP 22 by using different sediment types (100). One experiment was conducted on sandy sediments in plastic containers (0.5 m² and 0.2 m deep) in Kings Bay, Spitsbergen, Norway. Tidal flow was simulated with seawater from the adjacent fjord. Crude oil was applied to two tanks, one of which was treated with Inipol EAP 22; the other tank remained untreated as a control. The authors also reported the results from two similar experiments carried out on fine-grained lagoon shoreline sediments. Gas oil was applied at 6 liters/m² (6 mm thick) in the intertidal zone of the beach. In each case, an untreated control plot was compared with a plot fertilized with Inipol EAP 22 on the ebb tide. Presumably only one application of Inipol EAP 22 was made, although this was not stated.

The results of these studies suggested that applying Inipol EAP 22 to what are described as sandy and mixed-sand-and-gravel sediments stimulated oil biodegradation (100). After 50 days, there were reductions in both the pristane/C₁₇ and phytane/C₁₈ ratios in comparison with controls, suggesting enhanced biodegradation on the fertilized plots. However, Inipol EAP 22 addition did not encourage oil biodegradation in the fine-grained lagoon sediments. The authors suggested that the reason for these differences was related to the physical behavior of the Inipol EAP 22 on the two sediment types. They speculated that on the coarse sand the Inipol EAP 22 had a better opportunity to partition with the oil whereas on fine-grained material the fertilizer was more likely to be removed by the tide, presumably because of the high viscosity of the treatment. No nutrient measurements were reported to support this theory.

Inipol EAP 22 was also used in response to a small accidental gas spill (88,000 liters) from storage tanks into Kings Bay, Spitsbergen, in November 1985 (98, 100). The oil became stranded on coarse sediments and was initially allowed to weather for 2 months. One plot was then treated manually with Inipol EAP 22, and one plot remained untreated as a control. Four further experimental plots were oiled in the following summer; three were treated with Inipol, and the fourth remained untreated as a control. The ratios of normal to branched hydrocarbons (C₁₇/pristane and C₁₈/phytane ratios) were used once again to illustrate the success of the treatment. The authors estimated a two- to threefold increase in biodegradation of the oil on the fertilized portion of the slick in comparison with the unamended portion. After 90 days, the C₁₇/pristane ratio was less than 1.0 in the treated portion and greater than 2.0 in the control portion. In the trials conducted in the summer, stimulation of biodegradation was noted but was not as high as that found with the oil spilled over the winter months. However, the authors found that the addition of Inipol EAP 22 increased the total number of microorganisms within the beach material and noted that it caused a substantial increase in CO₂ production from the fertilized oily material in comparison with the unfertilized controls. This latter observation may be a result of increased oil mineralization or the degradation of the oleic acid portion of the nutrient. Nonetheless, these results appear to confirm the utility of Inipol EAP 22 for encouraging the biodegradation of petroleum that has spilled onto coarse oiled beach material.

An unnamed oleophilic fertilizer was used in an attempt to enhance biodegradation in a peaty mangrove soil (88). Two adjacent plots (2 m²) were treated with 5 liters of light Arabian crude oil per m². One of the two plots was also treated with an oleophilic fertilizer, while the other remained untreated as a control. At the time of oil addition, the soil was submerged beneath 30 cm of water. Generally, only the uppermost 1 cm of the soil was thought to be aerobic, except when the soil was

exposed to the air, which occurred periodically during the dry season.

During the first 3 months of the trial, which occurred toward the end of the dry season, the authors implied that the soil was exposed to the air regularly, although the length of time is not recorded (88). During this period, there was some evidence of enhanced oil biodegradation. The C_{17} /pristane and C_{18} /phytane ratios were reduced in the oil treated with fertilizer to a greater extent than in the control at the three depths measured within the soil. However, during the following 8 months, which were in the wet season, biodegradation was observed only in the uppermost layer (0 to 5 cm) of sediment. This was probably a result of oxygen deprivation in the lower layers, because the soil was completely covered with water during this period (88). Overall, even the fertilized oil decomposed slowly, suggesting that oil contamination is likely to persist in mangrove soil. Low oxygen concentrations may be one cause of the low rates. A second may be the low pH of the peaty soils, which was not recorded by the authors, and the possible strong adsorption of the oil onto the sediment, reducing its availability for microbial decomposition.

Studies of the biodegradation of a light crude oil (Scotian Shelf Condensate) have been conducted on a low-energy sand beach in a sheltered cove (Long Cove, Nova Scotia, Canada) (48, 52). The sand consisted of well-sorted, medium-fine material (diameter, approximately 250 μ m) and was oxygenated to a depth of approximately 1 m. Sand was placed in situ in stainless steel enclosures (surface area, 0.064 m²; volume, 0.006 m³). Two replicate sets of trays were buried in random order following experimental treatment, flush with the existing beach topography, midway between the high and low water marks, and secured to wooden stakes. Sand was mixed with 200 ml of oil (equivalent to 3.13 liters/m² of beach) and combinations of 20 ml of Inipol EAP 22 and 100 ml of hydrated bacteria (strains of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *Bacillus subtilis* grown on bran).

The authors found larger numbers of bacteria (measured as most probable numbers) on the oil-treated sands than on the untreated control but found little difference between the oiled controls and those treated with bacteria. The number of condensate-degrading bacteria increased after a 10- to 15-day lag period as a result of oil addition and of the addition of oil and nutrient. Addition of nutrient alone did not stimulate the numbers of oil-degrading bacteria. Similarly, seeding of unoled sand, with and without fertilizer, failed to encourage oil-degrading microorganisms. Differences in the growth response between the seeded oiled plots and the fertilized oiled plots led the authors to speculate that a different population of microorganisms was encouraged within the seeded plots, although taxonomic analysis was not carried out to confirm this hypothesis.

The lag period noted in this research suggested that the number of oil-degrading bacteria did not increase until toxic volatile hydrocarbons had evaporated. The Scotian Shelf Condensate used in this work was an unweathered light crude oil containing naphthalenic compounds and low-molecular-weight alkanes, both of which are known to be toxic to microorganisms. The unweathered nature of the crude oil was confirmed by the rapid loss of oil (71 to 82%) in all plots over the first 4 days (48). Unfortunately, owing to high inter- and intraplot variability (caused by tide-driven migration of sand in and out of the enclosures), no substantial differences in oil concentration could be detected between treatments.

Measurement of the C_{17} /pristane ratio showed that the plot seeded with bacteria and nutrient experienced substantial degradation of the condensate after 60 days of treatment. This was

not observed in the plot which received oil and Inipol EAP 22 only. The failure of the nutrient alone to increase the degradation of the condensate could be a result of the rapid decline in nitrogen levels recorded in the fertilized plots. The nitrogen content declined to background levels 2 days after application of Inipol EAP 22. On the basis of these initial observations, Lee and Levy (48) suggested that repeated additions of Inipol EAP 22 may be more successful at encouraging the biodegradation of Scotian Shelf Condensate on shorelines. This hypothesis was studied in the next experiment, which used sediment enclosures with similar dimensions constructed of Nitex (264- μ m mesh) to control the physical loss of oiled sediments attributed to tidal activity (52). Two concentrations of oil (3.13 and 0.31 liter/m²) were mixed with the sand, and the oiled sediment was allowed to weather for 19 days prior to nutrient treatment.

The effect of intermittent additions of Inipol EAP 22 was not to stimulate the degradation of oil when measured on the basis of changes in the C_{17} /pristane ratio. There were no differences between the decrease in the ratio measured in the unfertilized controls and those noted in the fertilized treatments. In fact, at the lower concentration of oil, the addition of Inipol EAP 22 appeared to reduce the oil biodegradation rate temporarily. The authors speculated that this may be the result of the microorganisms biodegrading preferentially the oleic acid portion of the fertilizer. This observation has since been confirmed in laboratory experiments by Rivet et al. (84), who observed that when Inipol EAP 22 was added with hexadecane to a culture of *Marinobacter hydrocarbonoclasticus*, the microorganism biodegraded the oleic acid in the fertilizer in preference to biodegrading hexadecane.

Lee and Levy (52) also monitored the mineralization of uniformly labeled [¹⁴C]hexadecane as an indication of changes in hydrocarbon degradation and found no consistent increases in hydrocarbon metabolism. In fact, the Inipol EAP 22 reduced hexadecane degradation in these field experiments. This suggests that when Inipol EAP 22 is added repeatedly, the microorganisms tend to concentrate on biodegrading organic constituents within the oleophilic fertilizer (e.g., fatty acids) in preference to aliphatic components of the oil. This potential drawback of the use of oleophilic fertilizers has been noted by others (17, 70, 71). Lee and Levy (52) also studied the oil-degrading population and noted that the addition of oil resulted in an increase in the number of potential oil degraders. However, there was no consistent significant difference between the unfertilized control and the fertilized plots.

Similar results were obtained when the experiment was repeated with both SSC and HCO (49). Regular addition of Inipol EAP 22 did not stimulate oil-degrading microorganisms or the rate of mineralization of hexadecane.

In summary, the results of these field trials with SSC and HCO (48, 49, 52) contrast with those described earlier, which demonstrated that Inipol EAP 22 enhanced oil degradation on shorelines (35, 98, 100). Both groups have used similar chemical analyses to draw their conclusions. The main differences were in the type and concentration of oil used and the type of shoreline sediments studied. These differences will be considered in turn.

It is possible that the SSC and HCO condensate is a more toxic oil than the Statfjord crude and gas oils studied in Norway, although this toxicity would have to persist even after weathering for 10 to 19 days, implying that such components were nonvolatile. Indeed, detailed physicochemical studies conducted by Strain (97) showed that the very light components of SSC, such as C_7 and C_8 acyclic and cyclic saturated hydrocarbons, can persist for more than 6 months within the

intertidal zone of the sandy beaches used in the studies by Lee and Levy (48, 49, 52). Furthermore, in addition to differences in the microbial populations, potentially toxic compounds could be generated as a result of photooxidation of the oil (100). Evidence in support of this hypothesis can be confirmed only by further research.

There were large differences in the oil concentrations used in these experiments. Lee and Levy (48, 52) used between 3.13 and 0.31 liter/m², whereas the Norwegian work was conducted at concentrations of 6 to 20 liters/m². Perhaps at these higher concentrations the nutrient deficiency encountered on the shore is more pronounced and therefore nutrient addition will have a more profound effect. However, without detailed analyses of the nutrient content on the beach, it is impossible to draw firm conclusions. Future research should record the nutrient content in the beach pore water to determine the success of the fertilizer application and to facilitate comparisons between different field trials.

Perhaps the most likely explanation may be the inability of Inipol EAP 22 to remain associated with the petroleum hydrocarbons on certain beach types. For example, Sveum and Ladousse (100) noted that Inipol EAP 22 was effective on cobble and coarse sand but not on fine-grained sediments. Similarly, Lee and Levy (48) found that the nitrogen in Inipol EAP 22 was lost within 2 days from the medium-fine sand (approximately 250 μ m in diameter) used in their field trials. Repeated addition of Inipol EAP 22 to maintain nitrogen contents on the beach not only failed to stimulate degradation but also appeared to inhibit the decomposition of oil (52). These authors suggested that the microorganisms may be preferentially biodegrading the oleic acid in the fertilizer rather than decomposing the oil, particularly as the fertilizer was being added repeatedly to the treated plots. Laboratory research suggests that microorganisms will initially biodegrade the fatty acids in the fertilizer preparation before degrading the oil (17, 26, 84), lending credence to the suggestions of Lee and Levy (52).

The relative persistence of Inipol EAP 22 on beach material may be related to its physical properties and the degree of wave and tidal energy impacting the treated beach (26). At low temperatures, it is a viscous liquid, and in the trials at Long Cove and subsequently in Prince William Sound, it had to be warmed before being applied to the beach (113). This viscosity may limit the penetration of the oleophilic fertilizer into medium- and fine-grained beach material. However, this hypothesis is speculative because detailed descriptions of the particle size of the beach material used in the field trials have not been reported, making comparisons difficult.

Lee et al. (56) studied the use of inorganic sources of nitrogen (ammonium nitrate) and phosphate (triple superphosphate) applied with and without a slow-release granulated agricultural fertilizer (sulfur-coated urea treated with a wax sealant and a little kaolin clay). Terra Nova crude oil (3% [vol/vol]) was mixed with sand, placed in Nitex bags, and buried 5 cm below the sediment surface in a line midway between the high and low water marks. The oil was allowed to weather for 6 days before the addition of granular fertilizers. The fertilizers were applied at different ratios and concentrations to different plots, and the nutrients were reapplied to the sediments after each sampling period.

There was no significant difference between the results obtained with the fertilizers used at the lowest concentration and those obtained at higher concentrations. Therefore, only the results at the lowest concentration were reported (56). At 50 days after treatment, the C₁₇/pristane ratio showed that more rapid biodegradation was found in each fertilized plot. The

best results were obtained in the plot treated with the highest concentration of the slow-release fertilizer (which had the lowest concentration of ammonium nitrate). Measurements of hexadecane mineralization showed similar results; all the fertilized plots had higher hexadecane mineralization rates than the unfertilized controls, but the highest rates were found with the highest content of sulfur-coated urea. These results were confirmed by reductions in the relative concentrations of the aliphatic and polynuclear aromatic hydrocarbons. Furthermore, fertilization stimulated the relative activity of heterotrophic bacteria (as measured by [¹⁴C]glutamic acid uptake) in comparison with oiled controls.

In this trial, the authors noted that the addition of nitrogen and phosphorus can stimulate oil biodegradation only to a fixed extent on a particular beach type. If this nutrient level is exceeded, no further enhancement of oil degradation is found. In such cases, other strategies for further enhancing oil degradation, such as addition of iron and other trace nutrients, would need to be considered.

There was no doubt that the form of the nutrient, even at the same N and P concentrations, affected the degree of stimulation of oil decomposition. The best results were noted when 90% of the total nitrogen was added in a slow-release form. These results suggest that nitrogen in this form benefits hydrocarbon degraders more than inorganic nitrogen addition does, presumably because the ammonium nitrate is more prone to being washed away from the oil. It is interesting to contrast this result with the data obtained with Inipol EAP 22, which also contains urea as a source of nitrogen (48, 49, 52). It seems likely that the combination of urea with other biodegradable organic sources in Inipol EAP 22 determines the success of this mixture on sandy beaches.

Organic fertilizers derived from natural products such as fish and meat meals have recently been evaluated as bioremediation agents for use in the marine environment. Basseres et al. (12) evaluated the efficacy of bioremediation agents composed of 60% animal proteins with a N/P ratio (molar ratio, 22:1) similar to that demanded by the bacteria (molar ratio, 16:1) to degrade a weathered Arabian Light crude oil in sandy beach sediments. These biodegradation studies were conducted in 600-liter outdoor tanks which were supplied with fresh seawater once a day to simulate tide conditions. In the first 60-day experiment under simulated supratidal zone conditions, bacterial numbers were found to be greater in the treated plot (animal meal added as 10% by weight of the hydrocarbon 1 week after oil addition) than in the control. Chemical analysis of the aliphatic fraction of the crude oil residues indicated that nutrient treatment enhanced the degradation of the hydrocarbons and was consistent with the observed increases in the population of hydrocarbon-degrading bacteria in the treated tank.

A second experiment, which simulated an intertidal zone under winter conditions, provided comparable results. Over the duration of this study, the development of aerobic heterotrophic bacteria and hydrocarbon-degrading bacteria was greater in the plot treated with animal meal than in the control plot for the supratidal, intertidal, and submerged zones within the sandy sediment. Reapplication of the nutrients after the bacterial population stabilized at approximately 38 days resulted in further stimulation. Furthermore, although the experiment was carried out during the winter, the development of hydrocarbon-specific bacteria (10⁹ bacteria per g of sediment) was 2 orders of magnitude higher than that previously observed in the summer.

However, before organic nutrients are accepted for use on a routine basis as bioremediation agents, further research must

be conducted to understand their mechanism of action and potential environmental impacts. A recent study by Lee et al. (55) compared the effects of inorganic (ammonium nitrate and triple phosphate) and organic (fish bone meal) fertilizers on the biodegradation rates of Venture Condensate within a sand beach environment. The study demonstrated that the organic fertilizer stimulated microbial growth and metabolic activity to the greatest extent; however, chemical analysis of residual oil concentrations and composition showed that the application of the inorganic fertilizer (at identical N and P concentrations) was the superior strategy. A single application of the fish bone meal fertilizer had little or no effect, and multiple applications of the organic fertilizer actually suppressed oil biodegradation rates. This paradox between the microbiological and chemical results obtained with the different nutrient formulations was attributed to selective growth of different bacterial populations. The authors speculated that a diauxic growth response occurred, in which the indigenous microflora preferentially utilized components within the organic fertilizer. Furthermore, biodegradation in the enclosures treated with periodic additions of the organic fertilizer may have been suppressed by the production of toxic metabolic by-products, such as ammonia, from the degradation of the organic fertilizer (54).

Use of seeding. There have been few evaluations of the effects of seeding on contaminated shorelines or in soil. Studies in soil have suggested that nutrient addition alone had a greater effect on microbial oil decomposition than did the addition of competent microorganisms (42). However, Vecchioli et al. (114) found that soil amended with hydrocarbon degraders was decontaminated more rapidly than fertilized controls. These latter experiments were conducted in the laboratory at the fairly high temperature of 30°C and with an oil concentration of 10% (wt/wt). The authors conclude that high oil concentrations and mixing of the inoculant into the soil may be important in encouraging success. At low oil concentrations (0.5% [wt/wt] oil), seeding had no beneficial effect (57). Hence, it is clear that experimental conditions have a large effect on the success of the treatment.

As discussed previously, Lee and Levy (48) showed only limited increases in biodegradation rates of SSC in sandy beach sediments seeded with a mixed culture of marine oil-degrading bacteria. These observations agree with those of Tagger et al. (106) in marine seawater enclosures, which showed that allochthonous microorganisms disappeared from the dominant microbial community after 60 days. This decline was attributed to their inability to compete with the indigenous microflora under changing environmental conditions, such as the decrease in seasonal temperatures. The authors noted that the number of microorganisms in the beach sediment with the capacity to degrade the oil increased in the presence of elevated oil concentrations without bacterial additions. Lee and Levy (48) concluded that biodegradation of SSC in sandy beaches could be enhanced by periodic nutrient additions after the indigenous populations had adapted to the contaminated sediments. Oil biodegradation was not limited by the need for a microbial inoculum.

Rosenberg et al. (86) have developed a urea-formaldehyde polymer (F-1) which is insoluble in water, binds to the oil-water interface, and can be depolymerized only by certain bacteria with enzymes bound to the cell wall. Experiments conducted in the laboratory suggested that the addition of hydrocarbon-degrading bacteria capable of depolymerizing the polymer together with F-1 could stimulate hydrocarbon biodegradation. After an accidental spill of 102 tonnes of heavy crude oil on a sandy shoreline in Israel, Rosenberg et al. (86)

evaluated the potential of fertilization with F-1 and seeding with depolymerizing bacteria to stimulate oil biodegradation.

Two 50-m² oiled plots on the beach were selected for the experiment. One was inoculated with 20 liters of a mixed culture containing three F-1-depolymerizing bacteria, referred to as RT (*Gluconobacter* sp.), RL4 (*Pseudomonas* sp.), and RL3 (*Pseudomonas alcaligenes*), and 38 kg of F-1 (0.76 kg/m²); the second plot was left undisturbed as a control. The experimental plot was raked daily and watered with 1.5 m³ of seawater. The oil removal rates were monitored by measuring the weight of oil extracted from the sand by using pentane. Oil contamination on the plots was relatively low (average of 3.8 mg of oil per g of sand), and it was fairly rapidly degraded by the treatment. After 25 days, 84.5% of the oil on the fertilized plot had been removed whereas the oil content in the control plot had declined by only 15.6%. While no attempt was made to quantify the significance of oxygenation and nutrient replenishment on biodegradation caused by tilling and watering the experimental plot, the experiment appeared to show a successful bioremediation application in a warm climate (ambient seawater temperature, 27°C). Prince (76) noted that the use of pentane as an extractant may not have removed the oil reproducibly from the sediment, particularly in the presence of the organic fertilizer F-1. He suggested that methylene chloride may have been a more appropriate solvent. Moreover, since there were no replicate plot treatments, it would have been helpful to measure the conserved biomarkers in the residual oil to verify the efficacy of this bioremediation strategy. While the experimental results suggested that the seed organisms degraded the oil, this was by no means demonstrated unequivocally. No attempt was made during the field study to monitor the survival of the seeded bacteria or to ascertain whether any members of the indigenous microbial community could depolymerize F-1. Further research is certainly required to determine whether the addition of F-1 is metabolized only by the seeded microorganisms and whether adding this combination of organic fertilizer and specific hydrocarbon-degrading microorganisms capable of utilizing the fertilizer confers any advantage over simply adding nutrients which are available to the entire indigenous hydrocarbon-degrading microbial population.

SPILL INCIDENTS

There have been several oil spill incidents in which bioremediation products have been used in an attempt to enhance oil biodegradation. In some cases, the response authorities have allowed products to be used for experimental purposes (38). However, in general, it is difficult to draw valid conclusions from many of these efforts because of the time constraints in planning experiments with appropriate controls after a major spill. Moreover, many of the results are reported secondhand with little reliable quantitative information. Despite these limitations, some of these spills have been given as examples of bioremediation success and therefore qualify for scientific appraisal.

One notable exception is the work carried out in the aftermath of the *Exxon Valdez* spill. The assessments of bioremediation products and techniques are based on experiments carried out with considerable scientific rigor, and the work after the *Exxon Valdez* incident is therefore given prominence in this review. The scientific results of this research have been only recently published in primary publications and conference proceedings. A majority of the papers were not peer reviewed prior to publication in the scientific literature (a fact that applies to much work conducted after oil spill incidents), and thus

the results from these studies should be assessed with caution. Also, it is important to emphasize that even in this case, there were significant limitations in the scope of the work. For example, the studies concentrated on North Slope crude oil on cobble shorelines in a high-latitude environment.

During the early 1990s, there was an increase in bioremediation field trials associated with accidental spills, largely as a result of the perceived success of the bioremediation program following the *Exxon Valdez* incident (38). These are mentioned herein, but many are characterized by having been carried out over a short period and, in some cases, with products in the early stage of development (3, 38).

Amoco Cadiz

On 16 March 1978, the tanker *Amoco Cadiz* containing 223,000 tonnes of Arabian Light and Iranian Light crude oil was wrecked off the coast of France. Rough sea conditions resulted in rapid emulsification of the spilled oil, resulting in an increase in the volume of pollutant. Despite efforts to treat the oil at sea, extensive contamination of the shoreline occurred. Most of the beach cleanup effort focused on pumping and mechanical recovery, particularly during the first few weeks of the operation when there was a thick emulsion on the sand and rocks and in the crevices between the rocks. These operations caused some oil to penetrate the sand. In some places, oily sand was overlaid with clean sand deposited as a result of natural coastal processes. Repeated ploughing and harrowing were used to clean the intertidal zone, and four different products were tested to assess the possibility of promoting the biodegradation of oil trapped in sand (14): (i) a commercial cleaning compound containing nutrients especially adapted to restore oiled soils; (ii) a mixture of lyophilized adapted bacteria, dispersant, and nutrient; (iii) a chemical fertilizer used in agriculture; and (iv) a talc treated with 0.1% of surfactant.

The approaching tourist season seems to have prevented extended experimentation, and other techniques were used to complete the cleanup operations. Hence, the limited results were inconclusive (14). Some changes in oil content were found in these experiments, but it was not clear if the removal was physically or biologically mediated.

Apex Barge

On 28 July 1990, the Greek tanker *Shinoussa* collided with two *Apex* tank barges in the Houston Ship Channel, Galveston Bay, Tex., causing a release of approximately 3,000 m³ of partially refined catalytic feedstock oil over 2 days, which spread onto the surrounding coastline. Alpha BioSea (Alpha Environmental, Houston, Tex.), a product composed of a lyophilized bacterial mixture and inorganic phosphorus and nitrogen nutrients, was applied 8 days after the spill in selected areas of Pelican Island and Marrow Marsh (62, 107). Two plots on the beach were treated, and two were left untreated as controls. The 15-m diameter experimental plots (separated by 45 to 75 m) were sampled on a routine basis (67).

The results of the detailed chemical analysis showed that there were no significant differences between pre- and post-treatment samples after 96 h of treatment with any of the selected methods. Although visual signs indicated that the condition of the marsh areas improved after treatment (34), there was no conclusive evidence to show significant degradation of the oil within the 4-day monitoring period.

Numerous compromises in the experimental design of this study have been identified (67). For example, the separation of treated and untreated plots and the booming methods used to isolate them may not have prevented mixing and cross-con-

tamination. Furthermore, our knowledge from previous laboratory studies and field trials suggests that the 96-h duration of the experiment was insufficient for a definitive test of bioremediation. Unfortunately, no attempt was made to establish which factor (if any) was limiting biodegradation and what the most appropriate bioremediation strategy might be.

Mega Borg

On 8 June 1990, the Norwegian tanker *Mega Borg* was carrying out a lightering operation with the Italian tanker *Fragmura* about 57 miles off the Texas coast. Following an explosion and fire, the *Fragmura* carried out an emergency breakaway operation from the *Mega Borg*, which resulted in the release of approximately 45 m³ of Angolan Palanca crude oil (58). The next day, further oil was lost before the situation was controlled. While it was initially predicted that no oil would reach the shoreline, the Louisiana coast was littered with tiny tarballs 16 days after the accident (58).

In terms of bioremediation strategies, the On-Scene Coordinator granted permission to conduct a field trial 1 day after the accident occurred. Two portions of the slick were treated with a product containing Alpha BioSea (108). A 16-hectare patch of slick located about 5 km from the *Mega Borg* was treated 7 days after the accident with 50 kg of microbial agent (Alpha BioSea) which had been rehydrated with seawater. The product was applied with the standard shipboard fire-hose system. The equipment and treatment preparation time of approximately 1 h (108) indicates that very little rehydration time was given to the product. Four traverses of the treatment area were made over a 30-min period.

Following large-scale application of the product at sea, visual observations indicated that the treated oil changed from a continuous film of brown oil and sheen to discrete areas of mottled brown and yellow material and sheen. An aerial reconnaissance 16 h after treatment was not able to detect oil in the area. However, there is considerable uncertainty about the fate of the treated oil (108).

The measurements on water samples from the treated slick showed no evidence of acute toxicity to marine life or significantly elevated levels of nutrients or total hydrocarbons. Attempts to assess the effect of the microbial agent from measurements of oil content in the emulsion samples were unsuccessful because of sample variability. By 8 h after treatment, the slick had largely broken up and dissipated. Although little change was observed in the control area, conclusive evidence of bioremediation effectiveness was not achieved because of limitations in the sampling strategy and the chemical evidence obtained.

This study demonstrated the potential problems with the application of bioremediation products at sea, including difficulties with uniform product application, representative sampling, and uncertainties about the ultimate fate of the oil. The short periods over which monitoring is often possible may not be sufficient to validate the presence and activity of oil-degrading bacteria or the effectiveness of bioremediation treatments. The observed visual effects may well have been caused by physical or chemical processes such as surfactant action associated with the treatment.

Prall's Island

In January 1990, fuel oil from a pipeline failure spilled into the Arthur Kill waterway in New Jersey and contaminated a gravel beach on the Prall's Island bird sanctuary. Mechanical methods were used to remove the bulk of the oil. Cleanup was suspended in March 1990 to minimize possible adverse effects

on migrating birds. However, Exxon was granted permission to carry out a bioremediation experiment on part of a contaminated beach.

Two shallow trenches were dug in the intertidal zone to bury bags of beach substrate containing known concentrations of oil and to help overcome possible problems of variable distribution of oil on the beach. A slow-release fertilizer (Customblen, Sierra Chemicals) (Table 1) was placed in the trenches to encourage biodegradation. Over a 92-day period, subsamples were periodically taken from the oiled bags, together with beach samples and water samples for analysis of total petroleum hydrocarbons, GC-MS detection of hydrocarbons, microbial counts, and water quality (nitrogen, phosphorus, ammonia, and dissolved oxygen) determination.

No clear trends of increased biodegradation from the fertilized plots could be identified during the experiment, and there was high variability in the levels of total petroleum hydrocarbons, which may have masked any effects of the treatment (37).

Seal Beach

On 31 October 1990, a well blowout off Seal Beach, Calif., resulted in the release of approximately 2 m³ of crude oil that contaminated 8,000 to 12,000 m² of marsh grassland in the Seal Beach National Wildlife Refuge. One week after the incident, the marsh was hand sprayed with a combination of a microbial product used in sewage treatment plants (INOC 8162) and a commercial fertilizer (Miracle Gro 30-6-6). Two weeks later, the fertilizer alone was applied. Oiled, oiled and treated, and unoiled samples were collected and analyzed for oil content by GC-MS (37). Measurements were also made of the microbial mineralization of the phenanthrene, microbial respiration, and biomass.

The results of a 35-day monitoring effort showed no differences between the treated and untreated oil plots. Subsequently, laboratory tests were carried out with the microbial product and Prudhoe Bay crude oil to compare the performance of the microbial product with nutrient-only controls. After 16 days of incubation, little or no difference was found between treated and control flasks. It was concluded that the microbial product was not effective in accelerating biodegradation of oil under controlled laboratory conditions (37). Moreover, the salt marsh environment may be difficult to bioremediate simply by adding sources of nitrogen and phosphorus. Oxygen depletion may have been a significant factor in the inhibition of oil biodegradation (51).

Exxon Valdez

Background. The tanker *Exxon Valdez* ran aground on Bligh Reef in the Gulf of Alaska on 24 March 1989, spilling approximately 41,000 m³ of Alaskan North Slope crude oil (primarily Prudhoe Bay crude oil). A major response effort was mounted at sea to recover the oil, but the prevailing conditions and circumstances resulted in the contamination of about 2,090 km of coastline (72). Some beaches were heavily oiled, particularly those on islands in Prince William Sound that were directly in the path of the slick.

Many techniques were adopted in a massive effort to clean up the shoreline of the Sound (72% rock face, 24% mixed boulder and cobble, 3.5% mixed cobble and pebble, and 0.5% fine-grain sand/mud or marsh). These included cold- and warm-water washing, steam cleaning, and manual oil recovery techniques. Initially, the main aim was to remove the heaviest concentrations of oil to minimize the impact on wildlife and fisheries (17, 72).

A bioremediation option based on nutrient enrichment was proposed shortly after the spill. However, it was thought necessary to carry out some research first to establish the potential for effective and safe use of this technique. The limited success of the initial field tests led to the approval of full-scale application in August 1989, and 119 km of shoreline was subsequently treated that year.

By 1990, the previous cleanup efforts and winter storms had greatly reduced the extent of shoreline oiling (41) and natural recovery processes were already well advanced (7, 8). The National Oceanic and Atmospheric Administration applied the concept of net environmental benefit analysis in an evaluation of the main alternative to bioremediation at this time, namely, excavation and rock-washing treatment (69). It was concluded that this technique would be particularly damaging to the environment. Bioremediation was therefore adopted as a prime cleanup strategy. In 1990 and 1991, bioremediation was used in combination with storm berm relocation, tilling, and manual pickup. On 12 June 1992, the U.S. Coast Guard and the State of Alaska declared the cleanup officially concluded on the basis that there would be no further net environmental benefit from continuing the effort.

Potential for bioremediation in Alaska. Shortly after the *Exxon Valdez* spill, it was suggested that bioremediation may be able to enhance the rates of oil removal from the contaminated beaches (79, 80). As a preliminary step, the number of oil-degrading microorganisms on oiled beaches in comparison with untreated controls was determined. Pritchard et al. (81) reported that the hydrocarbon-degrading microorganisms on oiled shorelines had increased by as much as 10,000 times to an average level of 10⁶ cells per g of beach material. Once it was clear that hydrocarbon degraders were present in abundance, it was necessary to establish which factors were likely to limit biodegradation and which specific hydrocarbon components were biodegradable.

The research was conducted in the laboratory with Prudhoe Bay crude oil weathered by distillation at 277°C to remove the volatile fraction. Biodegradation by indigenous microorganisms was monitored by noting changes in the concentration of components of the oil by GC-MS, by monitoring carbon dioxide evolution and oxygen consumption by the microorganisms, and by determining the evolution of radioactive ¹⁴C from specific ¹⁴C-labeled oil components such as phenanthrene (17, 65, 104, 105, 113).

The experiments demonstrated unequivocally that the microbial population in Prince William Sound could rapidly biodegrade the aliphatic and aromatic fractions of Prudhoe Bay crude in the presence of suitable nitrogen and phosphorus sources. The microbial community decomposed C₁ dibenzothiophene, C₂ fluorenes, C₃ naphthalenes, phenanthrene, and anthracene among others (113). Studies of CO₂ production suggested that the oil was not just being biotransformed but that it was being completely mineralized to CO₂ and H₂O. For example, over 30% of [U-¹⁴C]phenanthrene could be mineralized to ¹⁴CO₂ within 4.5 days when incubated with oil-contaminated beach material from Prince William Sound (81). The highest mineralization rates were noted in the test systems treated with the highest concentration of nitrogen.

From these results, it is clear that the main factor limiting the biodegradation of oil on the beaches in Prince William Sound was the concentration of nutrients, particularly nitrogen. A substantial microbial biomass had already developed in the contaminated areas of Prince William Sound which was able to decompose many components within the contaminant oil. Hence, addition of nutrients, and not seeding, was thought to be the most appropriate bioremediation strategy (17, 80).

Two sites were chosen for the initial field trials in 1989, Snug Harbor and Passage Cove on Knight Island in Prince William Sound (17, 32, 113).

Snug Harbor. Snug Harbor is located on the southeastern side of Knight Island in Prince William Sound (17). It was chosen because the oil contaminated a continuous band along the length of the shoreline, which was composed of a mixture of sand, gravel, and cobble. Six plots were established, three on mixed sand and gravel and three on cobble. Typically, each plot was at least 12 m wide and 21 m long across the intertidal zone (32).

One of the cobble plots at Snug Harbor was treated with Inipol EAP 22, a second was treated with Woodace briquettes (for their composition, see reference 87), and the third was left untreated as a control. The nutrients were selected on the basis of laboratory assessments (87). Exactly the same treatment strategy was used on the portion of the beach containing sand and gravel (32). Inipol EAP 22 was administered via backpack sprayers, just after the tide had passed below the lowest point on the plots. Because of its viscosity at low temperatures, the Inipol EAP 22 was heated to 11°C prior to application to prevent clogging of the sprayers. Two applications were made, the second 9 days after the first. Each time, Inipol EAP 22 was added at a concentration of 5% of the approximate weight of the oil on the plot (approximately 37 kg per plot). Woodace was placed in herring seine bags and secured on the beach to prevent tidal removal. The bags were placed at regular intervals over the beach, and each bag contained 14.85 kg of product. Each plot received a total of 360 kg of Woodace, equivalent to 45 kg of nitrogen and 10.8 kg of phosphate. The bags in the upper part of the plot were repositioned 10 days after the start of the test, because they were not being submerged regularly at high tide. An extra four bags of Woodace were also added at this point (32), increasing the total added nitrogen to 58.5 kg and the phosphate to 14.4 kg.

The cobble plot treated with Inipol EAP 22 showed striking visual changes within 8 to 14 days of the initial treatment. A clean rectangle corresponding to the treated region could be seen clearly (79, 80). While these observations showed the surface of the cobble to be almost completely free of oil, substantial amounts of subsurface oil still remained. Such a clear visual effect was not noted on the surface of the beach treated with Woodace briquettes (79, 80). The sand-and-gravel plot treated with Inipol EAP 22 showed some visual changes, but they were not as dramatic as those seen on cobble. No oil or oily materials were observed in the seawater following the application of fertilizers, and no oil residues were found in mussels confined in cages just offshore of the beaches (113).

Microbial numbers increased on all the plots during the experiments (85, 113). No significant differences were noted between the treatments and the controls. However, the levels at the start of the experiment were already high in response to the oil spillage. Nutrient measurements on the beach treated with Inipol EAP 22 showed that elevated levels of fertilizer remained for only about 3 days. This result is consistent with observations made by Lee and Levy (48) and those made in the laboratory (87). Thus, any increased rate of oil biodegradation must have been the result of the initial nutrient pulse rather than a sustained release.

The amounts of oil extracted from beach sediment were highly variable, making the results difficult to interpret (65, 81, 113). The oil was also partially biodegraded before the trial was conducted. For example, the C_{18} /phytane ratio in sediment at Snug Harbor decreased from 2 in North Slope crude to between 1 and 1.5 as a result of biodegradation within the 2.5-month period before the trial. Quantifying the C_{17} /pristane

and C_{18} /phytane ratios showed evidence of biodegradation on both treated and control plots. Analysis of the nutrient content in the control plots showed the presence of significant quantities of ammonia, nitrate, and phosphate, which may have accounted for the biodegradation noted in the control sediment. It was not possible to determine if these nutrients had leached from the treated plot or if they reflected normal conditions in Snug Harbor at that time of the year.

Further investigation indicated that the isoprenoid compounds pristane and phytane were not feasible for use as conserved biomarkers to determine oil biodegradation rates in this environment, since they were degraded readily by the indigenous microbiota. Phytane-degrading microorganisms were subsequently isolated from the Alaskan beaches (81). However, although not statistically significant, the decrease in the C_{18} /phytane ratio of the oil sampled from the Inipol EAP 22-treated cobble beach appeared to be 1.2 times faster than that in the control over the 90-day period of the test (81). The maximum difference between the control and treated plots over the duration of the experiment was observed between 16 and 78 days after the treatment. Few discernible differences in the C_{18} /phytane ratio were evident on the sand-and-gravel plots.

Because the best evidence for enhanced oil degradation was obtained on the cobble plot treated with Inipol EAP 22, the data were analyzed further to determine which other petroleum hydrocarbons were degraded (81). Samples that showed low C_{18} /phytane ratios 50 days after the treatment were analyzed, and the concentrations of specific residues were normalized against 17 α ,21 β -hopane (a polycyclic aliphatic hydrocarbon, resistant to biological attack), which has been used previously as an internal biomarker (15, 44, 82). The results provided evidence that the aliphatic and many polynuclear aromatic hydrocarbons were decomposed (81). While this observation did not conclusively show that bioremediation accelerated the degradation of these compounds (because no comparison was made with appropriate controls), it was consistent with such a hypothesis.

Pritchard et al. (81) also examined the relationship between the median reduction in oil residue content (calculated as a percent reduction from the median level at the start of the experiment) and the percent change in the C_{18} /phytane ratio on the cobble beaches. They found that there was a correlation between weight reduction and changes in the C_{18} /phytane ratio for both the control plot and the plot treated with Inipol EAP 22. The changes in oil residue weight were then evaluated directly as a measure of biodegradation. The rate of decrease of oil residue weight (assuming first-order kinetics) on the Inipol EAP 22-treated cobble beach was twice as high as that seen in the control 29 to 51 days after the addition of nutrients, although, again, this difference was not statistically significant. However, statistical analysis did show that the rate of decrease in oil residue weight in Inipol EAP 22-treated samples over the course of the experiment was significantly different from zero whereas the rate of decrease in the control samples was not.

Even though the chemical data are by no means conclusive, there was at least some evidence that the treatment of cobble with Inipol EAP 22 did encourage microbial biodegradation of oil. Pritchard et al. (81) studied the effect of Inipol EAP 22 on the metabolic decomposition of oil to CO_2 in laboratory experiments designed to simulate a beach environment. Enhanced oil mineralization rates were approximately equivalent to that obtained with water-soluble nutrients (providing an equal nutrient concentration). Enhanced oil decomposition was noted even after taking into account the contribution of CO_2 from the mineralization of the oleic acid in the fertilizer.

Experiments in small-scale microcosms using oiled beach material from Prince William Sound also showed that oil degraded rapidly in the presence of Inipol EAP 22 under simulated tidal conditions (23). The oil was not mobilized physically from the surface of the beach material by the oleophilic nutrient additions. In fact, it may have reduced the loss of oil from beach material by limiting natural clay-oil flocculation processes that mediate physical transport (17, 18).

The notion that Inipol EAP 22 may act as a chemical beach-cleaning agent on the beaches of Prince William Sound has been studied extensively (17, 81). Evidence to counter the chemical action of Inipol EAP 22 has been collected from large-scale laboratory tests with replicate Plexiglas experimental columns (0.91 m high and 0.31 m wide) containing 85 kg of oiled rock collected from Prince William Sound. These columns were designed to reflect a core midway through an idealized beach in Prince William Sound and were subjected to two tidal cycles per day at a vertical velocity of 0.61 m/h. One column was sterilized by the addition of a biocide. Both columns were treated with Inipol EAP 22, and the effect on oil mobility and biodegradation was noted (17). No oil was mobilized into the aqueous phase taken from either column during the 7-week experiment. In the nonsterile column, 24% of the oil had decomposed, whereas 2.5% was degraded in the sterile control (17). These results tend to confirm that Inipol EAP 22 acted as a bioremediation agent and not simply as a chemical cleaning agent.

Passage Cove. Passage Cove on the northwestern side of Knight Island was heavily contaminated with North Slope crude. It was flushed vigorously with water to remove the bulk of the oil before it was used for a bioremediation field trial (32). This site was composed primarily of cobble overlaying mixed sand and gravel, with oil penetrating to depths of 0.3 to 0.4 m (113).

Four plots (typically 28 by 21 m) were established at Passage Cove. One plot was left untreated as a control, two were treated with a mixture of Inipol EAP 22 and Customblen, and the fourth was treated with a water-soluble fertilizer (32). The total nitrogen addition to the Inipol-treated plots was 21.5 g of N per m² of beach. The aqueous fertilizer was sprayed daily through a lawn sprinkler system. The inorganic salts of nitrogen and phosphorus were dissolved in seawater and sprayed onto the beach to achieve concentrations in pore water of 7 mg of nitrogen per liter and 4 mg of phosphate per liter to a depth of 2 m.

Visually, the plots treated with Inipol EAP 22 and Customblen were appreciably cleaner than the controls within 10 to 14 days, and oil was found only in isolated patches down to a depth of 10 cm after 1 month. However, oil remained 20 to 30 cm below the treated plots at the end of the experiment, 45 days after initial treatment (113). Similar effects were noted on the irrigated plot, except that observable visual changes took 10 to 15 days longer (32). No visible changes were observed within the control plot at the end of the experiment.

More oil removal was observed from the cobble portion of the fertilized plots in Passage Cove than from the controls over the first 30 days of the experiment (79). No significant differences in oil levels were found between samples of sand and gravel taken from the same beach. Biodegradation of phytane within the sediments from this beach hampered the use of C₁₈/phytane ratios for the calculation of oil biodegradation rates. Nevertheless, on a qualitative basis, the ratio decreased in the samples obtained from each plot, but the lowest ratios were found on the plot treated with the fertilizer solutions. Analysis of the number of oil-degrading bacteria on the beaches in Passage Cove also showed little difference between

the control and fertilized plots. However, as with Snug Harbor, the concentrations at the beginning of the experiment were high, no doubt in response to the prolonged exposure to spilled oil (85).

On the basis of chemical and visual data, bioremediation stimulated the biodegradative loss of oil from the Passage Cove sediments (79). Inipol EAP 22 and Customblen appeared to stimulate oil biodegradation by a factor of 2 to 3, and additions of the inorganic fertilizer solution stimulated degradation by a factor of 4 to 5 (79). While the visual effects supported this conclusion, the chemical data were often not conclusive in this respect. This was primarily because of the large variability between samples from the same plot and because the branched-chain aliphatic biomarkers pristane and phytane were degraded almost as rapidly as their straight-chain analogs (79, 81). Nonetheless, the data obtained during these preliminary studies provided sufficient evidence of the potential of bioremediation to justify further field tests (17).

Knight Island 1990. In 1990, further field trials were conducted on Knight Island by using a modified experimental design (78). Changes in the oil concentration were monitored around the center of a fertilized beach and compared with those monitored in the center of an adjacent untreated control. The plots were situated in the intertidal zone, and sampling points were clustered within a 1-m radius of each of three sampling wells used to measure nutrients in the pore (interstitial) water (15, 16). On each sampling date, three surface and three subsurface samples were taken from around each sample well. A surface sample was defined as the top 2 to 5 cm of the fine-grained sediment underlying the large cobble. The subsurface sample was taken at a depth of 30 to 35 cm (15).

At each sampling time, beach material was removed and analyzed for the levels of petroleum hydrocarbons, the total number of microorganisms, and the ability of the microbial population to mineralize hexadecane and phenanthrene. The total number of oil-degrading microorganisms was determined by using the sheen screen method, which was based on a most-probable-number method, with weathered Prudhoe Bay crude oil as the sole carbon source (20). Total numbers of heterotrophic microorganisms were determined by the same method with marine broth as the growth medium (59). The extracted oils from each of the three surface and subsurface samples, taken around each well, were bulked together for detailed chemical analysis. Thus, analytical results were obtained on each sampling occasion for an integrated sample from the three beach surface and subsurface samples. The sampling wells were used to monitor interstitial water in the beaches. Each well consisted of a slotted steel pipe (5 cm in diameter and 70 cm long), which was driven into the sediment to a depth of about 65 cm. The wells were sampled just as the tide dropped below the level of the wells. Interstitial water was sampled for dissolved oxygen, salinity, pH, temperature, and nutrient levels.

Three beaches were selected for the 1990 field trials and termed KN-132, KN-135, and KN-211 (15). The beach substrate, degree of oil contamination, and nutrient applications made to each beach are summarized in Table 3. Note that overall, KN-135 received nearly twice as much nitrogen as did each of the other test beaches. The degree of degradation of the oil on the test beaches was estimated by comparing the 17 α ,21 β -hopane concentrations in the whole-oil residue removed in May 1990 with that recorded in the North Slope crude spilled in 1989 (16). The results showed that the oil on KN-132 was most weathered (67% $\pm$ 4.3%) at the start of the experiment.

The nutrient measurements in beach pore water gave a good

TABLE 3. Nutrient additions to test beaches in Alaska in summer 1990^a

Beach site	Fertilizer	No. of applications	Time (days) of application ^b	Beach environment	Amt of nitrogen added (g of N/m ²)
KN-135	Inipol EAP 22 Customblen	2	0, 53	Low energy, contained surface and subsurface oil	106 ^c
		3	0, 53, 72		
KN-211	Customblen	2	0, 44	High energy, contained subsurface oil	53
KN-132 ^d	Inipol EAP 22 Customblen	2	0, 44	Low energy, contained largely surface oil	56
		2	0, 40		

^a Modified from reference 19.^b Days after the start of the test (taken as day zero).^c For example, this consisted of an initial application of 361 g of Inipol EAP 22 per m² and 103 g of Customblen per m² followed by 303 g of Inipol EAP 22 per m² and 17 g of Customblen per m² and finally by 91 g of Customblen per m².^d A small portion of this beach was treated with bioremediation agents in 1989.

indication of whether the fertilizers had penetrated the beach. Data from KN-135 and KN-132 showed that there was an increase in total nitrogen levels (sum of ammonia, nitrite, nitrate, and organic nitrogen) directly after application (17). This increased level remained on KN-132 for approximately 10 days and on KN-135 for nearly 20 days, but little change was noted on KN-211, which had been treated with Customblen only. However, nitrogen levels rose in response to the second application of nutrients on all the treated beaches (17). No elevation in nitrogen levels was seen on the untreated beaches. Nutrient measurements of the inshore waters at KN-211 showed highly elevated levels of nitrogen and phosphorus directly after treatment, a phenomenon not seen at KN-132 and KN-135. Thus, it appears that the first nutrient addition of Customblen was rapidly washed off the beach but the second was not (17). KN-211 is a high-energy beach (Table 3), suggesting that under certain conditions, granular slow-release fertilizers such as Customblen can be removed from a beach before a significant amount of nutrient is released.

No increase in phosphate was recorded in the interstitial water on any of the beaches (17). Analysis of the Customblen removed from the beaches indicated that the phosphate had leached from the granules. The fate of the phosphate is therefore unclear, but it may have precipitated to form inorganic phosphates with calcium or iron (17, 89).

In a manner similar to that shown by visual observations in 1989, nutrient additions also reduced the amount of oil apparent on the surface of KN-135. Dissolved oxygen concentrations in the pore water of this beach decreased after the first addition of nitrogen. They began to rise again after 20 days and then fell in response to the second nitrogen addition. Hence, it appears that nitrogen addition stimulated microbial oxygen consumption in a manner similar to that seen in the column studies in the laboratory (17). However, the stimulation was not sufficient to render the pore water anaerobic at any point, implying that oil degradation rates were not limited by oxygen depletion.

The total number of marine heterotrophs tended to increase on the treated and control beaches over the course of the experiment (59). On most occasions, the heterotrophs were more abundant in the treated sediment than in the control on KN-132 and KN-135; the reverse was found for KN-211, possibly because the nutrients failed to remain on the beach. On a few occasions, the numbers on the treated beaches were significantly greater than on the control, but the differences were not consistent. Similarly, few significant differences in the number of oil degraders were noted between treatment and control experiments, except directly after the second fertilizer additions on KN-132 and KN-135.

Significant increases in the ability of sediment microorganisms to mineralize hexadecane were noted within 2 days on the fertilized portions of KN-132 and within 8 days on KN-135 (59). Even on KN-211, a significant increase in the mineralization was noted in the subsurface samples after 2 days. The increase was not as large as on the other fertilized beaches, possibly reflecting the failure of the first addition of nutrients to remain on the beach. After the second addition of nutrients, the ability of the microorganisms to mineralize hexadecane was significantly higher, on each treated beach, for the remainder of the experiment.

In contrast, the changes in the ability of the sediment microbiota to mineralize phenanthrene were not as consistent. On KN-135, significant increases in mineralization rate were noted on the fertilized plot, in the surface samples 15 days after nutrient addition and in the subsurface samples after 32 days. A significant increase in the ability to mineralize phenanthrene was then seen for most of the remainder of the experiment. On KN-211, significant increases in the ability to mineralize phenanthrene were found on the treated plot only after the second addition of nutrients. Significant elevations in the ability of the microorganisms to mineralize phenanthrene were found on KN-132 only 4 to 16 days after the nutrient addition.

Hence, the addition of nutrients to KN-132 and KN-135 stimulated the total number of heterotrophic microorganisms and the ability of sediment microorganisms to mineralize hexadecane and phenanthrene. The second, apparently successful addition of nutrient to KN-211 also stimulated the ability of the sediment microbiota to mineralize both hydrocarbons. Thus, there is evidence (supported by statistical analysis) in these trials that bioremediation does stimulate the microbial community to degrade hydrocarbons more rapidly. The depletion of dissolved oxygen in the beach pore water of the fertilized beach is consistent with this hypothesis. Whether the addition of nutrients results in more hydrocarbon-degrading microbial biomass or whether each bacterium responded by producing more hydrocarbon-degrading enzymes is not clear from the data. Certainly, if there were an elevation in the hydrocarbon-degrading population, it was masked by sample heterogeneity and the imprecision of the most-probable-number technique (59).

Each beach sample was analyzed for total extractable hydrocarbons, which is equivalent to oil residue weight analysis used in the 1989 program. The aliphatic fractions of total resolvable hydrocarbons and unresolved hydrocarbons were then determined. The sum of these values gave the total GC-detectable hydrocarbons (TGCDHC). Finally, the total amounts of polycyclic aromatic hydrocarbons and selected components were analyzed by GC-MS. Prior to bioremediation, the oil on KN-

135 consisted typically of 57.1% TGCDHC, 27.3% polar hydrocarbons (asphaltenes and resins), 15% high-molecular-weight hydrocarbons, and 0.6% other measured components such as 17 α ,21 β -hopane (at a level of 366 ppm). The oil on KN-132 was more biodegraded than that on KN-135 at the start of the experiment (16, 17).

Quantitative changes in the oil composition were evaluated by calculating the ratios of total detectable hydrocarbons against 17 α ,21 β -hopane (17). Inspection of the change in the ratio of TGCDHC to hopane suggested that the ratio decreased exponentially in the fertilized plot and hardly changed in the control, particularly for the oil extracted from the subsurface of KN-135. Linear regression analysis showed that the rate of decrease of this ratio was significantly different (with >99.9% confidence) from that noted on the untreated portion of the beach (17), suggesting that fertilizer treatment significantly increased the rate of oil biodegradation in the subsurface. The results were not as clear for samples taken from the surface of KN-135. From the regression analysis, the authors were able to estimate the amount of each oil fraction removed over the 109 days of the treatment (16). These data suggest that the microbial community on KN-135 was capable of biodegrading many components of oil provided that it was supplied with the appropriate nutrients. The microorganisms removed the resolvable aliphatic fraction more readily than the polycyclic aromatic hydrocarbons, confirming observations previously made in the laboratory (21, 46). Furthermore, the polar content of the oil (expressed as a fraction of the oil content) increased on the fertilized plot but not in the control. This suggests that the polar hydrocarbons are relatively non-degradable and that some of the oil may have been degraded to compounds which are retained in the polar fraction.

The rate of decrease of the ratios of polycyclic aromatic hydrocarbons (including the parent and the C₁ to C₄ substitutions) to hopane was also studied by linear regression analysis. The results showed that significant reductions occurred in the ratios of naphthalenes, phenanthrenes, and dibenzothiophenes to hopane but that the chrysene/hopane ratio showed little evidence of change (17). As expected, the naphthalenes were the most completely biodegraded (22).

Statistically significant differences in oil reduction were not recorded on KN-211 or KN-132. Although the CG results indicated some evidence of enhanced oil degradation on both beaches, this could not be verified. However, the natural biodegradation rate (as evidenced by the control plot) was more rapid on KN-211 than on KN-135 (16, 19). This information, coupled with the possible failure of the initial addition of Customblen to remain on the beach, may account for the differences between KN-135 and KN-211. However, the same explanation cannot be used for the failure to obtain significant results on KN-132. In this case, the oil was substantially biodegraded prior to the addition of fertilizer (17), leaving little readily biodegradable oil. This is illustrated by comparing the oil extracted from KN-132 before the addition of nutrients with fresh Prudhoe Bay crude oil. This comparison showed that 94% of the total resolvable hydrocarbons and 73% of the TGCDHC had been removed before bioremediation was attempted. In contrast, only 48% of the total resolvable hydrocarbons and 21% of the TGCDHC had been removed from the subsurface of KN-135. The nutrient measurements on KN-132 suggested that this beach had a naturally higher nutrient input than did KN-135, which may have caused the enhanced rate of degradation (17). The authors speculated that the source of the nutrients may be a freshwater stream which flows close to KN-132.

The results therefore suggested that the nitrogen addition

and the polar content of the oil affect the rate of oil biodegradation. Consequently, the authors proposed a model which contained terms for each of the factors thought to influence oil biodegradation rates: the ratio of nitrogen to oil, the polar fraction of the oil, and the time since the treatment. These statistical models were recently summarized to give an overview of the effect of bioremediation on the oiled Alaskan beaches (16). The authors concluded that the statistical analyses demonstrated that fertilizer addition significantly increased the rates of oil biodegradation on KN-135. The multiple-regression model also correlated well with the data from KN-211, confirming that the failure of the nutrients to remain on this beach may well have resulted in the low oil removal rates. The ratio of nitrogen addition to oil load and the extent of natural biodegradation of the oil were identified as primary factors which influence the success of bioremediation.

In the subsurface of the fertilized plot on KN-135, the weight loss of the oil was gravimetrically estimated as 71% of the starting value at the end of the study. However, by reference to 17 α ,21 β -hopane, it could be calculated that only 41.5% of the total extractable hydrocarbons had been removed. This suggested that biodegradation was not the only process affecting oil loss from the beach. One explanation is that the oil is physically washed from the beach, possibly as flocs of oil and clay (18). Alternatively, stimulation of oil biodegradation could have resulted in the increased microbial production of biosurfactants, which may have encouraged physical removal of oil.

Elrington and Disk Islands. Further evidence for the efficacy of bioremediation was provided in separate field trials during the summer of 1990 (80). Three separate trials were conducted, one on Elrington Island and two on Disk Island. The former was used to determine the effectiveness of different types of aqueous nutrient applications, while the latter was an attempt to study the effect of different application rates of Customblen and the efficacy of commercial inocula.

Subsurface beach material on Elrington Island was mixed to reduce heterogeneity and placed in sample baskets (80). The baskets were mesh containers with mesh lids. They were filled with 4 cm of sieved (12.5 mm), oiled beach material sandwiched between two layers of clean beach material (depth, 4 cm each). These fine sediments were placed in baskets and buried in trenches on the test and control beaches, 20 cm below the surface (80). Two types of aqueous treatments were used: multiple applications of fertilizer solutions from agricultural sprinklers (Sprinkler Beach) and a single application of aqueous fertilizer (Bath Beach). In both cases, the same inorganic fertilizer solution was used. The nutrient was mixed with seawater and applied over a 4-h period. Six applications were made on Sprinkler Beach, each providing 6.88 g of nitrogen (as ammonium nitrate) per m² and 1.37 g of phosphorus (as superphosphate) per m². On Bath Beach, one application of the same nutrients at 13.60 g of nitrogen per m² and 2.70 g of phosphorus per m² was made. Note that these concentrations are lower than those applied on Knight Island. On each sampling occasion, a set of baskets was removed for analysis. Oil reduction was monitored by studying the change in oil residue weight over the duration of the experiment.

Periodic nutrient measurements on Sprinkler Beach demonstrated that the nitrogen and phosphorus concentrations in the beach pore water were elevated by fertilizer additions. Peaks in the nutrient content were seen directly after the addition of fresh nutrients (80). The concentration of nutrients in the control beach remained low throughout the experiment.

Over the duration of the 1.5-month test on Sprinkler Beach, the oil load in the containers treated with nutrients steadily decreased whereas the oil load remained virtually constant in

the control. By using linear regression analysis, a statistically significant reduction in the weight of oil was found and the increase in oil biodegradation rates was higher than that recorded on the control plot by a factor of 6.4. The variation in oil concentration was considerably reduced as a result of homogenization. In contrast to the results on KN-135, the rate of oil loss was found to be linear (or zero order). The authors attributed this to the relatively high oil content at the start (approximately 12,000 mg/kg) and the end of the experiment (80). They suggested that an exponential loss of oil would occur at lower oil concentrations. Certainly, the starting oil concentration on KN-135 (approximately 4900 mg/kg) was lower than the concentration at the end of the experiment on Sprinkler Beach (approximately 8,500 mg/kg).

On Bath Beach, similar results were obtained, although the oil residue data were more variable. The rate of decrease in oil weight was consistent with a linear model, with the rate of degradation being a factor of 7.4 greater than that in the control. Thus, the addition of nutrients, either as a single addition or as a succession of additions, resulted in a significant enhancement of oil degradation.

Analysis of the weight data for the oil residue suggested that a single addition of nutrients resulted in a greater enhancement of degradation in comparison with repeated additions. However, studies of the change in alkane composition over the first 7 days normalized to the oil residue weight showed a different result. This analysis revealed that the alkanes (C_{18} to C_{27}) were degraded at an approximate rate of 28 mg/kg/day on Sprinkler Beach and 20 mg/kg/day on Bath Beach (80). Interestingly, nutrient addition did not stimulate alkane biodegradation as much as oil weight reduction did, suggesting that biodegradation was not the only mechanism for the loss of the oil from the beach. This finding was consistent with that noted on KN-135.

The high rate of alkane biodegradation noted on Sprinkler Beach correlated with the high concentrations of nutrient added to the beach. The nutrient delivery to the beach pore water averaged 3 ppm for the first 2 weeks (80), which was comparable to the nutrient addition to KN-135 (17). This finding supports the conclusion that the success of bioremediation is critically dependent on the successful delivery of the nutrients to the pore water on the beach. The main difference between the studies on Sprinkler Beach and KN-135 was that repeated applications of fertilizer were required to maintain the high level of nitrogen because the nutrients were lost relatively quickly from the beach. While comparison between the two studies is limited by differences in experimental design, the combination of Inipol EAP 22 and Customblen appeared to maintain a sustained supply of nutrients over a longer period (17). Peak concentrations on Sprinkler Beach were as high as 5.8 ppm, whereas the highest on KN-135 was about 2.8 ppm. These were still below the recommended limits for acute toxicity of ammonia (112).

Microbial activity and biomass were measured in samples taken from Elrington Beach (80). Oil-degrading bacteria constituted 1 to 10% of the total heterotrophic population in the study sites. No consistent effect on the number of oil degraders was found. Microbial activity was indirectly measured by monitoring the change in dissolved-oxygen concentration in the beach pore water (80). There was evidence that the oxygen content of the pore water decreased in response to nutrient addition, and this decrease was sustained on both fertilized beaches until the end of the experiment.

Microbial activity was also measured by studying the mineralization of radiolabeled phenanthrene and hexadecane to CO_2 in representative beach samples (80). Mineralization of

both radiolabeled compounds was enhanced by the addition of nutrients. Phenanthrene was mineralized more rapidly by samples from Bath Beach over the duration of the experiment, whereas hexadecane was mineralized more rapidly on Sprinkler Beach. These differences may be a result of the different initial concentrations of these compounds on the beach at the start of the test.

CO_2 production (an indicator of total microbial respiration) from sealed flasks containing beach material was determined in the laboratory. Over a 72-h period, the rate of production of CO_2 was approximately linear in samples taken from the treated beaches and the controls, corresponding to results of the oil chemistry analysis. After 10 days, the rate of CO_2 evolution was higher in samples taken from Bath Beach than in those from Sprinkler Beach. After 20 days, the situation reversed, with the material from Sprinkler Beach yielding a higher rate of CO_2 production, a phenomenon which continued for the remainder of the experiment. All the samples taken from the fertilized beaches respired at a higher rate than did material from the control beaches.

Thus, overall, the oil composition and microbial respiration suggest that nutrient addition to Sprinkler Beach stimulated microbial activity to the largest extent over the course of the trial. The rate of loss of oil residue weight on Bath Beach was slightly higher than that recorded on Sprinkler Beach, suggesting that the difference between the two treatments was relatively small. This is a surprising result which requires explanation. Pritchard et al. (80) suggested that nutrient-starved microbial communities rapidly assimilate added nutrients into biomass. The nitrogen and phosphorus fixed within the organic constituents of these microorganisms, which is subsequently released into the environment by nutrient recycling processes following cell death, may be rapidly reassimilated by the indigenous bacterial community and hence preserved in the ecosystem. Whether this process occurs sufficiently to maintain enhanced oil biodegradation rates within the environment remains to be verified experimentally.

The stimulation of oil biodegradation by fertilization on Sprinkler Beach and Bath Beach was not sufficient to explain the reductions in the oil residue weights, which is consistent with that observed on KN-135. Hence, it appears that nutrient addition not only stimulates oil biodegradation but also encourages oil loss from the beach surface. As noted above, oil biodegradation may encourage the formation of clay-oil flocs, which may mediate the removal of oil from the shoreline (18). Alternatively, an increase in microbial surfactant production may be responsible. This effect warrants further study, particularly if bioremediation is to be used as a routine tool to treat spill incidents.

The experiments on Disk Island gave an unexpected result. Four plots (3 by 3 m) were treated with four different concentrations of Customblen (50, 100, 500, and 1,000 g/m²). Two plots remained untreated as controls. The plots were maintained at least 4.6 m apart. For comparison, the equivalent amount of the mixture of Inipol EAP 22 and Customblen used on KN-135 was 100 g/m². Each plot consisted of homogenized oiled beach material in mesh baskets, placed so that the top of the basket was flush with the shoreline. The baskets were clustered around four wells on each of the plots used to sample the beach pore water and were completely filled with oiled beach material.

The results were disappointing, and the initial conclusions were that Customblen did not stimulate oil degradation despite the maintenance of sufficient nutrient levels (80). However, the pulse of nutrients was relatively short-lived, and less nutrient persisted in the pore water than was found on KN-135,

despite the higher nutrient application rates on some of the plots (17, 80). The oil chemistry results showed no evidence of enhanced oil degradation, but the number of oil-degrading microorganisms did increase on fertilized plots and there were measurable stimulation of hexadecane and phenanthrene mineralization rates by beach samples. Microbial respiration rates in beach samples were also stimulated. Thus, although no enhanced biodegradation could be determined chemically, the addition of Customblen had a small but measurable effect on oil-degrading microorganisms.

One explanation for this result may be that on Disk Island the Customblen was washed from the beach shortly after addition. A similar fate was observed for the slow-release nutrient on KN-211 (17). Differences in geomorphology may also have contributed to the lack of bioremediation success. The beach at this study site was substantially shallower than most other beaches in Prince William Sound, and the beach material was less porous (a sandy clay mix covered by an armor of cobble). Underlying much of the area was also an organic layer of peat, which probably contributed to the uncharacteristically low biodegradation rates observed (119). Alternatively, the oil may have been significantly degraded before the experiments, and hence the biodegradation was no longer limited by nutrient levels.

Seeding was evaluated in the field during the *Exxon Valdez* cleanup operations on Disk Island (115). The commercial inocula selected were previously proven in laboratory flask studies to stimulate the degradation of the alkane fraction within Alaskan North Slope crude oil to a greater degree than did the indigenous bacterial populations supplied with excess nutrients. In this experiment, a randomized block design was used. Four small plots consisting of a nonfertilized control, a mineral nutrient plot, and two plots receiving mineral nutrients plus the two products were laid out in random order on a beach. These four plots comprised a "block" of treatments, each block being replicated four times on the same beach. Four times over the 27-day period of the experiment, triplicate samples of beach sediment were collected for the analysis of oil residue weight and the determination of changes in alkane hydrocarbon profiles. No significant differences ($P < 0.05$) were observed among the four treatments. The failure to detect significant differences was attributed to variability in the data (identified by statistical power analysis), the highly weathered nature of the oil (the site was contaminated 16 months previously), the insufficient time for significant biodegradative changes to occur, and oxygen limitation (anaerobic odors were reported to emanate from some plots). No attempt was made to use hopane as the conserved biomarker to study the degree of oil biodegradation at the start and the end of the experiment.

Although it was not true for every case, the results of the field experiments in 1990 proved conclusively that bioremediation can enhance oil biodegradation on the contaminated shorelines of Prince William Sound. One factor critical to the success of the treatment is to ensure that the added nutrients elevated the nutrient levels within the pore water of the beach. Similarly, bioremediation is not successful if the oil is largely biodegraded before the treatment is initiated. However, on the basis of the limited success achieved, the authorities gave permission for bioremediation to be used extensively. During the summer, 1,426 individual treatments were conducted; a further 223 sites were treated in the summer of 1991 (17). Bioremediation was conducted with Inipol EAP 22 and Customblen.

Effect of bioremediation on the microbiota. From September 1989 to September 1990, a detailed investigation of the levels of oil-degrading microorganisms on fertilized and unfertilized shorelines in Prince William Sound and the Gulf of

Alaska was made (77). The aim was to determine whether large-scale use of bioremediation had an effect on the microbial populations on oiled shorelines in comparison with untreated controls. The results from the small field trials suggested that nutrient addition had little consistent impact on the microbial populations (59, 80). A total of 27 sites were monitored: 17 in Prince William Sound and 10 in the Gulf of Alaska (77). The microorganisms were enumerated by most-probable-number methods. Growth on weathered Prudhoe Bay crude oil was used as a measure of the oil-degrading capacity, and growth on marine broth was used as an estimate of total heterotrophs. These measurements will not give an absolute estimate of the number of hydrocarbon degraders and total heterotrophs, but they allowed the authors to compare the effect of nutrient addition on different microbial communities.

The results showed that in September 1989 and September 1990, the number of oil-degrading microorganisms and heterotrophs was significantly larger on the fertilized beaches than on the oiled controls. This effect was seen shortly after the addition of bioremediation agents and suggested that the effect of fertilizer addition lasted at least 1 month. The increase in the number of microorganisms was between 10 and 100 times greater than in the controls (76). This trend was observed on surface and subsurface samples and in the upper, middle, and lower parts of the intertidal zone. After the peak in numbers in 1989, no significant increase in microbial numbers was observed until after the addition of fertilizer in September 1990, suggesting that nutrient enrichment was the cause of the change. This is an interesting result, because there are few reported instances of nutrient addition stimulating hydrocarbon degraders more than in the oiled controls. Furthermore, the number of hydrocarbon degraders as a proportion of the population decreased dramatically over the winter of 1989 to 1990 on both the fertilized and unfertilized beaches. A similar effect of winter temperatures on oil degraders has been noted by other workers (51). This suggests that the seasonal decline in seawater temperatures in north-temperate (Nova Scotia) and Arctic (Alaska) environments may have a disproportionate inhibitory effect on oil-degrading microorganisms.

DISCUSSION

The results of controlled laboratory experiments and field trials following actual oil spills have shown conclusively that bioremediation can enhance the biodegradation of petroleum on contaminated shorelines. However, there is little convincing evidence to suggest that bioremediation is effective at sea. This is partly due to the logistical difficulties involved in conducting controlled open-sea trials (101). Further research is required to derive an effective bioremediation strategy at sea and to confirm the efficacy of such processes under field conditions.

The results of controlled field studies and opportunistic studies following accidental spills (particularly those following the *Exxon Valdez* incident) suggested that the natural rates of oil biodegradation on coastal shorelines can be stimulated two- to sevenfold by bioremediation strategies. However, these techniques do not result in a rapid removal of oil comparable to that achieved by intensive physical cleaning methods. They merely stimulate the natural biodegradative processes. Nevertheless, successful bioremediation is a fairly complete solution to oil contamination. This process leads to the conversion of oil to biomass, water, and gases, which form part of the carbon cycle (primarily carbon dioxide), whereas physical cleaning results only in the transfer of the oil from one compartment in the environment to another (e.g., while some oil recovered physically from beaches may be recycled, in many cases it is

stored in pits or landfills). Furthermore, in terms of the biota, bioremediation is one of the few processes that will actually remove toxic components from the environment.

Field trials have shown that successful bioremediation is complex. The success of the treatment depends particularly on the type of contaminated beach, the penetration of the beach material by fertilizer, the presence of biodegradable petroleum hydrocarbons, the nature of the bioremediation product, and the prevailing environmental conditions (particularly temperature and oxygen content). For example, Inipol EAP 22 does not appear to stimulate biodegradation on shorelines consisting of fine material such as sand but can be effective on the coarse cobble beaches of the Arctic (52, 100). In contrast, Rosenberg et al. (86) have demonstrated that bioremediation involving an oleophilic fertilizer, seeding, regular tilling, and watering stimulated oil degradation on a sandy beach in Israel. Furthermore, in Nova Scotia, inorganic nutrients that were shown to work well on an oiled sandy beach environment were found to be ineffective in an oiled salt marsh environment (51). Studies have also shown that "no bioremediation treatment" should be considered a recommended option, but this will depend on the type and concentration of the oil and the type of contaminated sediment (51).

Conducting research on bioremediation after a spill incident has proved difficult and costly. The researchers cannot control the heterogeneity in oil concentration on shorelines and therefore have to take this into account in their experimental design and analytical procedures. The results to date have been variable. In many cases (for example, in the trials conducted after the *Mega Borg* and *Apex* barge spills), bioremediation has not been shown to enhance oil biodegradation. The reason for these failures has tended to be either poor experimental design, inappropriate sampling and analytical procedures, or simply an inadequate period of monitoring.

However, the comprehensive field program initiated to respond to the *Exxon Valdez* incident produced some important results on the efficacy of bioremediation (17, 77, 80). The bioremediation strategy of adding nutrients to the beaches produced visual reductions in the amount of oil contaminating the beach. The enhanced rates of oil biodegradation were difficult to measure on Alaskan beaches by established methods based on the ratios of the alkanes $n\text{-C}_{17}$ and $n\text{-C}_{18}$ to pristane and phytane, respectively. This was attributed to the fact that phytane and pristane decomposed rapidly in the Alaskan coastal waters. A complex cyclic C_{30} aliphatic compound ($17\alpha,21\beta$ -hopane) was identified to be an excellent conservative biomarker. By using this biomarker, significant enhancement of the oil biodegradation rate was shown on one beach site (KN-135) following bioremediation treatment. On two other beaches on Elrington Island, oil reduction in response to bioremediation was also statistically significant. Enhanced rates of removal of oil residues were found as a result of fertilization on the cobble portion of the beach in Passage Cove. Several of the trials suggested that nutrient addition not only encouraged oil biodegradation but also increased the rate of oil loss by physical and chemical processes on the beach. Some suggestions have been made to explain this enhanced oil loss; for example, increased rates of biodegradation increase the amount of partially oxidized hydrocarbons, which can interact ionically with clay particles to form buoyant flocs, which are removed from the shore (18). Alternatively, enhancing the oil biodegradation rate may also lead to the increased production of biosurfactants which encourage the desorption of oil from the shoreline surface to the water column.

By using multiple regression analysis, a model was developed for the biodegradation of oil which was found to correlate

reasonably well with the experimental data (16). The results tended to confirm the hypothesis that the degree of biodegradation of the oil prior to the application of fertilizer (as measured by the proportion of polar compounds in the oil residue) and the ratio of nitrogen added per unit oil load were the most important factors governing the rate of oil removal. These parameters may form the basis of future models, in which the significance of beach type and climatic conditions may also be considered.

One of the main observations of this review is the limited use of statistical methods to verify conclusions based on oil chemistry results. While many research studies have used statistics for the direct interpretation of quantitative microbiological data, chemical interpretations of the data have been based on changes in the ratios of degradable hydrocarbons to persistent biomarkers when demonstrating bioremediation success. This method of treating the chemical results takes into account variations in oil distribution between samples and does not require analysis of the large numbers of replicate sediment samples for rigorous statistical analysis. From an operational standpoint, the results of numerous studies have consistently shown that oil biodegradation rates can be enhanced in many nutrient-depleted environments by the addition of nitrogen and phosphorus. Nevertheless, application of rigorous statistical methods is needed in future studies to analyze complex time course data sets, consisting of nutrient results, microbiological measurements, and oil chemistry analyses. In conclusion, the results of the field trials concur with those carried out in the laboratory (5, 11, 48) in that bioremediation is an effective technique for the encouragement of oil biodegradation on some contaminated shorelines. This approach can apparently be conducted without presenting a further hazard to the environment, provided that the most toxic component of the application is identified and its concentration is maintained within safe limits (86). However, the success of bioremediation has been judged on only a limited number of shoreline types (with various experimental designs), and it is by no means sure whether this technique is widely applicable. Moreover, the influence of oil-weathering processes on bioremediation success remains to be studied rigorously in the field. Evaporation is thought to remove compounds known to inhibit microbial activity (11, 76). Because the process is rapid, evaporation is likely to be complete before or shortly after oil impacts coastline ecosystems, and it may therefore have little influence on bioremediation success. However, the formation of viscous water-in-oil emulsions (chocolate mousses), which are often washed on to shorelines after spills in bad weather, may have a profound effect on bioremediation success and warrants further study. To address these issues, field trials with accepted statistical and analytical designs (to enable interlaboratory comparison of results) are now being carried out both in North America and Europe to verify the efficacy and operational limitations of bioremediation strategies (53, 63, 117, 119).

Oil-degrading microorganisms are ubiquitous in the environment (11, 36, 64). Only in a few specific environments has the absence of competent microorganisms been thought to limit oil biodegradation (11). Seeding or addition of competent microorganisms has not been carried out on a large scale in response to a recognized paucity of indigenous hydrocarbon degraders. Lee and Levy (48) demonstrated that inoculation with commercial strains of oil-degrading bacteria was ineffective. In this case, the added microorganisms could not compete with the indigenous microbial population, which adapted rapidly to the oil. In contrast, Rosenberg et al. (86) have shown that successful bioremediation occurs with the addition of microorganisms competent at decomposing a urea-formaldehyde

oleophilic polymer within the fertilizer formulation. Although the experiment implied that the added microorganisms were responsible for mobilization of the urea in the polymer, this was not unequivocally confirmed by the experiment, since the role of the added nutrients and possible presence of biosurfactants was not addressed. Further field research is required to elucidate the role of allochthonous microorganisms and the oleophilic nutrient added in this way.

Westlake (120) noted that no single microbial species has the enzymatic ability to metabolize more than two or three classes of compounds typically found in a crude oil. A consortium composed of many different bacterial species is thus required to degrade a crude oil spill significantly. The adaptation of natural bacterial populations to degrade different components of a crude oil in response to their presence suggests that the natural environment is not limited by the need for specific bacterial inocula (3, 50). Thus, except for specific isolated cases, seeding of oil spills will probably offer few advantages.

OPERATIONAL GUIDELINES

Much of the research conducted on bioremediation has concentrated on establishing the potential of the technology for dealing with oil pollution and on whether this methodology has any role as a response strategy for dealing with an oil spill incident. Relatively little attention has been directed toward determining when to use bioremediation and establishing guidelines for the application of the process (102). However, some operational guidance for the treatment of marine oil spills can be inferred from previous studies (47), and further information can be expected to evolve from future research and field testing.

It is unlikely that bioremediation will be used as a first response to spills at sea, particularly if such spills are close to the shoreline. Oil biodegradation rates are not sufficiently high, even when they have been stimulated by nutrients or by the addition of competent microorganisms. The authors consider the most likely use of bioremediation at sea to be the combined use of chemical and biological treatments (101), i.e., combining a bioremediation product (probably an oleophilic fertilizer) and a dispersant to encourage the biodegradation of oil after formation of an oil-in-water emulsion. This approach may reduce the life span of the oil in the water column and help reduce the accumulation of undegraded oil in marine sediments.

The field trials cited within this review have shown that bioremediation by nutrient enrichment may be an effective method of accelerating the rate of oil biodegradation on some shoreline environments. Nonetheless, this strategy is unlikely to lead to a rapid decontamination of beaches. If there are important environmental or political reasons to carry out a rapid decontamination, bioremediation is unlikely to be the first choice. However, it is recommended that treatment options be selected on the basis of a net environment benefit analysis, weighing the gains of oil removal against the consequences of a cleanup strategy (69). Bioremediation tends to perform well in such analyses, because its application can usually be conducted at low cost, with limited personnel, and with relatively little or no environmental impact on the shoreline (16, 37).

Decision To Use Bioremediation

When it can be shown that oil biodegradation on contaminated shorelines is limited by nutrient levels or the absence of a substantial indigenous population of hydrocarbon degraders,

bioremediation may be an appropriate oil spill countermeasure. However, a number of factors must be considered before bioremediation should be used in earnest, including (i) type and concentration of oil, (ii) prevalent climatic conditions, (iii) type of beach that has been contaminated, and (iv) nutrient content (e.g., nitrogen, phosphorus, and oxygen) and pH of the pore water in the beach.

High concentrations of oil may be difficult to treat by bioremediation, although the upper limit has not been quantified. Certainly on the shorelines of Prince William Sound, Alaska, concentrations of ≤ 15 g of oil per kg of beach material were successfully treated (80). Oil in a water-in-oil emulsion may be more difficult to treat than weathered unemulsified oil. However, successful trials have been conducted with oil emulsions. Readily biodegradable oils will be more amenable to bioremediation than those which have a small biodegradable component. There is also some evidence that oil that has already been extensively biodegraded may not be amenable to the simple addition of nutrients (16).

Climatic conditions will influence the choice of whether to use bioremediation. High wave and tidal energy may rapidly remove the oil from a contaminated shoreline, negating the need for shoreline bioremediation. High seas were certainly implicated in the rapid dispersal of oil after the *Braer* incident (60).

Oil biodegradation is reduced significantly at low temperatures (46). Hence, in Alaska, Nova Scotia, and Spitsbergen, bioremediation was most effective in the spring and summer months. Temperature, and not nutrient content (at least nitrogen and phosphorus), was found to limit the biodegradation rate in the winter (43, 49, 51). This will not be the case in tropical and many temperate environments. However, warm and dry weather may inhibit biodegradation by desiccating the beach material, in which case irrigation may be necessary (86).

Successful bioremediation field trials have been carried out on sand, salt marsh, and cobble shorelines. On salt marshes, only low concentrations of oil were treated successfully by nutrient addition (0.3% volume of oil per volume of beach material). At higher oil concentrations, at which the oil had penetrated into the anoxic layer of sediments, oxygen depletion restricted biodegradation (51). Under such circumstance, methods of adding oxygen may have to be considered as part of the bioremediation strategy. Furthermore, low pH in salt marsh sediments may also reduce oil decomposition. On the basis of results of trials with salt marshes, it may be feasible to bioremediate oil-contaminated fine sediments by using nutrient and oxygen amendments. This approach will have to be verified in future field trials. Similarly, shingle beaches may be amenable to bioremediation, particularly to encourage the biodegradation of oil that has entered the subsurface of the shore, although this requires confirmation in future field trials. Oleophilic sources of nutrient may encourage biodegradation on the surfaces of rocks, but this remains to be demonstrated. Alternatively, the use of biosurfactants to help wash the oil from the beach may be worthy of consideration.

On the basis of limited data, Sveum and Bech (99) have recently speculated that bioremediation should be targeted primarily at the oil that is sorbed to beach sediment. They note that physical removal of oil, particularly from the beach surface and from the sediment pores, is likely to be significant immediately after oil addition (3, 30). During this period, conditions for oil biodegradation may be suboptimal. Hence, bioremediation should be focused on the oil remaining on a beach after physical removal processes are largely complete. This approach could be widely applied to different shoreline types.

Bioremediation will be successful only if hydrocarbon-de-

grading microorganisms are present in the contaminated environment and if they have the degradative potential to deal with a high proportion of the spilled hydrocarbons. Hydrocarbon degraders are ubiquitous in the environment, and only a few specific ecosystems have been found to be depleted of competent microorganisms (11). Furthermore, studies have shown that a single microbial species can degrade only one or two classes of hydrocarbon within a crude oil. Consortia of microorganisms are required to significantly biodegrade a large fraction of crude oil (77). If under certain circumstances the indigenous microflora is deficient in hydrocarbon-degrading potential, seeding may be considered, although it should be noted that allochthonous microorganisms may not always survive in the contaminated ecosystem (3, 48). For example, the pH of the ecosystem may be too low for the added microorganisms (particularly for salt marshes) or the organisms may not be adapted to the tidal cycles, the salinity, or the oligotrophic conditions of many beach environments.

Choice of Bioremediation Product

Nutrient products are varied and can be applied as briquettes, granules, or liquid mixtures. Slow-release briquettes tend to decompose through hydrolysis and tidal action (87). It is not generally adequate to place individual briquettes in proximity to the oil contaminating a shoreline because, typically, they can be moved independently relative to the oil by the action of the tide and waves. Briquettes used during the *Exxon Valdez* spill were contained in mesh bags that were tethered to steel bars secured in the beach (32). However, one of the problems with this approach is the positioning of the bags so that they are regularly submerged by successive high tides within the spring to neap tidal cycle. There is also the possibility that the dissolved nutrients will channel down the beach from the bags rather than spreading laterally. Briquettes should be of sufficient density and must be appropriately secured (113).

Granular fertilizers are favored for their ease of application. Solid, slow-release fertilizer granules release their nutrients by dissolution when contacted by seawater or rain. Inorganic nutrient sources such as ammonium nitrate, calcium phosphate, and ammonium phosphate may be used. One such product (Customblen) has a granule coating of vegetable oil to create the slow-release effect (32). Customblen was found to stimulate oil biodegradation (17, 80) on the shorelines of Prince William Sound, particularly in combination with an oleophilic fertilizer. However, on certain beaches, the small granules were washed away before any significant enhancement of bioremediation was recorded. These and subsequent studies (26, 56, 103) have shown that differences in shoreline exposure and tidal energy must be considered with the application of each proposed nutrient formulation.

Oleophilic nutrients are thought to partition preferentially with the oil to promote the growth of the local populations of hydrocarbon-degrading microorganisms at the oil-water interface. Inipol EAP 22 is an example of such a product, in which oleic acid gives the material its hydrophobicity, lauryl phosphate and 2-butoxy-1-ethanol act as surfactants, and urea provides a source of nitrogen (100) (Table 1). Some reports indicate that Inipol EAP 22 has difficulty partitioning with the oil on beaches that contain small particulate material such as sands and fine sediments (48, 52, 100). However, it was effective when added to the cobble beaches of Prince William Sound, particularly when used in combination with slow-release granules. A second disadvantage with oleophilic and other organic fertilizers is that they contain other organic car-

bon sources, which may be biodegraded in preference to the petroleum hydrocarbons.

Inorganic sources of nitrogen and phosphorus, dissolved in seawater and sprayed along the shoreline, have been proven effective on Elrington Island in Prince William Sound (80). However, in addition to highly variable results owing to nutrient washout (121), this technique has a number of disadvantages. First, inorganic nutrients have to be added carefully to ensure that toxic levels of fertilizer components (such as ammonia) are not reached in the beach pore water. Second, repeated additions of fertilizer to the same part of the beach may encourage unnatural levels of algal growth on the shoreline and may lead to eutrophication of nearshore waters. Such an effect was not reported in Prince William Sound, but the risk should not be discounted. Third, this form of application is likely to be more labor and energy intensive and hence expensive than the periodic addition of slow-release fertilizers. However, a single addition of a high concentration of a water-soluble inorganic nutrient has been shown to be effective at encouraging bioremediation (55, 80). This approach is relatively inexpensive and could be combined with later additions of slow-release nutrients.

Monitoring of Bioremediation

Once the bioremediation agents have been applied, careful monitoring is required. Most importantly, the pore water of the beach should be monitored to ensure that the treatment has permeated the subsurface. Regular monitoring of the nutrient levels in the pore water and of microbial activity within the sediments will help determine when additional nutrient applications are required. Fertilizer additions should be made to maintain high but environmentally safe levels of nutrients.

It is recommended that visual and photographic records of the treatment be kept and that periodic chemical analyses be conducted. The degree of decomposition of the oil can be ascertained by relating the levels of biodegradable hydrocarbons to those of conserved biomarkers such as 17 α ,21 β -hopane and by monitoring the change in oil residue weight. These analyses are expensive but will help confirm the efficacy of the treatment and determine attainment of a suitable end point.

Toxicity and Health Considerations

Bioremediation has generally received a positive response from the public. Nevertheless, there are still concerns about potentially adverse effects associated with the application of bioremediation agents on contaminated marine environments. Among these are the possibility that the addition of fertilizers or the generation of metabolic byproducts from oil degradation will cause eutrophication, leading to algal blooms and oxygen depletion. In addition, components of the fertilizer formulations and/or oil-degrading bacterial strains could induce a toxic response in humans and the marine biota (38, 39, 54, 111).

Before products are used in the field, they should be tested to ensure that they have a low toxicity to the environment and that they are efficacious. There are basically two approaches to achieve these aims: a licensing procedure which ensures that a product is nontoxic and attains a basic level of efficacy before it may be considered for use, and a testing methodology to generate impartial data relevant to oil spill treatment specialists, who will decide when and where to use bioremediation. Licensing is under consideration in the United Kingdom, while a testing methodology is being developed both in Canada (13) and in the United States (68, 116, 118). The aim of these

approaches is to prevent the inadvertent use of toxic materials or products with limited efficacy during a spill incident.

Many of the experiments carried out in response to the *Exxon Valdez* incident dealt with the toxicity of the products and subsequent effects on the natural environment (17, 24, 101). Careful laboratory tests defined the toxicity of the products before they were used in the field. These experiments set acceptable limits for the bioremediation products released in the environment. A detailed monitoring program was then initiated to confirm that these limits were adhered to during the field trials.

While adverse effects have not been observed in actual field operations to date (76), the possibility in future incidents does indeed exist. For example, a recent experimental field study has shown that periodic additions of organic fertilizers may suppress oil biodegradation rates because of the development of anoxic and potentially toxic conditions (55). With regard to an identified need in future guidelines, there is now an effort to identify and refine potential microscale toxicity tests which can be used to monitor the effectiveness of bioremediation strategies in reducing the toxicity of oiled shorelines (54, 61). Furthermore, the results of such tests may also serve as an indicator of when remediation programs should be terminated (54).

Finally, it is most important to quantify any hazard to human health and safety from bioremediation products before an application is made. This will involve setting guidelines for the use of protective clothing and training of personnel in the application procedures and may include the monitoring of human health during and after use (15). Microbial products should contain no human pathogens, and it is recommended that they contain no opportunistic pathogens (e.g., category II microorganisms [1]). Any microbial preparation will have to be carefully characterized before use in the field.

CONCLUDING REMARKS

From the results presented in this paper, we conclude that bioremediation is a potential new tool for the cleaning of certain oil-contaminated shoreline types. As bioremediation encourages natural processes, it may well have a low environmental impact compared with physical and chemical removal of oil. The toxicity of the oil spill is reduced by converting numerous components into recyclable products such as carbon dioxide, water, and biomass. However, bioremediation is not a rapid cleanup process, and visual effects may not be evident for at least 15 days after treatment (79).

Even though the potential of bioremediation to treat oil-contaminated shorelines has been established, it is still a new technology for which relatively few operational guidelines exist. In this paper, we have been able to propose some guidance for the use of bioremediation, but this is very much a first attempt. Further research is required, particularly in the field, to establish the limits of bioremediation; precisely when, how, and what to use; and, perhaps most importantly, when not to use such a methodology. These investigations should be carried out in the field and in mesocosms to be relevant to oil spill incidents. There is also a need for new statistical techniques to analyze the relationships between chemical and microbiological data over time. New innovative bioremediation products which are tailored to specific contaminated environments are required. Simple and rapid chemical and microbiological tools are required to monitor bioremediation efficacy. The results will provide important information on effective ways of dealing with oil pollution and will give microbial ecologists further

insight into the response of microbial communities to perturbations by pollution.

Finally, we note with some concern that much of the literature reported from field trials is reported in journals and conference proceedings not subject to rigorous peer review. This makes critical appraisal of the results difficult and hides the research from fellow scientists and potential users of the technology. Studies on soil and groundwater bioremediation have not suffered in this regard, and thus it is hoped in future that more field research will be published in the open scientific literature.

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