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Effectiveness and Safety of Strategies for Oil Spill Bioremediation: Potential and Limitation, Laboratory to Field

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Abstract

Several additional research efforts were identified during the development of test systems and protocols for assessing the effectiveness and environmental safety of oil spill commercial bioremediation agents (CBAs). Research that examined CBA efficacy issues included: (1) development of oil-degrading microbial assemblages for use as positive controls or indigenous microbial flora, (2) assessment of the effect of oil quantity on extent of oil biodegradation, (3) investigation of an apparent anomaly in relative susceptibility of classes of hydrocarbons to biodegradation, and (4) evaluation of the effect of emulsification on oil biodegradation. Environmental safety research explored the use of toxicological endpoints as an alternative to analytical chemical endpoints in addition to techniques for investigating the toxicity of water-soluble fractions of oil. Molecular microbiological tools were developed to study the microbial ecology of oil spill habitats, to detect potential indicators of oil/CBA effects on key ecological processes, such as nitrogen fixation in the rhizosphere, as well as to enumerate indigenous microorganisms important for bioremediation efficacy (i.e., hydrocarbon-degrading bacteria). Finally, field studies allowed assessment of oil biodegradation efficacy in a more realistic context without the constraints of laboratory test systems.

Introduction

Over the last 10 years, an increase in the development of commercial bioremediation agents (CBAs) designed for cleaning up oil spills has provided a variety of choices to on-scene oil spill coordinators, but no standardized procedures existed for selection of appropriate technologies. Two of the more important issues in the selection process are the effectiveness and environmental safety of the CBA. In an earlier project, we developed flow-through test systems that modeled oil spills on open-water and sandy beaches in order to evaluate CBAs. The open-water test system consisted of a 500-ml sealed glass jar with a constant flow of seawater under a slick of weathered oil. The beach test system provided a sandy beach substratum, colonized by seawater microorganisms, inside a 250-ml fluorocarbon beaker receiving two tidal cycles per day. Effluents from both systems were collected for oil residue analysis and toxicity determinations. Gravimetric and gas chromatographic-mass spectrometric analyses (GC/MS) of residues extracted from the test systems provided endpoints for comparing the effectiveness of biodegradation of oil by various CBAs with untreated controls. Coupled with the development of efficacy protocols that used these test systems were environmental safety protocols, designed to evaluate the risk of CBA use to marine and estuarine fauna. Survival and growth of a crustacean *(Mysidopsis bahia,* mysid) and a fish *(Menidia beryllina,* inland silverside) were measured in a 7-day exposure to a CBA by itself, as well as to the CBA in the presence of a sublethal water-soluble oil fraction. The mysid test included a measure of fecundity. To evaluate the possibility of increased toxicity as a result of CBA and oil interaction (e.g., increased oil availability or toxic oil metabolites), mysids were exposed for 7 days to effluent from the efficacy test systems.

During the development of CBA protocols, factors were identified that limit oil degradation effectiveness, and approaches were developed to better assess CBA efficacy and safety. This project summarizes the results of research to address these questions. The studies have been grouped into four broad categories: factors that affect or limit bioremediation efficacy,

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environmental safety endpoints, microbial ecology of oil spill habitats, and field assessments of bioremediation.

This project was a cooperative research effort between the University of West Florida Center for Environmental Diagnostics and Bioremediation and the U.S. Environmental Protection Agency, Gulf Ecology Division. Collaboration with a subcontractor, AEA Technology in the United Kingdom, provided bioremediation research in a field setting, as well as related research with microcosms, characterization of oil-degrading microbial communities, and effects of emulsification.

Factors Affecting/Limiting Efficacy

As evaluation of the test systems progressed, it became apparent that the CBAs selected to test the efficacy protocols did not seem to cause substantial losses of petroleum hydrocarbons. This raised a number of issues regarding factors that may affect or limit bioremediation or our ability to measure bioremediation effectiveness. A significant fraction of the research studied some of these ancillary issues.

Through the use of simple shake-flask systems, more complex open-water and sandy beach "microcosms," and actual field trials, it was possible to address various environmental factors that influence biodegradation of oil in environmental spills. This section focuses on research of those factors: artificial assemblages of microorganisms for development of positive controls and artificial seawater; the order of degradation of oil components; and the extent of biodegradation as influenced by the amount of oil, or emulsification.

Artificial Microbial Assemblages for Positive Controls or as Components of Artificial Seawater [1,2]

We developed a set of standard bioremediation treatments to be used as "positive controls" in order to investigate the effects of environmental parameters on biodegradation of oil in the elected standard environments. The ability to promote consistent biodegradation of target analytes of crude oil, including polycyclic aromatic hydrocarbon (PAH) constituents, was a major requirement of positive control regimes. Selected microorganisms that were applied along with inorganic nutrient supplements (nitrogen and phosphorus) was one type of positive control. The microorganism fraction included strains that degrade PAHs combined with strains selected for degradation of *n*-alkanes and production of biosurfactants. The selected strains were tested for interstrain compatibility. A non-microbial positive control consisted of only inorganic nutrients. These controls were used to refine test systems and examine the effects of environmental parameters. These same microbial strains may also appropriately serve as surrogate indigenous background flora to be included in an artificial seawater to reduce the dependence on collection and shipment of natural seawater for CBA testing. A collaboration with Environment Canada was established to allow exchange of oil-degrading microbial strains for evaluation at each laboratory.

Relative Susceptibility to Biodegradation of Hydrocarbon Compound Classes [3,4]

Much of the oil biodegradation literature supports the concept that PAHs are generally more recalcitrant than the more easily degraded *n*-alkanes. However, we observed substantial depletion of fluorene, phenanthrene, dibenzothiophene, and other PAHs in the active control treatments of test systems that simulated oiled beaches. These active controls consisted of Gulf of Mexico seawater (with no added microorganisms or nutrients) pumped through the test systems in simulated tidal cycles over a 28-day period. One possibility was that these PAHs, which are orders of magnitude more soluble than the *n*-alkanes, dissolved in the seawater and were washed out of the test systems with the tides, perhaps aided by biosurfactants produced by oil-degrading microorganisms. However, PAHs were not detected in the pooled test system effluents. The greater disappearance of PAHs relative to *n*-alkanes was enhanced by the addition of nutrients (inorganic N and P). Microcosm sediment core experiments performed by collaborators in the United Kingdom produced a similar pattern of degradation, only to an exaggerated degree: nutrient-treated cores showed moderate *n*-alkane depletion, but levels of PAHs were below the detection limit of GC/MS.

In a further attempt to resolve the issue of PAH loss via degradation versus wash-out (facilitated by biosurfactants), we examined oiled beach microcosms with sterile synthetic seawater. Triplicate treatments included sterile control, 10 ppm of a bacterially produced rhamnolipid biosurfactant added to the seawater, or biweekly inoculation of the microcosms with two marine bacteria that produce biosurfactants but degrade only *n*-alkanes. Test systems inoculated with the alkane-degrading microorganisms exhibited depletion of the *n*-alkanes, but essentially all of the aromatic analytes were still recoverable from the oiled sand; we were able to recover both alkane and PAH analytes from the other two treatments. This suggests that the compound class of lower PAHs is preferentially degraded by microorganisms indigenous to natural seawater under aerobic conditions.

Effect of Amount of Oil on Biodegradation [2,5]

The degree of oil biodegradation in the open-water and sandy-beach systems was evaluated with a range of oil doses. We used periodic applications of a positive control that supplied inorganic nitrogen and phosphorus and two marine bacteria capable of degrading *n*-alkanes and a range of aromatic compounds. The amount of oil typically used (referred to here as "high-oil") modeled a slick of 0.5-mm nominal thickness: 1.9 ml for the beach and 2.5 ml for the open-water systems; the respective "low-oil" doses were 0.38 ml and 0.25 ml. Gravimetric results indicated that after 28 days, the beach low-oil inoculated treatment lost an average of 22.5% weight, while the high-oil, inoculated treatment lost only 11.3%. The open-water, low-oil inoculated treatment lost 19.1%; the high-oil, inoculated lost 2.9%. Thus, the lower doses of oil were more highly degraded in terms of total oil weight lost. In addition, more of the recalcitrant GC/MS analytes were affected, and to a greater degree, by this positive control treatments than in high-oil dose treatments.

Effects of Emulsification on In Situ *Oil Biodegradation* [6]

Depending to a large extent on weather conditions, spilled oil may undergo emulsification to varying degrees as wind and wave action mix seawater into the oil slick. The effect of emulsification on the biodegradation rate of Arabian Light crude oil was studied by dosing microcosms designed to mimic a fine sediment beach with two oil-in-water emulsions: 25% and 50% artificial seawater:oil (v:v). The bioremediation strategy incorporated the weekly additions of inorganic sources of nitrogen and phosphorous. The results showed that emulsions with a higher concentration of water were more resistant to biodegra-

dation and that addition of external sources of nitrate and phosphate was not effective in enhancing rates of biodegradation over background rates. Conversely, emulsions with a lower water content were more amenable to biodegradation and the rate of breakdown could be significantly enhanced by the use of inorganic fertilizers. This suggested that emulsification may be a key factor influencing the rate at which oil spilled at sea is biodegraded, when it is subsequently washed ashore. The ability of responders to enhance this degradation by using bioremediation will depend on the level of emulsification.

Environmental Safety Research

It is important to assess the environmental impact of the application of biotechnology products to oil spills in marine environments. CBAs contain a variety of components, including fertilizers, microorganisms, surfactants, enzymes or combinations of these ingredients, and may themselves be toxic to resident organisms.

Various inert particulates (e.g., clay) used as carriers may also be harmful. Indirect effects of CBAs could include oxygen depletion through eutrophication or increased activity of oil-degrading microorganisms, increased bioavailability of toxic oil components from CBA-associated microbiologically generated surfactants, or enhanced production of toxic oil degradation metabolites. Research described here examines the use of toxicology as an alternate endpoint to analytical chemistry for evaluating efficacy, as well as selection of oil:water ratios for preparing a water soluble fraction (WSF) of oil for toxicity testing.

Use of Toxicological Endpoints as Alternatives to Bioremediation Efficacy Endpoints [1,7]

A 10-day amphipod *(Leptocheirus plumulosus)* sediment toxicity test (American Society for Testing and Materials, E1367-92) was adapted to evaluate increased toxicity that might be associated with the formation of toxic metabolites in the beach test system following the 28-day CBA efficacy test. The test has two endpoints: survival and the amphipod's ability to rebury itself at the end of the 10-day exposure period. However, we observed that oiled sediment, whether subjected to bioremediation or not, was toxic to this test organism, thus preventing accurate assessment of any added toxicity due to metabolites from bioremediation.

Further research examined whether reduction in the toxicity of oiled sediments through bioremediation could be used to reduce effective mortality (reburial) as an alternative to chemical analysis of oil residues. The addition of as little as 40 or 100 mg of oil to beach test systems increased the effective mortality of *L. plumulosus* to 69% and 79%, respectively. Oiled beach test systems that were treated with oil-degrading microorganisms and nutrients showed significant losses of oil residue weights relative to the untreated control; moreover, such treated microcosms showed substantial and significant reductions in the target analytes as determined by conventional GC/MS analyses, indicating that the remediation was a "success." However, we could find no differences in the effective mortality of the test organisms between the bioremediated systems and the untreated, oiled controls. It could be that oil components had been metabolized to equally toxic compounds, or that reburial of the amphipods was influenced by characteristics of the oil that may be unaltered by the bioremediation treatment (e.g., the ability of resins and asphaltenic compounds to stick to the amphipods). Thus, although the results indicate that significant reductions in analytical chemical endpoints do not necessarily correlate with decreased toxicity and perhaps should be reexamined, additional research will be required to develop the very sensitive amphipod test into a useful indicator of efficacy.

Preparation of Water-Soluble Fractions (WSFs) of Crude Oil for Toxicity [8]

The toxicity of crude oil components occurring in the aqueous environment is of special importance in the consideration of the environmental impact of crude oil spills on water. Although considerable research has been conducted to determine the toxicity of aqueous solutions containing dissolved and/or particulate oil to aquatic organisms, methods for preparing aqueous media in these studies vary substantially. Mixing time, mixing energy, oil properties, oil-to-water ratio, temperature, light conditions, and properties of the water used may affect the composition of oil components in the water phase. Most studies prepare a WSF by layering oil on water and mixing the two phases together for a designated period; after separation, the water phase is removed as the test solution. A clear relationship between the oil-to-water ratios used for WSF and the chemical and toxicological effects is not apparent in the current literature. We prepared WSFs of both weathered and fresh Alaskan North Slope crude oil with a range of oil-to-water ratios. Toxicities of WSFs in a series of acute and short-term chronic toxicity exposures of the mysid, *Mysidopsis bahia,* were tested, resulting in no apparent differences among oil-to-water ratios ranging from 1:9 to 1:499. This study suggests that petroleum hydrocarbon components distributed into the water column reach saturation, and their effects on submerged aquatic biota would not be expected to change substantially over a wide range of oil pollution levels, and that high oil:water ratios may be unnecessary.

Microbial Ecology of Oil Spill Habitats

The microbial ecology of areas that may be impacted by oil spills and, potentially, CBAs relates to both an environmental safety issue (environmentally significant communities that could be adversely affected) as well as an efficacy concern (presence of oil-degrading microorganisms). This section describes the development of tools to assess diversity of microbial communities with respect to important ecological functions, such as nitrogen-fixation, and to enumerate hydrocarbon-degrading microorganisms whose activity could be stimulated with appropriate amendments.

Effects of Oil Pollution/Bioremediation on Bacterial Diversity [9,10,11]

Salt-marsh and wetlands ecosystems comprise some of the more sensitive and biologically active ecosystems on earth. The proximity of salt marsh ecosystems to oil-related activities increases the potential for contamination in these ecologically sensitive habitats. We studied the effects of sediment oiling on marsh plants and their rhizosphere microflora by growing *Spartina alterniflora* seedlings for 4 weeks in autoclaved or unautoclaved artificial sediments mixed with oil and inoculated with a characterized microbial rhizoflora culture from a naturally occurring S. *alterniflora* colony. At harvest, we examined the short-term physiological adaptation of the rhizosphere by microbial fatty acid profiles and the genetic diversity and activity of ecologically significant enzymes (glutamine synthetase, glutamate synthase, and glutamate dehydrogenase). Strongly conserved genetic regions for glutamine synthetase and

glutamate dehydrogenase have been identified for probing restriction-digested DNA. Effects on the plants were assessed by measuring chlorophyll quantity and quality, root and shoot dry weights, and mortality. Branched-chain fatty acid methyl esters representative of inoculated rhizosphere communities decreased in oiled sediments. Plants grown in oiled sediments had significantly reduced biomass and chlorotic leaves, and autoclaved sediments supported more vigorous plant growth.

Gene probe analyses and other methods were developed in order to detect changes in diversity of subsets of microbial communities associated with the rhizosphere of wetlands in response to stress of oil contamination or bioremediation efforts. Microorganisms that catalyze nitrogen fixation were studied, since these populations were among those likely to be affected by remediation strategies involving application of bioavailable nitrogen. Although we used these technologies on model wetlands systems, they would be applicable to other matrices. We attempted to correlate measurements of the appropriate microbial activities (e.g., acetylene reduction, ammonium assimilation, environmental nitrogen fluxes) with oil dose.

A method to assess the community structure of nitrogen-fixing bacteria in the rhizosphere was developed. Total DNA was extracted from the macrophytic plants' root zones *(Spartina alterniflora and Sesbania macrocarpa)* by bead beating and was purified by CsCl-EtBr gradient centrifugation. The average DNA yield was 5.5 $\mu g\ g^{-1}$ of soil and was of sufficient purity for PCR amplification of *nif*H. [α-^{32}P] dCTP was incorporated into the PCR reaction and *nif*H PCR products were restriction digested. Restriction Fragment Length Polymorphism (RFLP) analysis of the amplified sequences revealed differences in the community structure of nitrogen-fixing rhizobacteria of the field-collected salt marsh plant, *Spartina alterniflora, and* of a laboratory cultured *Sesbania macrocarpa*. Soil inoculation experiments were used to determine the efficiency of the methods, and amplified *nif*H DNA could be detected when 10^4 cells each of *Vibrio natriegens* and *Azotobacter vinelandii* were added per gram of soil. Restriction patterns produced by each species were detected at 10^6 cells g^{-1} soil. These results indicate that RFLP analysis of amplified *nif*H sequences from rhizosphere communities may provide information on species composition and reveal shifts in diversity.

Development of Molecular Methods to Monitor Hydrocarbon-Degrading Bacteria [12]

Methods were developed for the molecular biological analysis of hydrocarbon-degradation genes during an oil spill bioremediation field trial at Stert Flats, Somerset, UK (see next section, Field Research). PCR primers were developed that would specifically amplify a diverse range of *meta*-cleavage dioxygenase genes (*xyl*E, *nah*C, *bph*C, *mpc*I, *mpc*II and a gene encoding a component of the alkane monoxygenase gene (*alk*B) from cultivated microorganisms and from nucleic acids extracted from environmental samples. (These primers can also be used to generate polynucleotide gene probes useful for the analysis of cultured bacteria and environmental nucleic acids.) We were unable to enumerate toluene- or naphthalene-degrading bacteria from an oil spill site by dilution plate methods, suggesting that the populations of these organisms at the Stert site were low. Our initial plan to concentrate on the diversity of *meta*-cleavage genes in these bacteria was therefore modified to encompass the analysis of total hydrocarbon-degrading bacteria obtained either by dilution plate methods or by most probable number (MPN) methods. We could not successfully use the gene probe methods on samples from the MPN plates and thus used dilution plating on oil agar, followed by colony blot procedures to examine the hydrocarbon degradation genes in the cultivated fraction of hydrocarbon-degrading bacteria. This revealed a predominance of bacteria containing only *alk*B-like genes; no aromatic ring-cleavage dioxygenase genes were ever detected in colony blots.

A method to extract DNA suitable for enzymatic amplification was developed and used on samples of beach sediment from the Stert site. When this had been achieved successfully, DNA isolated from selected plots at the Stert site was challenged with our complete suite of primers and probes specific for catabolic genes involved in aromatic and aliphatic hydrocarbon degradation. The results obtained were strikingly different from the colony hybridization procedures. It proved very difficult indeed to detect *alk*B-like genes using a combined PCR-gene probe assay, while *xyl*E-like genes were readily detectable in the beach sediments. Other *meta*-cleavage dioxygenases were less widespread and *nah*C-, *mpc*I- and *mpc*II-like genes were not detected. Some plots were shown, however, to contain genes similar to the *bph*C gene.

As part of the method development for the project, a large number of naphthalene- and toluene-degrading bacteria were isolated from river water and sediments and subjected to PCR and gene probe analysis with primers and probes specific for *xyl*E-, *nah*C- and *bph*C-like genes. In addition, the bacterial strains were characterized using random amplification of polymorphic DNA-PCR (RAPD-PCR). This showed that considerable diversity existed in the naphthalene and toluene-degrading bacteria from the river water and sediments. However, almost all the strains characterized harbored genes homologous with the well-characterized *xyl*E *and nah*C genes.

With the development of nucleic acid-based methods to study microbial ecology here and in other laboratories, there is potential to expand our knowledge of both the microbial populations involved in bioremediation and their catabolic genes. While detection of specific genes associated with particular catabolic activities is useful, determining the expression and activity of these genes would be of far greater value. Methods to do this have been developed recently by others. Application of this to petroleum hydrocarbon bioremediation offers exciting possibilities for the elucidation of changes not only in microbial populations but also their activities and how these relate to observed changes in hydrocarbon degradation.

Field Research [6,13,14,15,16]

Laboratory research on bioremediation efficacy strategies may suffer from the limitations of laboratory constraints. With this in mind, field studies guided by results from sediment column microcosm experiments were conducted. These studies incorporated an additional endpoint, respirometry, to assess the effectiveness of stimulating indigenous oil-degraders with nutrients.

A field evaluation of the use of bioremediation to treat oiled fine sand in the intertidal zone of Stert Flats (Somerset, UK) was conducted, and the use of *in situ* respirometry and analytical chemistry to monitor bioremediation success was evaluated. Early experimental studies had shown that superficial oil is rapidly removed from Stert Flats, with tidal action removing or depositing 0.05 - 0.10 m of fine sand in a single tidal cycle.

Thus, only oil found at depth as a result of penetration or burial by sediment deposition is persistent. To evaluate the feasibility of bioremediation to treat this stranded subsurface oil, a subsequent field trial was conducted using inorganic sources of nitrogen and phosphate. Arabian light crude oil (weathered and emulsified with 25% seawater) was added to selected plots at a coverage of 4 $l \cdot m^{-2}$. Regular addition of nutrients (sodium nitrate and potassium dihydrogen orthophosphate) was made throughout the 3-month experiment, beginning 1 week after oil application. The application rate was determined by separate laboratory studies using columns of sediment from the field site. The success of the bioremediation strategy was determined by chemical analysis of the residual hydrocarbons and monitoring of carbon dioxide evolution *in situ*. The results suggest that inorganic fertilizer did stimulate the biodegradation and mineralization of oil buried in the aerobic zone of fine sediments.

Conclusions

Earlier studies indicated that application of bioremediation technologies to simulated oil spills in open-water and beach model systems did not result in high oil biodegradation rates. Further advancement of this technology requires a full understanding of its limitations as well as sound approaches to overcome them. We hope these studies will advance more valid criteria for assessing the effectiveness and safety of bioremediation approaches to environmental oil spills, and that our conclusions can be extrapolated from these laboratory model systems and field trials to actual environmental spills. We believe that the results of the development of a consensus for efficacy and safety criteria endpoints will provide better guidelines for developers of CBAs to improve their products.

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