Bioremediation of Fossil Fuel Contaminated Soils

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BIOREMEDIATION

Bioremediation involves the use of microorganisms and their biodegradative capacity to remove pollutants (Atlas & Pramer 1990). The byproducts of effective bioremediation, such as water and carbon dioxide, are nontoxic and can be accommodated without harm to the environment and living organisms. Using bioremediation to remove pollutants has many advantages. This method is cheap, whereas physical methods for decontaminating the environment are extraordinarily expensive. Over $1 million a day was spent in an attempt that was only partially successful to clean up the oiled rocks of Prince William Sound AK using water washing and other physical means after an Exxon tanker ran aground there. Neither government nor private industry can afford the cost to clean up physically the nation's known toxic waste sites. Therefore, a renewed interest in bioremediation has developed (Beardsley 1989). Whereas current technologies call for moving large quantities of toxic waste and its associated contaminated soil to incinerators, bioremediation can be done on site and requires simple equipment that is readily available. Bioremediation, though, is not the solution for all environmental pollution problems. Like other technologies, bioremediation has limitations.

Studies on the microbial degradation of hydrocarbons, including determination of the effects of environmental parameters on

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biodegradation rates, elucidation of metabolic pathways and genetic bases for hydrocarbon assimilation by microorganisms, and examination of the effects of hydrocarbon contamination on microorganisms and microbial communities, have been areas of intense interest and the subjects of several reviews (Atlas 1981, 1984; Bartha 1986; Colwell & Walker 1977; Leahy & Colwell 1990; NAS 1985). Rates of biodegradation under optimal laboratory conditions have been reported to be as high as 2,500 to 100,000 $g/m^3/day$ (Bartha & Atlas 1987). Under *in situ* conditions petroleum biodegradation rates are orders of magnitude lower. *In situ* natural rates have been reported in the range of 0.001 to 60 $g/m^3/day$ (Bartha & Atlas 1987). The microbial degradation of petroleum in the environment is limited primarily by abiotic factors, including temperature, nutrients such as nitrogen and/or phosphorus, and oxygen (Atlas 1981, 1984; Leahy & Colwell 1990).

The biodegradation of petroleum and other hydrocarbons in the environment is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the indigenous microbial community (Atlas 1981; Leahy & Colwell 1990). Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions (Rontani *et al.* 1985; Shiaris 1989), high molecular weight aromatics, resins, and asphaltenes are generally considered to be recalcitrant or to exhibit only very low rates of biodegradation. In aquatic ecosystems, dispersion and emulsification of oil in slicks appear to be prerequisites for rapid biodegradation; large masses of mousse, tarballs, or high concentrations of oil in quiescent environments tend to persist because of the limited surface areas available for microbial activity. Petroleum spilled on or applied to soil is largely adsorbed to particulate matter, decreasing its toxicity, but possibly contributing to its persistence.

ASSESSING BIOREMEDIATION TECHNOLOGIES

Laboratory Efficacy Testing

To demonstrate that a bioremediation technology is potentially useful, it is important that the ability to enhance the rates of hydrocarbon biodegradation be demonstrated under controlled conditions.

This generally cannot be accomplished *in situ* and thus must be accomplished in laboratory experiments. Laboratory experiments demonstrate the potential a particular treatment may have to stimulate the removal of petroleum pollutants from a contaminated site (Bailey *et al.* 1973; Chianelli *et al.* 1991; Venosa *et al.* 1991). Laboratory experiments that closely model real environmental conditions are most likely to produce relevant results (Bertrand *et al.* 1983; Bragg *et al.* 1990; Buckley *et al.* 1980). In many cases this involves using samples collected in the field that contain the indigenous microbial populations. In such experiments it is important to include appropriate controls, such as sterile treatments, to separate the effects of the abiotic weathering of oil from actual biodegradation. Such experiments do not replace the need for field demonstrations, but are critical for establishing the scientific credibility of specific bioremediation strategies. They are also useful for screening potential bioremediation treatments.

The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial populations, rates of microbial respiration (oxygen consumption and/or carbon dioxide production), and rates of hydrocarbon degradation (disappearance of individual hydrocarbons and/or total hydrocarbons). The methodologies employed in these measurements are critical. It is assumed, for example, that bioremediation of oil pollutants will result in elevated populations of hydrocarbon degraders. Many of the organisms that form colonies on agar-based hydrocarbon media grow on contaminants rather than on hydrocarbons. Therefore, confirmatory tests are needed. In some studies isolates have been tested in liquid culture with more rigorous criteria, such as measuring actual hydrocarbon disappearance, to establish that particular organisms are, in fact, hydrocarbon degraders. These tests often have shown that less than 30 percent of the organisms that form colonies on oil agar actually are capable of metabolizing hydrocarbons. To overcome these limitations, methods have been developed that utilize indicators of hydrocarbon metabolism. For example, dyes can be used to demonstrate the actual metabolism of aromatic hydrocarbons by specific organisms on agar plates or in liquid culture in microtiter plates (Shiaris & Cooney 1983). Colony hybridization procedures have also been employed to identify positively the colony forming units with the genetic capacity for degrading specific aromatic hydrocarbons, for example, by using gene probes for the naphthalene catabolic genes (Sayler *et al.* 1985). The production of radiolabeled carbon dioxide from radiolabeled hydrocarbon substrates also can be used to demonstrate hydrocarbon utilization (Caparello & LaRock 1975). Such production of $^{14}CO_2$ from radiolabeled hexadecane

has been used with a most probable number format to enumerate hydrocarbon degraders with certainty that the number of organisms determined reflects the real number of hydrocarbon degraders in the sample (Atlas 1979).

Oxygen consumption rates have been used as a measure of microbial utilization of hydrocarbons (Gibbs *et al.* 1975; Venosa 1991). The measurement of such rates, however, may reflect the utilization of contaminants in the sample. This is especially critical since the addition of oil to the environment may result in the death of some organisms and the input of organic carbon from such organisms would be a ready source of carbon for aerobic microbial respiration. Since the consumption of oxygen can produce erroneous results, the production of carbon dioxide, particularly from radiolabeled hydrocarbon substrates, has been used as a more definitive measure of rates of microbial hydrocarbon metabolism (Atlas 1979; Caparello & LaRock 1975). If experiments are performed using simulated field conditions, the measurement of such respiration rates can be extrapolated to rates of hydrocarbon metabolism likely to appear at contaminated sites. Measurements of respiration, however, require that the system be closed. This, of necessity, creates an artificial condition that makes simulation of flow-through systems difficult. The actual measurement of the disappearance of hydrocarbons has been considered a definitive test of whether or not biodegradation has occurred. Interpretation of such data, however, depends upon the actual analytical procedures employed. Methods that measure gravimetrically the loss of hydrocarbons are difficult to interpret. This is possibly because of the inclusion of water in the extract, leading to underestimation of the result; the production of products, leading to a similar underestimation; or the failure to extract efficiently the sample so that an excessive loss of hydrocarbons is erroneously measured.

Most studies have turned to more definitive analytical procedures, such as gas chromatography and mass spectrometry (Fedorak & Westlake 1981; Schwall & Herbes 1979). Gas chromatographic analyses of the aliphatic fraction, often following column chromatographic separation of this fraction from other hydrocarbon fractions, are used most frequently. Such analyses permit the determination of specific hydrocarbon losses so that the degradation of individual hydrocarbons and individual classes of hydrocarbons can be determined. These detailed analyses, particularly when performed using capillary chromatography columns, allow for the inclusion of recovery standards so that the efficiency of hydrocarbon extraction and analysis can be determined. The aromatic hydrocarbon fraction can similarly be analyzed using

capillary column gas chromatography. Most often these analyses are coupled with mass spectrometry using the selected ion monitoring mode to determine the fate of individual aromatic hydrocarbons and classes of aromatic hydrocarbons.

The problems with such analyses, however, are that not all compounds in an oil mixture can be resolved, so a significant unresolved hydrocarbon complex remains to be dealt with. Also, high molecular weight polynuclear aromatic hydrocarbons are very difficult to analyze. In some studies, high-pressure liquid chromatography has been used to assay the biodegradation of such compounds (Heitkamp & Cerniglia 1989).

Field Evaluations

The evaluation of hydrocarbon biodegradation *in situ* is far more difficult than in laboratory studies. Analyses that require enclosure, such as respiration measurements, typically are precluded from such field evaluations. Field evaluations, therefore, have relied upon the enumeration of hydrocarbon-degrading microorganisms and the recovery and analysis of residual hydrocarbons. This is especially complicated since the distribution of oil in the environment typically is patchy; therefore, a very high number of replicate samples must be obtained to yield statistically valid results. Even in partially enclosed containers, the patchiness of hydrocarbon distribution requires the analysis of multiple replicates (Haines & Atlas 1982). Movement of macroorganisms, such as polychaete worms, through sediments creates zones where oxygen incorporation favors biodegradation, while adding to the physical patchiness of the oil distribution. In open water situations, it is difficult to ascertain that appropriate sites are being resampled, especially when time-course determinations are being made to measure rates of hydrocarbon biodegradation.

Because of the problems with quantitation of hydrocarbon recovery from field sites, ratios of hydrocarbons within the complex hydrocarbon mixture have been used to assess the degree of biodegradation (Atlas *et al.* 1981). In particular, the fact that hydrocarbon-degrading microorganisms usually degrade pristane and phytane at much lower rates than straight-chain alkanes has permitted the use of pristane or phytane as internal recovery standards. These measurements assume that pristane and phytane remain undegraded; therefore, by determining the ratio of straight-chain alkanes to these highly branched alkanes, it is possible to estimate the extent to which

microorganisms have attacked the hydrocarbons in the petroleum mixture. In situations, however, where pristane or phytane is degraded at rates similar to straight-chain alkanes, this assumption is invalid, and alternative internal standards, such as hopanes, are required (Atlas *et al.* 1981; Prince *et al.* 1990; Pritchard 1990).

Of equal importance to the problem of determining the appropriate measures to be used in assessing the effectiveness of a bioremediation treatment is the experimental design that includes appropriate controls. Often in a field bioremediation situation, the necessity for cleaning up the pollutants overshadows the need for leaving an untreated reference site that is comparable to the site being treated. Thus at the end of many bioremediation efforts, all areas have been treated, leaving no basis for comparison with what would have happened had no bioremediation treatment been employed. Given the natural degradation capacity of the indigenous microorganisms, this leaves in question the effectiveness of many bioremediation treatment strategies.

Ecological Effects Testing

In addition to demonstrating efficacy, it is essential to demonstrate that bioremediation treatments do not produce any untoward ecological effects (Colwell 1971; Doe & Wells 1978; O'Brien & Dixon 1976). The focus of ecological effects testing of bioremediation has been on the direct toxicity of chemical additives, such as fertilizers, to indigenous organisms. Standardized toxicological tests are used to determine the acute toxicities of chemicals. Chronic toxicities and sublethal effects may also be determined. Generally toxicity tests are run using a bivalve larvae, such as oyster larvae, and a fish, such as rainbow trout. Sometimes regionally important species, such as salmon or herring, are included. Additionally, tests are run to assess effects on algal growth rates to determine what levels of fertilizer application will stimulate oil biodegradation without causing algal blooms. No test protocols have been developed and implemented for testing the potential pathogenicity of seed cultures. Concerns have been voiced that seed cultures could cause disease among humans or plant and animal populations. These concerns have been put aside when indigenous microbes—to which these populations are naturally exposed—are employed.

APPROACHES TO ENHANCING MICROBIAL DEGRADATION

The microbial degradation of petroleum in aquatic and soil environments is limited primarily by nutrients, such as nitrogen and phosphorus, and oxygen availability. The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases for which molecular oxygen is required (Atlas 1984). Aerobic conditions are, therefore, necessary for this route of microbial oxidation of hydrocarbons in the environment. Conditions of oxygen limitation normally do not exist in the upper levels of the water column in marine and freshwater environments (Cooney 1984; Floodgate 1984). The availability of oxygen in soils, sediments, and aquifers is often limited depending on the type of soil and whether the soil is waterlogged (Bossert & Bartha 1984; Jamison *et al.* 1975; von Wedel *et al.* 1988). Anaerobic degradation of petroleum hydrocarbons by microorganisms also occurs (Grbić-Gallić & Vogel 1987; Vogel & Grbić-Gallić 1986; Ward & Brock 1978; Zeyer *et al.* 1986). The rates of anaerobic hydrocarbon biodegradation, however, are very low, and its ecological significance appears to be minor (Atlas 1981; Bailey *et al.* 1973; Bossert & Bartha 1984; Cooney 1984; Floodgate 1984; Jamison *et al.* 1975; Ward *et al.* 1980).

Several investigators have reported that concentrations of available nitrogen and phosphorus in seawater are severely limiting to microbial hydrocarbon degradation (Atlas & Bartha 1972; Bartha & Atlas 1973; Floodgate 1973, 1979; Gunkel 1967; LePetit & Barthelemy 1968; LePetit & N'Guyen 1976). Other investigators (Kinney *et al.* 1969), however, have reached the opposite conclusion: i.e., that nitrogen and phosphorus are not limiting in seawater. The difference in results is paradoxical and appears to be based on whether the studies are aimed at assessing the biodegradation of hydrocarbons within an oil slick or the biodegradation of soluble hydrocarbons. In an oil slick, a mass of carbon is available for microbial growth within a limited area. Since microorganisms require nitrogen and phosphorus for incorporation into biomass, the availability of these nutrients within the same area as the hydrocarbons is critical. When considering soluble hydrocarbons, nitrogen and phosphorus are probably not limiting since the solubility of the hydrocarbons is so low as to preclude establishment of an unfavorable C/N or C/P ratio. Investigators considering the fate of low-level discharges of hydrocarbons (soluble hydrocarbons), thus, have properly concluded that available nutrient concentrations are adequate to support hydrocarbon biodegradation.

Oxygenation to Enhance Oil Biodegradation

Biodegradation of petroleum hydrocarbons at rapid rates requires molecular oxygen. Under anaerobic conditions, hydrocarbons are not biodegraded in the environment at rates that can be used to remediate polluted sites. Therefore, for effective petroleum biodegradation, it is necessary to ensure an available supply of oxygen (Floodgate 1973; ZoBell 1973). The use of forced aeration and nutrient supplementation to stimulate the biodegradation of gasoline in groundwater has been reported by Jamison *et al.* (1975, 1976). This study was conducted on a groundwater supply that had been contaminated with gasoline following a pipeline break. The reservoir was found to contain microorganisms capable of growth on the hydrocarbons that are found in gasoline as the sole carbon source. It was the conditions for growth and metabolism of oil that were found to be limiting. Ammonium sulfate (58 metric tons), monosodium phosphate, and disodium phosphate (29 metric tons) were added to the groundwater. Air was also pumped into the groundwater with a small compressor. This treatment increased the number of hydrocarbon-utilizing microorganisms, and gasoline was degraded. It was estimated that 2×10^5 L of gasoline were removed by stimulated degradation and that the use of forced aeration and nitrogen and phosphorus addition significantly reduced the time necessary to remove the spilled gasoline from this groundwater reservoir. A patent was issued to Raymond (1974) for reclamation of hydrocarbon-contaminated groundwater using stimulated microbial degradation.

To overcome oxygen limitation, hydrogen peroxide may be added in appropriate and stabilized formulations (API 1987). The decomposition of hydrogen peroxide releases oxygen that can support aerobic microbial utilization of hydrocarbons. At concentrations that are too high, however, hydrogen peroxide is toxic to microorganisms and will actually lower rates of microbial hydrocarbon biodegradation. Also, hydrogen peroxide typically is not stable and decomposes rapidly upon addition to contaminated environments. Nevertheless, this treatment has been used effectively to stimulate microbial degradation of environmental hydrocarbon contaminants.

Berwanger and Barker (1988) investigated *in situ* biorestoration involving stimulating aerobic biodegradation in a contaminated anaerobic, methane-saturated groundwater situation using hydrogen peroxide as an oxygen source. Batch biodegradation experiments were conducted with groundwater and core samples obtained from a Canadian landfill. Hydrogen peroxide, added at a nontoxic level,

provided oxygen that promoted the rapid biodegradation of benzene, toluene, ethyl benzene, and *o*-, *m*-, and *p*-xylene. In winter of 1983, Frankenberger *et al.* (1989) found a flow of approximately 4,000 L of diesel fuel along an asphalt parking lot of a commercial establishment toward a surface drain near an open creek. Investigations led to the discovery of a leaking underground diesel fuel storage tank. Hydrocarbon quantities ranged up to 1,500 mg/kg of soil. A laboratory study indicated fairly high numbers of hydrocarbon-oxidizing organisms relative to glucose-utilizing microorganisms. Bioreclamation was initiated in April 1984 by injecting nutrients (nitrogen and phosphorus) and hydrogen peroxide and terminated in October 1984 upon detection of no hydrocarbons (< 1 mg/kg). A verification boring within the vicinity of the contaminated plume confirmed that residual contamination had reached background levels.

Nutriation to Enhance Oil Biodegradation

Landfarming. Much interest has developed in the disposal of oily wastes by soil cultivation. Soil microorganisms have a high capacity for degrading petroleum hydrocarbons (Bossert & Bartha 1984). Several investigators have examined the feasibility of using landfarming for the removal of oily wastes (Bartha & Bossert 1984; Dibble & Bartha 1979a,b; Francke & Clark 1974; Gudin & Syratt 1975; Huddleston & Cresswell 1976; Jones & Greenfield 1991; Kincannon 1972; Lehtomake & Niemela 1975; Maunder & Waid 1973, 1975; Odu 1978; Raymond *et al.* 1976a,b). There have been some reports on mobilization of oil into the soil column (Verstraete *et al.* 1975), but in most cases little evidence has been found for significant downward leaching of oil (Dibble & Bartha 1979a,b; Raymond *et al.* 1976a,b).

Cook & Westlake (1974) reported a series of studies in which northern crude oils were spilled on soils in northern Alberta, Canada. The biodegradation of several different oils was examined. Oil biodegradation was found to be stimulated by application of nitrogen and phosphorus fertilizer. This conclusion was based on observed increases in microbial numbers and chromatographic analysis of the residual oil. For soil application many nitrogen- and phosphorus-containing fertilizers are available (Dotson et al. 1971) that can be tilled into the soil. The best fertilizers for soil application are forms of readily useable nitrogen and phosphorus and slow-release forms that provide a continuous supply of nutrients that are not leached from the oil-soil interface. Kincannon (1972) reported that biodegradation rates of heavy oily

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wastes in soils were as high as 21 L/m^2/month. The addition of nitrogen and phosphorus fertilizer resulted in a doubling of the oil biodegradation rate to 16 kg/m^3/month. Fertilizer was added before the oil and rototilled into the soil. It was recommended that monthly determinations of nitrogen and phosphorus levels in the soil and periodic fertilizer application when necessary would optimize the degradation process. Neither leaching of oil nor applied fertilizer into the soil column was observed. The cost of soil disposal of oily wastes was estimated at $0.02/per liter.

Wang and Bartha (1990) recently studied the effects of bioremediation on residues of fuel spills in soil (2.3 mL cm^3 of jet fuel, heating oil, and diesel oil). Persistence and toxicity of the fuel increased in the order of jet fuel < heating oil < diesel oil. Bioremediation treatment (fertilizer application plus tilling) strongly decreased fuel persistence and toxicity and increased microbial activity as compared to contaminated but untreated soil. Good correlations were found among fuel residue decline, microbial activity, and toxicity reduction. These findings indicate that bioremediation treatment can restore fuel spill contaminated soils in 4 to 6 weeks to a degree that can support plant cover. Recovery of the soil is complete in 20 weeks.

Wang *et al.* (1990) continued their studies on bioremediation treatment to remove the polycyclic aromatic hydrocarbon (PAH) components of diesel oil in soil. Bioremediation treatment, while increasing the rate of total hydrocarbon degradation, had an even greater effect on PAH persistence, almost completely eliminating these compounds in 12 weeks. Without bioremediation, 12.5 to 32.5 percent of the higher weight PAH was still present at 12 weeks. After substantial initial mutagenicity and toxicity, the contaminated soil approached the background level of uncontaminated soil after 12 weeks of bioremediation. Detoxification was complete in 20 weeks.

Marine Oil Spills. The spill of more than 4×10^7 L of crude oil from the oil tanker *Exxon Valdez* in Prince William Sound AK on March 24, 1989 (Hagar 1989), as well as smaller spills in Texas, Rhode Island, and the Delaware Bay (Anon. 1989), has focused attention on the problem of hydrocarbon contamination in marine and estuarine environments and the potential use of bioremediation to remove petroleum pollutants. The ability of environmental modification to stimulate microbial degradation of oil in marine ecosystems by indigenous microorganisms has been demonstrated in several cases. Atlas and Bartha (1973) developed an oleophilic nitrogen and phosphorus fertilizer that Atlas (1975), Atlas and Busdosh (1976), and Atlas and Schofield (1975) tested for its ability

to stimulate petroleum degradation by indigenous microorganisms in several environments. This oleophilic fertilizer, paraffinized urea and octyl phosphate, has been tested in near-shore areas off the coast of New Jersey, in Prudhoe Bay, and in several ponds near Barrow AK, including *in situ* as well as *in vitro* experiments in each case. Each site included a naturally occurring microbial population that was capable of petroleum biodegradation when this oleophilic fertilizer was added to the oil, and in each case, the addition of oleophilic fertilizer stimulated biodegradative losses. The amount of stimulation varied for different crude oils tested, but oil degradation generally was 30 to 40 percent higher in oleophilic-fertilized oil slicks than in unfertilized slicks. Application of oleophilic fertilizer was not found to lead to undesirable algal blooms or to produce effects toxic to invertebrate bioassay organisms. Dibble and Bartha (1976) found additional stimulation with some crude oils when oleophilic iron and ferric octoate were added along with nitrogen and phosphorus. Greater stimulation was observed in nonpolluted than in polluted near-shore waters. Addition of oleophilic iron is likely to result in even greater stimulation of petroleum biodegradation in open ocean areas where iron concentrations are particularly low.

The *Exxon Valdez* spill formed the basis for a major study on bioremediation and the largest application of this emerging technology (Pritchard 1990; Pritchard & Costa 1991). The initial approach to the clean-up of the oil spilled from the *Exxon Valdez* was physical. Washing of oiled shorelines with high-pressure water was expensive, and cleaned shorelines became reoiled, forcing recleaning. Bioremediation, therefore, was considered as a method to augment other clean-up procedures. The U.S. EPA and Exxon entered into an agreement to explore the feasibility of using bioremediation. This historic effort considered a variety of approaches to optimizing microbial degradation of oil. The project focused on determining whether nutrient augmentation could stimulate rates of biodegradation. Three types of nutrient supplementation were considered: water soluble (23:2 N:P garden fertilizer fomulation); slow release (isobutylenediurea); and oleophilic (Inipol EAP 22 = oleic acid, urea, lauryl phosphate) (Chianelli *et al.* 1991; Safferman 1991; Tabak *et al.* 1991). Each was tested in laboratory simulations and in field demonstration plots to show the efficacy of nutrient supplementation. Consideration was also given to potential adverse ecological effects, particularly eutrophication from algal blooms and toxicity to fish and invertebrates. Application rates were adjusted to minimize undesirable ecological impact. Using a sprinkler system periodically to apply the water-soluble fertilizer at low

tide stimulated rates of biodegradation without causing excessive algal growth. The application of the oleophilic fertilizer produced very dramatic results, stimulating biodegradation so that the surfaces of the oil-blackened rocks on the shoreline turned white and were essentially oil-free within 10 days after treatment. The use of Inipol and Customblen was approved for shoreline treatment and was used as a major part of the clean-up effort. A joint Exxon-U.S. EPA-Alaska monitoring effort followed the effectiveness of the bioremediation treatment, which was estimated to increase the rates of biodegradation at least threefold (Chianelli *et al.* 1991; Prince *et al.* 1990).

Microbial Seeding to Enhance Oil Biodegradation

Seeding involves the introduction of microorganisms into the natural environment for the purpose of increasing the rate or extent, or both, of biodegradation of pollutants. The rationale for this approach is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in such complex mixtures as petroleum. However, the premises that the microorganisms naturally present in an environment subjected to contamination with oil would be incapable of extensively degrading petroleum and that added microorganisms would be able to do a superior job should be examined carefully. The criteria to be met by effective seed organisms include ability to degrade most petroleum components, genetic stability, viability during storage, rapid growth following storage, a high degree of enzymatic activity and growth in the environment, ability to compete with indigenous microorganisms, nonpathogenicity, and inability to produce toxic metabolites (Atlas 1977).

Some individuals have proposed that hydrocarbon-degrading microorganisms and their enzymatic capabilities may be critical limiting factors in the rates of hydrocarbon biodegradation (Atlas 1977). Clearly, there is an adaptive process following the introduction of oil into the environment, and if metabolically active hydrocarbon utilizers, capable of utilizing hydrocarbons in the petroleum pollutant, could be added quickly, the lag period before the indigenous population could respond would be reduced. Even if these organisms were subsequently replaced by competition with indigenous hydrocarbon utilizers, there might be some benefit to such seeding operations. However, if freeze-dried or otherwise metabolically inactive organisms were to be added or if delay were necessary to culture such organisms, the benefit of reducing the

lag period before the onset of rapid hydrocarbon degradation might be negated. Additionally, there is the problem of finding microorganisms with the right metabolic capabilities to augment the activities of the indigenous populations, and the problem of adding microorganisms that could survive and favorably compete with the indigenous organisms.

Terrestrial ecosystems differ from aquatic ecosystems in that soils contain higher concentrations of organic and inorganic matter and, generally, larger numbers of microorganisms and have more variable physical and chemical conditions (Bossert & Bartha 1984). The microbial community of soils usually includes a significant hydrocarbon-utilizing component, which readily increases in response to hydrocarbon contamination (Atlas *et al.* 1980; Jensen 1975; Llanos & Kjoller 1976; Pinholt *et al.* 1979). The presence of indigenous microbial populations highly adapted to a particular soil environment would be expected to influence negatively the ability of seed microorganisms to complete successfully and survive; for this reason, soils are not widely considered to be amenable to improvements in rates of biodegradation through seeding alone (Atlas 1977; Bossert & Bartha 1984). Other potential problems associated with the inoculation of soils, as reviewed by Goldstein *et al.* (1985), include inadequate (i.e., extremely low) concentrations of the chemical of interest, the presence of inhibitory substances, predation, preferential metabolism of competing organic substrates, and insufficient movement of the seed organisms within the soil.

Many investigators have suggested that complex mixtures of hydrocarbon degraders would be necessary to degrade effectively all of the hydrocarbons in a complex petroleum mixture. Others have attempted to isolate organisms, which could be stockpiled for use in case of an oil spill or in the treatment of oily wastes, that are particularly effective at degrading hydrocarbons. In particular, some investigators have sought organisms capable of degrading specific components within an oil that usually are only slowly degraded. For example, some investigators have sought organisms that specifically degrade four-ring aromatic hydrocarbons, which are among the more resistant compounds found in petroleum (Heitkamp & Cerniglia 1989).

Microbial seeding of petroleum-contaminated aquatic environments has been attempted, with mixed results. Tagger *et al.* (1983) observed no increase in petroleum degradation in seawater inoculated with a mixed culture of hydrocarbon-degrading bacteria. Atlas and Busdosh (1976) reported increased degradation of oil in a saline Arctic pond after inoculation with an oil-degrading *Pseudomonas* sp., but no

improvement in a freshwater pond. Horowitz and Atlas (1980) found that greater losses of oil in seawater in an open flow-through system occurred when octadecane-coated bacteria were applied 2 weeks after the addition of an oleophilic fertilizer to the system, than when the fertilizer alone was added. In the same study, no significant increases in the loss of gasoline from freshwater sediment were produced by seeding. Venosa *et al.* (1991) found several commercial cultures ineffective at degrading oil but that a few had some potential; field studies, however, could not demonstrate effectiveness of microbial seeding over the biodegradation capacities of indigenous marine microorganisms.

Mixed cultures have been most commonly used as inocula for seeding because of the relative ease with which microorganisms with different and complementary hydrocarbon-degrading capabilities can be isolated. A special culture collection was begun as a depository for hydrocarbon-utilizing microorganisms (Cobet 1974). Several commercial enterprises began to market microorganism preparations for removing petroleum pollutants. Commercial mixtures of microorganisms are being marketing for use in degrading oil in waste treatment lagoons. These commercial microbial seed mixtures are also intended for use in other situations for the removal of oil pollutants. The applicability of seeding-selected bacteria and fungi to oil spills has been patented by Azarowicz (1973). The literature supplied with seed bioremediation products is often the only information available about them. The full claims of their effectiveness remain to be proven.

Biotreatment of the *Mega Borg* spill off the Texas coast consisted of applying a seed culture with a secret catalyst produced by Alpha Corporation to the oil at sea (Mangan 1990). Claims were made that the treatment was successful at completely removing the oil (Mauro 1990a, b), but the effectiveness of the Alpha seeding to stimulate biodegradation has not been verified, nor has the effectiveness of the culture been confirmed by the U.S. EPA in laboratory tests (Fox 1991).

Another approach has been to engineer microorganisms genetically with the capacity to degrade a wide range of hydrocarbons. The potential for creating, through genetic manipulation, microbial strains able to degrade a variety of different types of hydrocarbons has been demonstrated by Friello *et al.* (1976). They successfully produced a multiplasmid-containing *Pseudomonas* strain capable of oxidizing aliphatic, aromatic, terpenic, and polyaromatic hydrocarbons. The genetic information for at least some enzymes involved in alkane and simple aromatic hydrocarbon transformation occurs on plasmids (Chakrabarty 1974; Chakrabarty *et al.* 1973; Dunn & Gunsalus 1973).

The use of such a strain as an inoculum during seeding would preclude the problems associated with competition between strains in a mixed culture. However, there is considerable controversy surrounding the release of such genetically engineered microorganisms into the environment, and field testing of these organisms must therefore be delayed until the issues of safety, containment, and potential for ecological damage are resolved (Sussman *et al.* 1988). A hydrocarbon-degrading pseudomonad was engineered by Chakrabarty and was the organism that the U.S. Supreme Court in a landmark decision ruled could be patented (Anon. 1975). The organism engineered by Chakrabarty is capable of degrading a number of low molecular weight aromatic hydrocarbons, but does not degrade the higher molecular weight persistent polynuclear aromatics, and thus has not been used in the bioremediation of oil spills.

Given the current regulatory framework for the deliberate release of genetically engineered microorganisms, it is unlikely that any such organism would gain the necessary regulatory approval in time to be of much use in treating an oil spill. Such organisms, however, could be useful in enclosed oily waste treatment systems that could be used to replace landfarming as an option for disposing of such residual oils.

SUMMARY

Microorganisms clearly have the potential for degrading a substantial portion of, but not all, the components of an oil that may pollute the environment. Demonstrating the effectiveness of bioremediation generally involves laboratory tests to show potential and field tests to confirm applicability. The rates of microbial hydrocarbon degradation can be enhanced several fold which forms the basis for bioremediation of oil polluted environments. Methods that overcome environmental limitations have proven most effective. These methods include oxygenation and nutriation. For the most part seeding with microbial cultures has produced ambiguous results.

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