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Bioremediation of Hazardous Wastes

Research, Development, and Field Evaluations

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BIOREMEDIATION OF HAZARDOUS WASTES: Research, Development, and Field Evaluations

Biosystems Technology Development Program
Office of Research and Development
U.S. Environmental Protection Agency

U.S. Environmental Protection Agency
Ada, OK; Athens, GA; Cincinnati, OH; Gulf Breeze, FL;
and Research Triangle Park, NC

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Executive Summary

The U.S. Environmental Protection Agency's (EPA's) Office of Research and Development (ORD) hosted the eighth annual Symposium on Bioremediation of Hazardous Wastes: Research, Development, and Field Evaluations in Rye Brook, New York, August 8-10, 1995. More than 250 people attended, including leading bioremediation researchers, field personnel from federal, state, and local agencies, and representatives from industry and academia. Three speakers opened the symposium with introductions and background information on bioremediation research.

Fran Kremer, Coordinator of the Bioremediation Field Initiative and Symposium Chairperson, began by introducing several members of the scientific steering committee of the Biosystems Technology Development Program (BTDP). The BTDP draws on ORD scientists who possess unique skills and expertise in biodegradation, toxicology, engineering, modeling, biological and analytical chemistry, and molecular biology.

George Pavlou, Deputy Director of EPA's Region 2 Emergency and Remedial Response Division in New York City, presented a summary of recent advances in bioremediation technology. Mr. Pavlou noted that new techniques have increased the availability of in situ contaminants to biological degradation, and they have also increased the range of contaminants that can be treated biologically. EPA scientists have learned that microorganisms are not only able to transform simple hydrocarbons but also such toxic and resistant contaminants as chlorinated aromatics and heavy metal salts. Mr. Pavlou provided a regional perspective on these developments, describing how bioremediation has been put to use in Region 2.

Timothy Oppelt, Director of the National Risk Management Research Laboratory in Cincinnati, Ohio, discussed the future of bioremediation on the national level. He explained that bioremediation has been essential to the development of cost-effective cleanup technology and that it has already been used at more than 450 sites. This technology could potentially save hundreds of millions of dollars in future cleanup costs. While bioremediation is now predominantly used for petroleum decontamination, it will be applied to a wider range of sites in the future. Mr. Oppelt concluded by describing the reorganization of research under ORD and by warning that funding shortages may hinder the development of bioremediation technology.

The 33 papers and 22 posters presented at the conference highlighted recent program achievements and research projects aimed at bringing bioremediation into more widespread use. Taken as a whole, these topic areas represent a comprehensive approach to bioremediation of hazardous waste sites. The presentations were organized into five key research and program areas:

- *Bioremediation Field Initiative:* This initiative was instituted in 1990 to collect and disseminate performance data on bioremediation techniques from field application experiences. The Agency assists regions and states in conducting field tests and in carrying out independent evaluations of site cleanups using bioremediation. Through this initiative, tests are under way at Superfund sites, Resource Conservation and Recovery Act corrective action facilities, and underground storage tank sites. Three papers presented at the symposium were devoted to this key program area.
- *Field Research:* Once a bioremediation approach has proven effective in a laboratory or pilot-scale treatability study, it must be monitored and evaluated at a field site. The objective of this level of research is to demonstrate that the particular bioremediation process performs as expected in the field. For most bioremediation technologies, certain key factors concerning applicability, such as cost effectiveness, cannot be thoroughly evaluated until the approach is scaled up and field tested. Ten papers and two posters provided information on recent field research.
- *Performance Evaluation:* Performance evaluation involves assessing the extent and rate of cleanup for particular bioremediation processes as well as monitoring the environmental fate and effects of contaminants and their biological byproducts. Two papers and one poster addressed this area.
- *Pilot-Scale Research:* Pilot-scale research provides information on the operation and control of bioremediation technologies and the management of process-related residuals and emissions to enable the full-scale application of a technology. Given the expanding base of experience with various bioremediation methods, the need for pilot-scale research is

increasing. Ten papers and three posters were presented concerning research based on microcosms of field sites.

- Process Research: Process research involves isolating and identifying microorganisms that carry out biodegradation processes as well as developing techniques for modeling and monitoring such processes. This research is fundamental to the development of new biosystems for treatment of environmental pollutants in surface waters, sediments, soils, and subsurface materials. Nine papers addressed this critical area, focusing on the role of metals and chlorinated organics in bioremediation. In addition, six poster presentations discussed process research.

- Hazardous Substances Research Centers: In addition to presentations on research being carried out under the BTDP, the symposium included nine poster presentations from the EPA Hazardous Substance Research Centers (HSRC). The scientists and engineers involved in HSRC conduct EPA research sponsored by the following centers: the Northeast Hazardous Substance Research Center (Regions 1 and 2), the Great Lakes and Mid-Atlantic Hazardous Substance Research Center (Regions 3 and 5), the South/Southwest Hazardous Substance Research Center (Regions 4 and 6), the Great Plains and Rocky Mountain Hazardous Substance Research Center (Regions 7 and 8), and the Western Region Hazardous Substance Research Center (Regions 9 and 10).

Introduction

Bioremediation is one of the most promising technological approaches to the problem of hazardous waste. This process relies on microorganisms such as bacteria or fungi to transform hazardous chemicals into less toxic or nontoxic substances. There are several reasons why such biological transformation is often more attractive than direct chemical or physical treatment. Microorganisms typically:

- Directly degrade contaminants rather than merely transferring them from one medium to another.
- Employ metabolic degradation pathways that can terminate with benign waste products (e.g., carbon dioxide and water).
- Derive the food energy necessary to degrade contaminants from the contaminants themselves.
- Can be used in situ to minimize disturbance of the cleanup site.

For these reasons, microorganisms can be effective, economical, and nondisruptive tools for eliminating hazardous chemicals. Until recent years, however, the use of bioremediation was limited by the lack of a thorough understanding of biodegradation processes, their appropriate applications, their control and enhancement in environmental matrices, and the engineering techniques required for broad application of the technology.

Because the U.S. Environmental Protection Agency (EPA) believes that bioremediation offers an attractive alternative to conventional methods of hazardous waste cleanup, it has developed a strategic plan for its acceptance and use by the technical and regulatory communities. The Agency's strategic plan is centered on site-directed bioremediation research to expedite the development and use of relevant technology. EPA's Office of Research and Development (ORD) developed an integrated Bioremediation Research Program to advance the understanding, development, and application of bioremediation solutions to hazardous waste problems threatening human health and the environment.

Related bioremediation studies are being carried out at five EPA Hazardous Substance Research Centers (HSRCs) under the direction of ORD's National Center for Extramural Research and Quality Assurance (NCERQA). EPA was authorized to establish these centers by provisions in the 1986 amendments to the Superfund law calling for research into all aspects of the "manufacture, use, transportation, disposal, and management of hazardous substances."

EPA's bioremediation research efforts have produced significant results in the laboratory, at the pilot scale, and in the field. The many accomplishments include aquifer restoration, soil cleanup, process characterization, and technology transfer. Research also focuses on extending the range of substances that can be treated with biological agents. This symposium was held to present and discuss recent developments in bioremediation research undertaken during 1994 and 1995 under the Biosystems Technology Development Program.

In this document, abstracts of paper and poster presentations from the symposium are organized within five key research and program areas:

- Bioremediation Field Initiative
- Field research
- Performance evaluation
- Pilot-scale research
- Process research

The last section of this document includes abstracts of presentations on bioremediation research performed as part of the HSRC program.

Section 1
Bioremediation Field Initiative

The Bioremediation Field Initiative is one of the major components of EPA's Bioremediation Research Program. The Initiative was undertaken in 1990 to expand the nation's field experience in bioremediation techniques. The Initiative's goals are to more fully assess and document the performance of full-scale bioremediation applications, to create a database of current field data on the treatability of contaminants, and to assist regional and state site managers using or considering bioremediation. The Initiative is currently tracking bioremediation activities at more than 400 sites under government and private-sector jurisdiction, in both the United States and Canada. Performance evaluations are currently being conducted at nine sites, three of which were reported on at this symposium.

Data were presented from work at the St. Joseph, Michigan, Superfund site on the use of iron as an electron acceptor and the potential for natural attenuation of chlorinated solvents. A presentation was given on the modeling of the natural attenuation of solvents at the ground-water and lake interface. Studies also were carried out on the design and field applications of bioventing in the bioremediation of jet fuel spills.

Intrinsic Bioremediation of Trichloroethylene at the St. Joseph Aquifer/ Lake Michigan Interface: A Role for Iron and Sulfate Reduction

Jack Lendvay, Mike McCormick, and Peter Adriaens
University of Michigan, Ann Arbor, Michigan

Introduction

The anaerobic aquifer at the St. Joseph, Michigan, National Priorities List (NPL) site was contaminated with trichloroethylene (TCE), which has been shown to have dechlorinated to cis- and trans-dichloroethylene (DCE), vinyl chloride (VC), ethylene, and ethane. These products occur as a result of natural attenuation processes, presumably under methanogenic conditions (1).

The flux of all alkyl halides into Lake Michigan is of major public concern because of the suspected carcinogenicity of VC. As the plume moves toward the aerobic surface water, the dominant redox conditions can be expected to change because of wave action and vertical seepage, which promote the interchange of oxygen-rich lake water and anaerobic ground water. This presentation provides preliminary results of laboratory investigations geared toward determining the prevailing redox processes and the potential for natural attenuation of chlorinated solvents at the interface.

Background

Three of the most important redox processes in the natural anaerobic environment are the coupling of the oxidation of organic matter to iron (Fe) (III) reduction, to sulfate reduction, and to methanogenesis. These three processes are considered mutually exclusive which, in the anaerobic subsurface environment, results in the development of spatially or temporally distinct redox zones. It has been demonstrated in aquatic sediment and aquifer samples that Fe(III)-reducing bacteria can outcompete sulfate reducers, as well as methanogens, for organic matter (2, 3).

Mineral-bound Fe(III) has been shown to contribute significantly to the total oxidation capacity of both pristine and contaminated aquifers, as it often represents the most abundant anaerobic terminal electron acceptor (4, 5). The speciation of iron in aquifer solids is greatly influenced by microbial processes, particularly under redox conditions favoring sulfate- and Fe(III)-reduction. Depending on the temporal or spatial succession in the development of subsurface redox conditions, Fe(II) produced by iron reducers precipitates as iron sulfides once sulfate-reducing conditions develop, or as iron oxides such as magnetite (Fe_3O_4) in the absence of sulfide. Alternatively, biogenically produced sulfide may precipitate as $FeS_{(1-x)}$ (mackinawite) after reductive dissolution of Fe(III) minerals. The iron sulfide and iron oxide minerals thus formed may then contribute significantly to the reduction capacity of aquifer solids and, in turn, play a major role in the fate of organic contaminants. The presence of these precipitated minerals is direct evidence for past or present iron- or sulfate-reducing conditions.

Materials and Methods

A three-stage iron analysis was performed on sediments collected from the same depths in sampling wells 55AB (upstream) and 55AD (near shore) to evaluate the occurrence of oxidized and reduced iron minerals, and to calculate inorganic reducing equivalents present in aquifer materials.

- *Bioavailable iron (Fe(III)):* Microbial Fe(III)-reduction has been shown to predominantly use amorphous oxyhydroxides and goethite as terminal electron acceptors. Quantitation of these minerals can be approximated by extracting sediments according to Lovley and Phillips (6).
- *Ferrous monosulfides and amorphous iron oxides (Fe(II)):* Reduced Fe(II) minerals resulting from microbial iron and sulfate reduction, predominantly ferrous sulfides and iron oxides, can be quantified using a 24-hr extraction with 0.5 M hydrochloric acid (HCl) (7). As this extraction removes bioavailable iron as well, FeS can be approximated by substration.
- *Siderite, crystalline iron oxide, and magnetite (Fe(II)):* This extraction represents the precipitated ferrous

iron fraction in the absence of sulfate reduction. Wet sediment samples were extracted with 5 M HCl for a 21-day period, and analyzed according to Heron and Christensen (5).

This information was then used in conjunction with available data on ground-water chemistry (pH, redox, carbonate, sulfate, sulfide) and compiled in a chemical equilibrium model (MINEQL+) to predict speciation of iron. Precipitation of ferrous iron solids was based on stability constants from the literature.

Results

The analysis of samples from wells 55AB and 55AD indicated the presence of similar concentrations of bioavailable Fe(III) (5 mmol equiv./kg) (Table 1). Whereas precipitated iron oxides and ferrous monosulfides increased dramatically toward the shore, however, solids representative of the absence of sulfate reduction significantly decreased (12 versus 5 mmol equiv./kg aquifer material, after subtraction of bioavailable iron). This observation, based on iron extraction data, was confirmed by chemical modeling, which predicted a predominant occurrence of siderite in the sampling point farthest from the lakeshore and increasing occurrence of iron sulfide and mackinawite closer to shore and into the lake. Iron- and sulfate-reducing activity was relatively easily stimulated in these sediments.

Ongoing dechlorination experiments under sulfate- and iron-reducing conditions, using TCE and VC, have resulted in the production of t-DCE from TCE under iron-reducing conditions. Whether the production of the trans rather than the cis isomer is indicative of an abiotic dechlorination mechanism (by Fe(II)) remains to be elucidated. VC did not dechlorinate under sulfate-reducing conditions during the 2 months monitored to date.

Information available from previous field studies in collaboration with the Robert S. Kerr Laboratories (Dr. John Wilson) suggested that neither methanotrophic nor methanogenic activity predominates in the contaminant plume under the shoreline and Lake Michigan, based on redox potential and oxygen and methane measurements. Redox potentials and an increase in soluble iron concentrations between both shore samples and below the Lake Michigan bottom, however, suggest that iron reduction may be the dominant process in regions near plume emergence (8).

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Table 1. Determination of Iron and Sulfate Equivalents in St. Joseph Sediments

Sample Location and Analysis	Chemical Species Analyzed	Chemical Concentration	Redox Equivalents
Well 55AB	**Iron Extractions**	**(mg/kg soil)**	**(mmol equiv./kg soil)**
1-hr/0.5 M HCl	Limited Fe(II)	219.2 ± 15.8	3.9 ± 0.3
1-day/0.5 M HCl	Limited Fe(II)	210.6 ± 30.6	3.8 ± 0.6
1-hr/0.25 M HCl and 0.25 M $NH_2OH \cdot HCl$	Limited Fe(II) and Bioavailable Fe(III)	269.6 ± 29.7	4.7 ± 0.5
21-day/5.0 M HCl	Limited Fe(II)	881.8 ± 66.2	15.8 ± 1.2
	Sulfate Extractions	**(mg/L)**	**(mmol equiv./L)**
Ion chromatography	Soluble sulfate	25.8 ± 3.5	2.2 ± 0.3
Well 55AD	**Iron Extractions**	**(mg/kg soil)**	**(mmol equiv./kg soil)**
1-hr/0.5 M HCl	Limited Fe(II)	333.4 ± 11.5	6.0 ± 0.2
1-day/0.5 M HCl	Limited Fe(II)	445.5 ± 16.9	8.0 ± 0.3
1-hr/0.25 M HCl and 0.25 M $NH_2OH \cdot HCl$	Limited Fe(II) and Bioavailable Fe(III)	270.8 ± 8.2	4.9 ± 0.2
21-day/5.0 M HCl	Limited Fe(II)	735.9 ± 48.7	13.2 ± 0.9
	Sulfate Extractions	**(mg/L)**	**(mmol equiv./L)**
Ion chromatography	Soluble sulfate	29.3 ± 4.2	2.4 ± 0.4

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Modeling Intrinsic Remediation as Ground-Water Discharges to a Lake: The Trichloroethylene Plume at St. Joseph, Michigan

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Introduction

Contamination of ground water by chlorinated solvents is widespread and has been in the forefront of public and regulatory concern for the last decade. As a result, considerable research efforts, both in the laboratory and in the field, have addressed the potential for using biological processes to degrade these pollutants via either in situ or onsite bioremediation technologies. Natural attenuation has been observed to be responsible for removal or partial transformation of both chlorinated and nonchlorinated organic contaminants. At several sites, naturally occurring reductive dechlorination has been found to be responsible for the anaerobic transformation of trichloroethylene (TCE) to lesser chlorinated intermediates, such as c- and t-dichloroethylene (DCE) and vinyl chloride (VC), and to ethylene (1, 2). Because aquifers become oxygenated near groundwater and lake interfaces, concerns have been raised with respect to surface water contamination by VC. In the current study, a modeling approach addresses the fate of TCE and its lesser chlorinated transformation products at this anaerobic/aerobic interface. The modeling effort describes and predicts the fate of the chlorinated solvents at and near the interface, taking into account groundwater flow rates and microbial degradation rates, as well as the oxygenating effects of wave action and lake water intrusion near the shore line.

Numerical Model

The numerical model for the simulation of the hydrodynamic, physicochemical, and biological processes that take place at the lake-aquifer interface is validated based on specific data from an application site at St. Joseph, Michigan. Although the hydrodynamic processes are truly three-dimensional, most of the phenomena of interest, such as migration of TCE, DCE, and VC into the lake and transfer of dissolved oxygen into the aquifer from water infiltrating through the surf region, can be modeled by a two-dimensional model on the vertical plane.

Model Components

Due to the significant time-scale difference between the near-shore circulation and wave runup and breaking in the lake compared with the flow in the porous media, two separate models are constructed for the corresponding hydrodynamic phenomena. The resulting flow fields are then integrated in a single mass transport and contaminant fate model. All three components of the model are two-dimensional, covering a vertical plane extending from a location inland where uniform flow and mass flux are observed in the aquifer to a distance inside the lake where most near-shore current activity has diminished. The various modules are verified by analytical solutions and intermodel comparisons.

Ground-Water Module

In the porous media, a finite-element module for variably saturated flow has been constructed on a vertical plane. This module uses pressure heads calculated by the lake module as a boundary condition at the lake-aquifer interface. For the St. Joseph site, this module has been used in conjunction with two separate grids to take advantage of naturally defined boundary conditions.

A coarse grid has been developed extending from Lake Michigan to a ground-water divide approximately 900 m inland. Zero flux boundaries are prescribed at the ground-water divide and an underlying clay layer. A seasonally varying flux boundary condition at the ground surface reflects recharge from rainfall. To focus on the lake-aquifer interface region, a refined grid has been developed extending from Lake Michigan to a point approximately 100 m inland. One year's output from running this module on the coarse grid defines a sea-

sonally varying inland boundary condition for the refined grid.

Lake Module

The near-shore/free-surface flow simulation is based on the numerical solution of the Navier-Stokes equations by means of the finite-element method. For turbulent flow, a widely accepted two-equation closure model is employed together with certain approximations near the bed and free-surface boundaries. At high Reynolds numbers, an upwind formulation known as the Petrov-Galerkin method of weighted residuals is introduced for the suppression of nonlinear instabilities. The model can predict the vertical structure of the flow from the seepage face between the aquifer and the lake to the free surface. Wave action is incorporated, and special attention is focused on wave runup and breaking. The beach is assumed to be a porous bed so that water from the surf and break region is allowed to infiltrate and reach the aquifer.

For the complete formulation of the problem, bed permeability resulting in seepage through the surf region would be computed by simultaneous solution of the free-surface flow problem in the lake with the associated unsaturated flow problem in the subsurface domain. In this model, bed seepage is introduced as a boundary condition. This eliminates the difficulty of having to deal simultaneously with two time scales without affecting the robustness of the model. The bed seepage is averaged over time to provide an interface boundary condition to the ground-water flow module.

Contaminant Transport Module

The contaminants are assumed to be well mixed laterally. The contaminant fate and transport module uses a finite-element model to solve the two-dimensional transport equation based on the flow fields computed by the ground-water and lake modules. This accounts for contaminant transport due to advection and dispersion in the aqueous phase and for interphase mass transfer due to sorption and volatilization.

Microbial transformations are incorporated using modified Monod kinetics to describe a source/sink term in transport equation. Rate constants have been estimated by a parallel experimental effort focusing on microbial interactions. The microbial biomass is assumed to be immobile below a limiting concentration at which sloughing occurs.

Conclusion

A two-dimensional finite element has been developed to simulate the transport and biodegradation of chlorinated solvents at and near ground-water and lake interfaces. Example simulations consider the effects that factors such as heterogeneities in the porous media, uncertainties in parameter estimation, and varying recharge through the beach have on the location and concentration of a plume of chlorinated solvents.

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The U.S. Environmental Protection Agency's Development of Bioventing

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Research conducted in the mid to late 1980s by the U.S. Air Force (1, 2), researchers in the Netherlands (3-6), Texas Research Institute (7, 8), Battelle Memorial Institute (2, 9, 10, 11), Utah State University (11), and the U.S. Environmental Protection Agency (EPA) (12), among others, suggested that delivering air to the vadose zone to promote biodegradation could be a low-cost means of cleaning fuel-contaminated vadose zone soils. This approach was motivated by the attempt to solve two different remediation development problems:

- Soil vacuum extraction for treatment of contaminated vadose zones involved costly off-gas treatment and only removed the volatile fraction of the contamination.
- Oxygen delivery to the vadose zone to promote aerobic biodegradation using the approaches attempted in promoting biodegradation in ground water (namely, delivering oxygen saturated water or aqueous solutions of hydrogen peroxide or nitrate to the contaminated area) was neither efficient nor cost-effective.

A process was needed that could deliver oxygen by introducing air into the vadose at a rate that minimized volatilization of the contamination. Several groups simultaneously developed what is now known as bioventing.

EPA recognized the potential cost savings of such a technology over traditional remediation approaches and began an aggressive bioventing development program in 1990. The mission of the program, in essence, was to develop bioventing so that it could be applied at as many contaminated sites as possible. To date, EPA's program has demonstrated or is currently developing the use of bioventing for the following situations:

- For operation with air injection.
- In cold climates.
- With soil warming.
- For jet fuel/aviation fuel.
- For nonfuel contaminants such as acetone, toluene, polycyclic aromatic hydrocarbons (PAHs), and trichloroethylene (TCE).

Table 1 provides a list of EPA's involvement in bioventing research and development. The cumulative knowledge of EPA, the Air Force, and Battelle Memorial Institute regarding bioventing of fuel-contaminated sites was distilled in *Principles and Practices Manual for Bioventing*, to be released in late 1995.

The next frontier for aerobic bioventing is the application of the process to sites contaminated with chlorinated solvents. EPA is currently involved in two laboratory and field projects to develop "co-metabolic bioventing." Co-metabolic bioventing is the promotion of the aerobic biodegradation of chlorinated solvents, such as TCE, in the vadose zone by delivering oxygen and, if necessary, a volatile co-metabolite to the contaminated site. EPA projects will consider two scenarios: 1) the co-metabolite is a co-contaminant of the chlorinated solvent, and thus only air must be delivered; and 2) the co-metabolite must be delivered with the air stream and must therefore be volatile.

In summary, EPA and its collaborators, primarily the Air Force, the U.S. Coast Guard, and Battelle, has been successful in developing bioventing into an inexpensive, robust process, applicable to the cleanup of many contaminated sites.

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Table 1. Summary of EPA Bioventing Research and Development

Project/ Location	Dates	Contaminants	Scale	Approach/Results	References
Coast Guard Station, Michigan	1990-1991	Aviation fuel	Lab, pilot	First test of air-injection bioventing. Showed that air injection near the water table could induce biodegradation in the vadose zone and in ground water.	13-16
Hill AFB, Utah	1990-1994	Jet fuel (JP-4)	Full	First full-scale air-injection bioventing. Showed that low-rate air injection could supply biodegradation oxygen demand and produce no measurable surface emissions of volatile organic compounds.	17
Eielson AFB, Alaska	1991-1994	Jet fuel (JP-4)	Lab, pilot	Showed that bioventing in cold climates is feasible and that simple soil warming techniques can increase the rate of biodegradation when bioventing, thereby decreasing the time required for remediation.	18
Reilly Tar SF Site, MN	1992-	PAHs	Pilot	Attempting to show that PAHs at wood treating sites can be remediated with bioventing.	20
Greenwood Chemical SF Site, Virginia	1993-1995	Acetone, toluene, others	Pilot	Attempting to show that bioventing of sites contaminated with non-fuel, aerobically biodegradable organics and in low permeability soils is feasible.	N/A
Dover AFB, Delaware	1995-	TCE	Lab, pilot	Attempting to show that TCE can be treated with bioventing if the necessary co-metabolite is present, either as a co-contaminant or delivered as a volatile organic in the injected air stream.	N/A
Principles and Practices Manual	1995 release	Fuels	Two volumes	*Volume 1: Principles of Bioventing* *Volume 2: The Practice of Bioventing*	N/A

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Section 2
Field Research

Field research is essential for evaluating the performance of full-scale bioremediation precesses and for conducting accelerated testing on technologies that are appropriate for scaled-up application. For example, problems associated with the use of bacteria used in the laboratory include optimizing the activity of the organism under site conditions and defining the risks associated with introducing a non-native microorganism to the site. The objective of this level of research is to demonstrate that the particular bioremediation process performs as expected in the field. Researchers at the symposium provided information on several ongoing field experiments.

Field studies conducted at St. Joseph, Michigan, and Rocky Point, North Carolina, sought to determine the extent of intrinsic bioremediation in subsurface contaminant plumes. EPA found extensive dechlorination of trichloroethylene contamination by endogenous microorganisms at St. Joseph. At Rocky Point, researchers found considerable agreement between laboratory models of hydrocarbon biodegradation and field observations.

At Pensacola, Florida, researchers obtained data from a site contaminated with creosote to construct a model of toxic inhibition of bioremediation. Another group of scientists constructed a set of models to better understand the processes of soil vapor extraction and bioventing.

Research also was performed on the use of a mixed oxygen/nitrate electron acceptor condition to degrade aromatic hydrocarbons.

Finally, a group of presentations dealt with the use of bioremediation to clean petroleum contamination in beaches and wetlands. Researchers performed studies to determine how often nutrients should be applied to beaches to support bacterial growth, as well as directly studied the process of bacterial petroleum degradation on beaches. Another study examined what environmental influences might affect the biodegradation of petroleum in wetland areas.

There were two field research poster presentations. The first of these described the National Center for Integrated Bioremediation Research and Development (NCIBRD) and its work at Wurtsmith Air Force Base in Michigan. The second presentation described use of plant growth to enhance bioremediation in the field.

A Review of Intrinsic Bioremediation of Trichloroethylene in Ground Water at Picatinny Arsenal, New Jersey, and St. Joseph, Michigan

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Reductive dechlorination occurs frequently in large trichloroethylene (TCE) plumes. TCE is transformed largely to cis-dichloroethylene (cis-DCE), then to vinyl chloride, and finally to compounds that do not contain organic chlorine. This abstract evaluates the rate and extent of natural reductive dechlorination of TCE in two large plumes with similar properties.

Description of the Plumes

Both plumes originated in a release of liquid TCE. The plume at St Joseph, Michigan, originates from an industrial park, while the plume at the Picatinny Arsenal, New Jersey, originates in a release from a degreasing vat at a plating shop. Cross sections of the plumes are depicted in Figures 1 and 2. Both plumes have high concentrations of TCE in the core of the plume (over 25,000 µg/L), are devoid of oxygen or nitrate, contain low concentrations of iron (II) and methane (generally less than 10 mg/L), and have relatively low concentrations of sulfate (generally less than 15 mg/L). Both plumes have concentrations of dissolved organic carbon that are elevated over background. The ground water in both plumes is cold (near 10°C). The water is hard, with pH near neutrality.

Both plumes discharge to surface water. The interstitial seepage velocities of the plumes are very similar. The seepage velocity of the TCE plume at St. Joseph (corrected for retardation) is near 0.1 m/day, while the velocity of the plume on the Picatinny Arsenal varies from 0.3 to 1.0 m/day. For purposes of calculation, 0.3 m/day is used in this abstract.

Monitoring

The plume at St. Joseph was characterized by four transects that extended across the plume, perpendicular to ground-water flow. At each point in each transect, water was sampled in 1.5-m vertical intervals extending from the water table to a clay layer at the bottom of the aquifer. Each transect contains at least 20 sampling points. Table 1 compares the average concentration of TCE, cis-DCE, and vinyl chloride in each transect, as well as the highest concentration encountered. The most distant transect was sampled from the sediments of Lake Michigan. The plume was encountered approximately 1.5 m below the sediment surface, 100 m from the shore line.

The plume of TCE at the Picatinny Arsenal is monitored by a series of well clusters installed along the centerline of the plume. Table 1 presents data from the monitoring well in a cluster that had the highest concentration of TCE. The data were collected in 1989.

Extent of Attenuation

Dechlorination in the plume at St. Joseph is extensive. Vinyl chloride and cis-DCE accumulated near the spill, then were degraded as the plume moved downgradient (Table 1). Dechlorination in the plume at Picatinny Arsenal was also extensive. Comparing the location of the highest concentration with the point of discharge, dechlorination destroyed approximately 90 percent of the TCE. Vinyl chloride and cis-DCE did not accumulate to an appreciable extent. Because the plume at Picatinny Arsenal discharged to surface water before dechlorination was complete, the U.S. Army installed and continues to operate a pump-and-treat system on the plume.

Comparison of Attenuation due to Dilution and Dechlorination

The plume at St. Joseph has high concentrations of TCE at its core, while the concentration of chloride in the aquifer is low. This makes it possible to estimate the

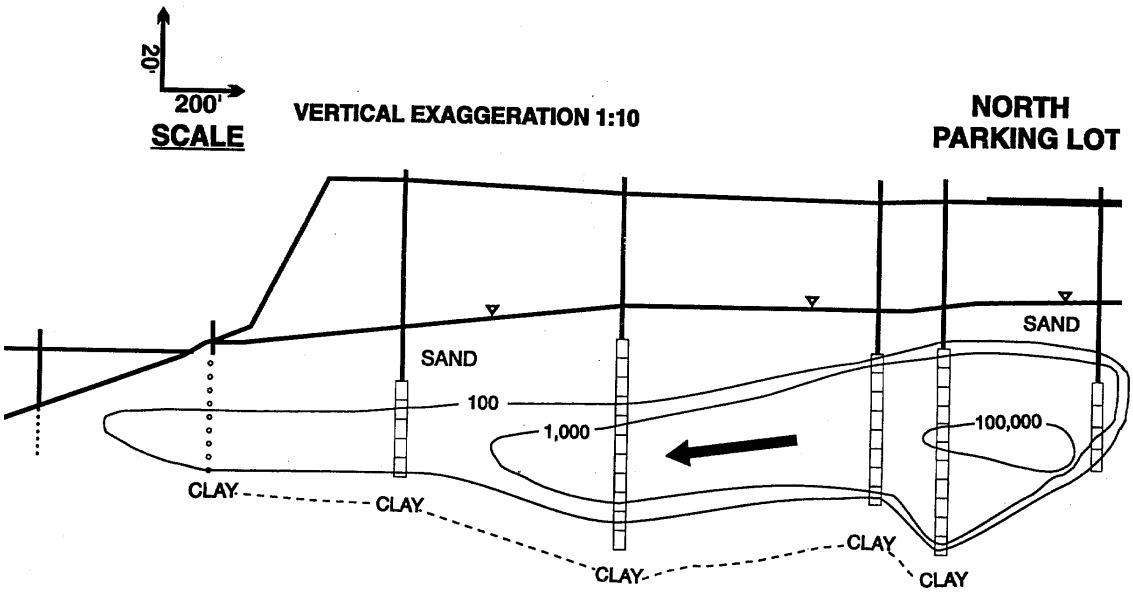

Figure 1. Cross section of the plume at St. Joseph, Michigan, as it leaves the industrial park and enters the sediments under Lake Michigan. Concentrations are in µg/L total chloroethenes.

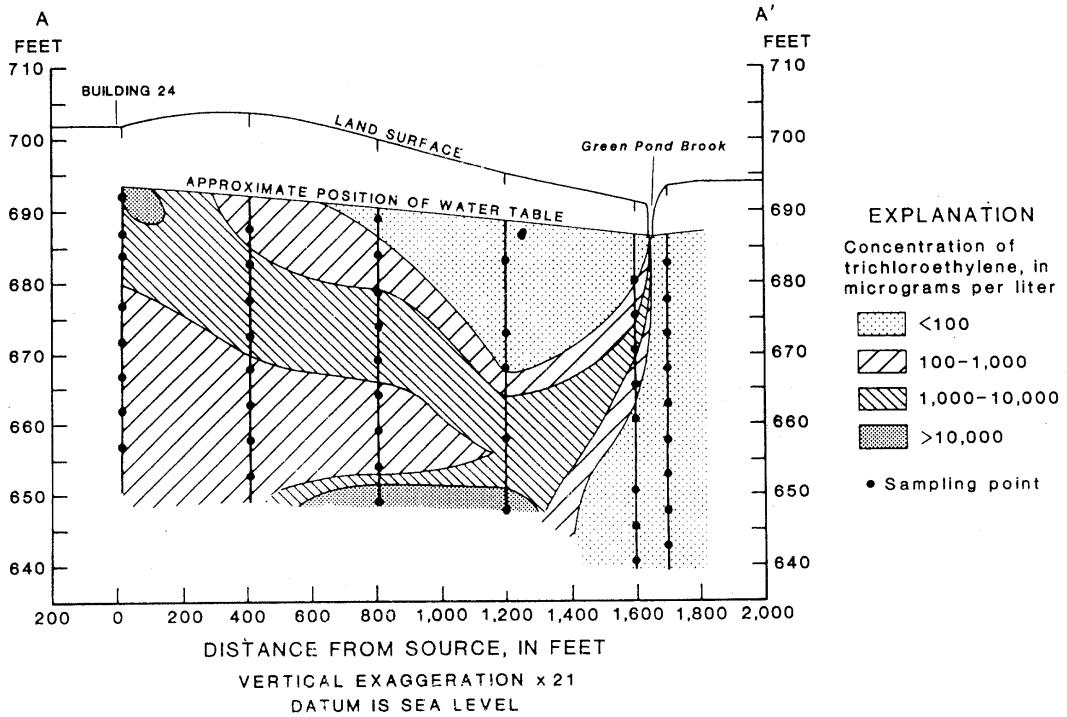

Figure 2. Cross section of the plume at Picatinny Arsenal, New Jersey, as it moves from its source near Building 24 and discharges at Green Pond Brook.

contribution of dilution by comparing the accumulation of chloride from reductive dechlorination to attenuation of chloroethenes. Table 2 portrays the accumulation of chloride and reduction of total organic chlorine along the flow path. Table 2 compares water from the most concentrated sample in each transect. Based on K_{oc} relationships and the fraction of organic carbon in the aquifer, approximately 60 percent of the TCE in the aquifer should be in solution. TCE was largely depleted, and sorption of cis-DCE and vinyl chloride in the aquifer

Table 1. Attenuation of TCE in Ground Water With Distance From the Source and Residence Time in the Aquifer (1-3)

Location	Distance From Source (m)	Time in Aquifer (y)	Average conc. (μg/L) *Highest conc. (μg/L)* TCE	cis-DCE	Vinyl Chloride
St. Joseph	130	3.2	6,500 *68,000*	8,100 *128,000*	930 *4,400*
	390	9.7	520 *8,700*	830 *9,800*	450 *1,660*
	550	12.5	15 *56*	18 *870*	106 *205*
	855	17.9	< 1 *1.4*	< 1 *0.8*	< 1 *0.5*
Picatinny	240	2.2	25,000	220	4
	320	2.9	10,000	35	1
	460	4.2	1,400	310	6

Table 2. Comparison of the Relative Attenuation of TCE, cis-DCE, and Vinyl Chloride With the Attenuation of Chloride in the Plume at St. Joseph, Michigan (1, 2)

Distance From Source (m)	Chloride Ion (mg/L)	Organic Chlorine (mg/L)	Highest concentration TCE (μg/L)	c-DCE (μg/L)	Vinyl Chloride (μg/L)
Background	14				
130	55	104	4,000	128,000	4,400
390	109	15	8,700	9,800	1,660
550	71	0.8	11	828	205
855	57	< 0.1	1.4	0.8	0.5

should be minimal. We will assume that the organic chlorine in ground water represents the pool of chlorine available for dechlorination to chloride.

Near the source, the concentration of chloride plus potential biogenic chloride minus background chloride was 145 mg/L. Only 38 percent of this quantity was actually chloride. Total organic and inorganic chlorine attenuated with distance downgradient. By the time the plume reached the lake, the concentration of total chlorine (minus background) was 43 mg/L, which is significantly higher than background. Apparently the plume was attenuated three- to four-fold due to dilution. Total attenuation of chloroethenes was at least 100,000-fold.

Kinetics of Reductive Dechlorination in Ground Water

Table 3 compares first-order rate constants calculated between transects in the plume at St. Joseph and between monitoring wells in the plume at Picatinny Arsenal. Field-scale estimates of rates are also compared with attenuation in microcosms constructed from material collected along the flow path at Picatinny Arsenal. There is surprising agreement in the rates of dechlorination of TCE within the same plume, between plumes, and between microcosm studies and field-scale estimates. Nine separate estimates vary less than an order of magnitude. The rates of degradation of vinyl chloride and cis-DCE were comparable to the rates of degradation of TCE (Table 3).

The rates of attenuation in the two plumes are as slow as humans experience time. In particular, they are slow compared with the time usually devoted to site characterization. In plumes with a long residence time, on the order of decades, however, they have significance for protection of waters that receive the plumes.

Table 3. Rates of Reductive Dechlorination of TCE, cis-DCE, and Vinyl Chloride in Ground Water (residence time refers to time in the segment of the plume being described, or incubation time of microcosms) (1-5)

Location	Distance From Source (m)	Time From Source (yr)	Residence Time (yr)	Apparent Loss Coefficient (1/yr)		
				TCE	cis-DCE	Vinyl Chloride
Field Scale Estimates						
St. Joseph	130 to 390	3.2 to 9.7	6.5	0.38	0.50	0.18
	390 to 550	9.7 to 12.5	2.8	1.3	0.83	0.88
	550 to 855	12.5 to 17.9	5.4	0.93	3.1	2.2
Picatinny	240 to 460	2.2 to 4.2	2.0	1.4	Produced	Produced
	320 to 460	2.9 to 4.2	1.3	1.2	Produced	Produced
	0 to 460	0.0 to 4.2	4.2	1.0		
	240 to 320	2.2 to 2.9	0.7		1.6	
	0 to 250	0.0 to 2.3			0.5	
Laboratory Microcosm Studies						
Picatinny	240	2.2	0.5	0.64	0.52	
	320	2.9	0.5	0.42	9.4	
	460	4.2	0.5	0.21	3.1	

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Intrinsic Bioremediation of a Gasoline Plume: Comparison of Field and Laboratory Results

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Introduction

Assessing the potential for natural bioremediation in the subsurface is complicated by site-specific conditions and the methods used to estimate biodegradation rates. Controlled laboratory experiments often are necessary to verify biological loss of a compound and to assess factors that influence biodegradation. The effect of removing samples from such a stable environment and placing them in laboratory microcosms, however, is not understood. In situ columns have been used to measure biodegradation on a limited basis, and little is known about their reliability. In this paper, we use laboratory microcosms and in situ column experiments to estimate intrinsic biodegradation rates of benzene, toluene, ethylbenzene, and xylene (BTEX) isomers in the subsurface.

Site Background

This research was conducted at a petroleum-contaminated aquifer in the southeastern coastal plain near Rocky Point, North Carolina. The plume is characterized by negligible dissolved oxygen and redox potentials of -100 to -200 mV due to intrinsic biodegradation of BTEX (1). The dominant electron acceptors within the plume are sulfate and iron (1). The midpoint of the plume is characterized by high dissolved iron (Fe)(II) (greater than 40 mg/L) and low SO_4-2 concentrations (less than 4 mg/L). Toluene and o-xylene are nearly depleted (less than 20 μg/L), whereas high quantities of benzene, ethylbenzene, and m-,p-xylene remain (greater than 500 μg/L).

Experimental Methods

Laboratory Microcosms

Multiple replicate microcosms with no headspace were constructed in an anaerobic chamber under aseptic conditions using blended aquifer sediment and ground water recovered under anaerobic conditions. Microcosm preparation was designed to simulate ambient conditions to the maximum extent possible. Microcosms were spiked with approximately 10,000 μg/L BTEX (2,000 μg/L of each compound) and incubated in anaerobic containers stored at the ambient ground-water temperature, 16°C. Because it cannot be distinguished from m-xylene by the analytical procedure used, p-xylene was not added. BTEX loss was monitored by destructively sampling three live and three abiotic microcosms at monthly intervals for 300 days. A final time point was taken 100 days later (after 400 days).

In Situ Columns

The in situ columns were similar to a system used previously (2). Each column consisted of a 1-m long chamber where sediment and ground water were isolated from the surrounding aquifer. Two sets of columns, Group A and Group B, were installed at the midpoint area of the plume. Each set contained three individual columns: two live and one abiotic control. After installation, the columns were filled with anaerobic ground water containing BTEX, which had been recovered from nearby wells. The contaminant concentrations in water added to Group A were 1,300, 40, 1,800, 700, and 15 μg/L for benzene, toluene, ethylbenzene, m-,p-xylene, and o-xylene, respectively. The approximate concentrations of compounds added to Group B columns were 200, 200, 600, 1,600, and 800 μg/L for benzene, toluene, ethylbenzene, m-,p-xylene, and o-xylene, respectively. The abiotic control columns were prepared by adjusting the pH to less than 2 with hydrochloric acid (HCl). Tracer tests were conducted on all columns before each experiment began to ensure that they were properly installed.

Results

A distinct order of compound disappearance was measured in the laboratory incubations: m-xylene degradation began with no lag period, followed by toluene,

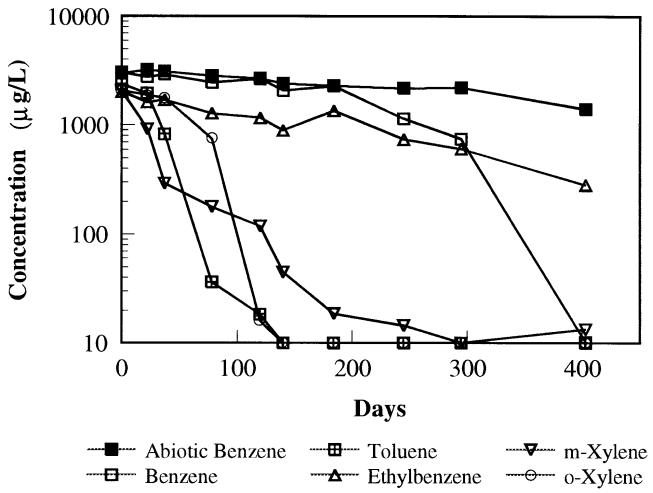

Figure 1. BTEX biodegradation in laboratory microcosms.

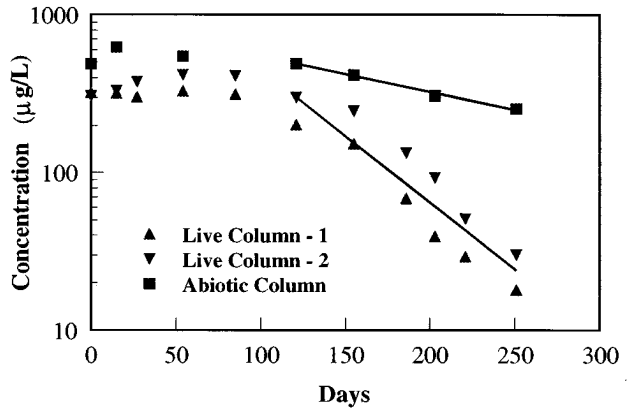

Figure 2. m-,p-Xylene biodegradation in Group A in situ columns.

o-xylene, benzene, and ethylbenzene (Figure 1). The rate of m-xylene loss slowed once toluene loss began; once toluene and o-xylene were below 20 µg/L (120 days), the rate of m-xylene loss increased. The aquifer material was obtained in an area of the plume where toluene and o-xylene concentrations were very low (less than 50 µg/L) but significant quantities of m-,p-xylene remained (greater than 1,000 µg/L). Thus, the microbial population appeared to have an initial preference for m-xylene, but switched to toluene and o-xylene after a 22-day acclimation period. Benzene began to biodegrade once m-xylene was depleted and was at or below 10 µg/L in all microcosms at the final sampling (403 days). First-order decay rates (K) were determined during the time of loss for each compound (Table 1).

All live and abiotic in situ columns exhibited an initial concentration decrease of several hundred micrograms/per liter between the injection water and the first sample taken from the chamber. This initial loss is attributed to sorption. After the sorption loss, the initial compound concentrations were less than 500 µg/L in most columns. The concentrations of hydrocarbons in the abiotic columns remained fairly constant or declined slowly after the initial decrease, indicating biological activity or short circuiting did not occur in the control columns.

Table 1. Comparison of Microcosm and In Situ Column Biodegradation Rates[a]

Compound	Laboratory Rate (percent -day^{-1}) (time interval in days)	In Situ Column Rate (percent -day^{-1}) (time interval in days)
Benzene	2.37 (184 to 403)	0.41 (121 to 251)
Toluene	4.46 (22 to 120)	1.15 (13 to 75)
m,p-xylene	2.04 (0 to 184)	1.43 (121 to 251)
o-xylene	5.59 (37 to 120)	NS
Ethylbenzene	0.19 (0 to 403)	NS

[a] Rate calculated is the difference between the live and abiotic loss rates assuming first-order model.
NS = The difference between the live and abiotic loss rate is not significant at the 95-percent confidence level.

In Group A, benzene and m-,p-xylene exhibited significantly higher losses in the live columns relative to the abiotic columns. The concentration of m-,p-xylene decreased in the live columns after an initial lag of 85 to 121 days (Figure 2). Benzene concentrations remained constant in both live columns for 155 days, after which time decreases attributed to biological activity were measured (data not shown). Initial toluene and o-xylene concentrations were too low (less than 50 µg/L) to accurately measure concentration changes. Figure 2 shows the measured m-,p-xylene loss in the Group A columns and illustrates the timeframe used to calculate the decay rates. Results from the two live columns were pooled to estimate the live decay rate. In Group B, significant biological loss of toluene occurred with no apparent lag time. The short sampling period of 75 days was not adequate to measure losses of the other compounds.

Comparison of In Situ Columns and Laboratory Microcosms

Biological loss for three of the five BTEX compounds occurred over similar periods in the laboratory and in situ experiments. In both cases, toluene degradation was followed by m-,p-xylene and benzene. This order is consistent with previous field investigations (1). Ethylbenzene loss was minimal in the laboratory microcosms during the 400 days of incubation, and no ethylbenzene degradation was measured during the 7 months of in situ monitoring. Loss of o-xylene was not observed in the Group B columns, but fairly rapid depletion concurrent with toluene loss was measured in the laboratory. The initial concentration of o-xylene (less than 500 µg/L)

was possibly too low to stimulate in situ degradation, or the 75-day monitoring period could have been too short.

Although the monthly sampling frequency was consistent for both types of measurements, the length of monitoring was shorter in the in situ columns due to the limited sample volume available. Thus, direct comparison of decay rates between the two types of measurements is difficult. Given these limitations, the measured rates are comparable in both columns and microcosms. The slightly lower decay rates measured in the in situ columns may be due to the lower initial concentrations used in these experiments. Biological decay was demonstrated in the controlled column and microcosm experiments. Use of in situ columns could provide a practical link between laboratory evaluations and full-scale field studies.

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Toxicity Effects on Methanogenic Degradation of Phenol in Ground Water

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Introduction

At an abandoned creosote works located near Pensacola, Florida, the shallow ground water is contaminated with phenolic compounds, heterocyclic compounds, and polyaromatic hydrocarbons. Based on the use of unlined disposal ponds during the 80 years of operations at the plant, the contaminants have probably been present in the ground water for several decades. A methanogenic consortium in the aquifer is degrading some of the compounds, and the concentrations of the degradable fraction drop to less than 1 percent of the source values by 100 to 150 m downgradient from the nonaqueous phase (1). In spite of the long exposure time to a continuous source, the results of acridine orange direct counts and most probable number determinations indicate a low, uniform microbial density (1). The continued existence of low microbial numbers suggests that some factor is limiting growth. Several possibilities, such as microbial transport, nutrient limitation, predation, and toxicity, have been examined. Of these, toxicity appears to be the most promising explanation. The toxicity of creosote compounds to various organisms has been studied for a long time (2). Very little work has been done, however, on the effects of creosote on methanogenic consortia known to be especially sensitive to toxic compounds (3). The results of our work indicate that the dimethylphenols and methylphenols present in the ground water at this site inhibit the degradation of phenol. Furthermore, incorporating these inhibition effects into a one-dimensional model of the aquifer predicts a steady-state degradation profile and zero net growth of the active methanogenic consortium.

Toxicity Assay Results

A serum bottle assay similar to that described by Owens et al. (3) was performed to determine which of the compounds present in the ground water might be toxic to the methanogenic consortium. These compounds were grouped by class and added at three concentration levels equivalent to 1.5, 1.0, and 0.5 times the highest measured field value. The classes of compounds tested were 1) indene, benzothiophene, 2-methylnaphthalene, biphenyl, flourene, and 2-naphthol; 2) 2-, 3-, and 4-methylphenol; 3) 2,4- and 3,5-dimethylphenols; 4) quinoline and isoquinoline; 5) 2(1H)-quinolinone and 1(2H)-isoquinolinone; and 6) all of the preceding compounds combined. Duplicate 100-mL serum bottles containing the target compounds, enriched methanogenic culture derived from the aquifer, mineral salts, and phenol as the growth substrate (at the highest concentration observed in the field) were prepared in an anaerobic glove box and capped. The volume of gas produced in each bottle was monitored by allowing a wetted glass syringe inserted through the septum to equilibrate with atmospheric pressure. Figure 1 shows the gas production in the bottles containing Mixtures 2, 3, 4, and 6 at the 1.5 times concentration as well as a control containing only phenol. Methylphenols and dimethylphenols showed a substantial toxicity effect, whereas nitrogen heterocycles had a smaller effect and polyaromatic hydrocarbons had no measurable effect (data not shown).

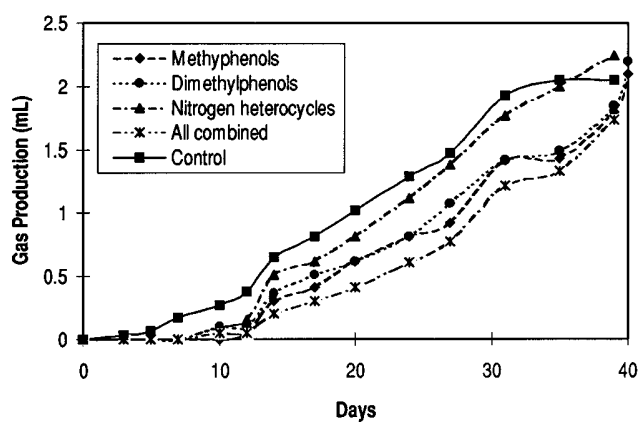

Figure 1. Gas production by the aquifer microbes with phenol as the growth substrate and various inhibitor concentrations equal to 1.5 times the maximum value observed in the aquifer. The results are not shown for quinolinone, which was similar to quinoline, or for polyaromatic hydrocarbons, which were similar to the positive control.

In the bottles with concentrations equal to those in the field, dimethylphenols had a substantial effect, and methylphenols had a smaller effect. In the 0.5 times concentration bottles, methylphenols and dimethylphenols had a slight effect. Preliminary results showing the buildup of fatty acids suggest that the intermediate steps in the degradation process are being inhibited.

Model Results

The monitoring of ground-water concentrations of the degradable compounds for more than 12 years shows that the concentration profiles are constant in time. The existence of a steady-state degradation profile of each substrate together with a low, uniform microbial density indicates that the microbial numbers do not change with time. In theory, the functional form of the Monod growth expression cannot be balanced by a constant decay rate. To address this problem, toxicity effects are incorporated into the following equations for one-dimensional substrate transport with degradation and microbial growth:

$$R\frac{\partial S}{\partial t} = D\frac{\partial^2 S}{\partial x^2} - v\frac{\partial S}{\partial x} - \frac{\mu_m}{Y}\frac{B}{\theta}\left[\frac{S}{K_s\left(1+\frac{S_c}{K_c}\right)+S+\frac{S^2}{K_i}}\right] \quad \text{(Eq. 1)}$$

$$\frac{dB}{dt} = \left\{\mu_m\left[\frac{S}{K_s\left(1+\frac{S_c}{K_c}\right)+S+\frac{S^2}{K_i}}\right] - k_d\right\}B \quad \text{(Eq. 2)}$$

where R is the retardation factor, S is phenol concentration, v is the flow velocity, D is dispersion, μ_m is the maximum growth rate, Y is the yield, B is biomass, θ is porosity, K_S is the half saturation constant, K_i and K_C are haldane and competitive inhibition constants, S_c is the concentration of the inhibiting compound, and k_d is the biomass decay or maintenance rate. When only the toxicity of phenol is incorporated using the Haldane inhibition model, the predicted growth is about 50 percent lower but still much higher than the only published decay rate (4). In addition, the equations do not produce a steady-state solution. Incorporating the effect of the dimethylphenol toxicity produces a steady-state solution for phenol and microbial concentrations that matches the character of the data (Figure 2). The values of the parameters used in the solution are given in the figure caption. The model results show that the aquifer concentrations take about 6 years to evolve to a steady state, while the microbial population takes about 25 years. The population of aquifer microorganisms oscillates as they adjust their distribution to account for two competing effects: 1) the maximum concentration of phenol near the source should lead to maximum growth and substrate utilization there, and 2) the maximum concentrations of dimethylphenols near the source lead to maximum inhibition of growth and substrate utilization. The result is a tradeoff between a location where the growth substrate concentrations are higher versus one farther from the source where the inhibitor concentrations are lower. The final microbial concentrations stabilize at about an order of magnitude higher 50 m from the source than immediately adjacent to it.

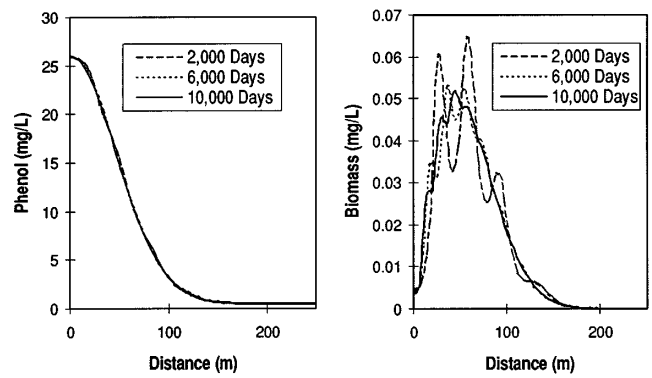

Figure 2. Solution to Equation 1 for phenol after 2,000, 6,000, and 10,000 days (left), and coupled solutions to Equation2 for microbe concentrations at the same times. The values of the parameters used were R = 1.01, S(0) = 26 mg/L, v = 1.0 m/d, D = 1.0 m^2/d, μm = 0.111, Y = 0.013, B_{init} = 0.005 mg/L(1.6 x 10^6 per 100g), θ = 0.38, K_s = 1.33, K_i = 250 mg/L, K_c = 0.52 mg/L, S_c = 23 exp(x^2/(2 $(47)^2$)) mg/L (an empirical fit to the observed dimethylphenol concentrations), and k_d =0.0326 d^{-1}

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A Multiphase, Multicomponent Numerical Model of Bioventing With Nonequilibrium Mass Exchange

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Introduction

Soil vapor extraction (SVE) and bioventing (BV) are common remediation practices for unsaturated soils contaminated with volatile organic compounds (VOCs). These methods have been demonstrated to be effective at comparatively low costs. The efficiency of these techniques is known to be restricted by soil characteristics; by mass transfer limitations between phases, including liquid/solid, liquid/gas, and liquid/microorganism phases; by the availability of oxygen; and by system design and operation parameters (1). Assessment of SVE/BV systems is often hindered by the complex interplay of physical, chemical, and biological processes. Consequently, design and operation of these systems are typically based on engineering experience and/or simple design equations. Numerical models of SVE/BV systems can be valuable tools for the investigation of the effects of various processes on system performance and for optimal system design. In this work, a numerical model is presented that has been specifically developed to incorporate the complete range of processes occurring at the field scale and to include interphase mass transfer rate limitations.

Model Formulation

Three fluid phases are modeled: gas, aqueous, and a nonaqueous phase liquid (NAPL). The gas and aqueous phases may flow simultaneously in response to applied pumping/injection or density gradients. The movement of these phases is described by standard macroscopically averaged flow equations (2). The NAPL phase is assumed to be at an immobile residual saturation. Changes in NAPL saturation, therefore, result solely from interphase mass transfer.

The NAPL may be a mixture of an unrestricted number of organic components. The gas phase is assumed to be composed of nitrogen and oxygen (the two major constituents of air), water vapor, volatile components of the NAPL, and a single limiting nutrient. The aqueous phase is composed of water, oxygen, soluble components of the NAPL, and the limiting nutrient. Sorption to the soil particles is restricted to components of the NAPL. The migration of each component in each phase is described by standard macroscopically averaged transport equations (2).

Quantification of the biotransformation processes follows the conceptual approach of Chen et al. (3). Biodegradation is assumed to occur only within the aqueous phase by an indigenous, spatially heterogeneous, mixed microbial population that is present as attached microcolonies. There is no biomass transport or detachment or sloughing of the microcolonies, and biomass growth does not affect permeability. Monod-type kinetic expressions are employed to describe biophase utilization of substrates, oxygen, and a limiting nutrient, as well as growth of the microbial population. Additionally, a minimum biophase concentration reflecting the indigenous population is maintained when growth is restricted due to oxygen, substrate, or nutrient limitations.

A linear driving force expression is used to model nonequilibrium interphase exchange. Interphase partitioning processes included in the model are: volatilization and dissolution of components from the NAPL; gas/aqueous exchange of oxygen, water vapor, and the components of the NAPL; sorption of the NAPL components to the soil particles through the aqueous phase; and rate-limited uptake by the biophase of oxygen, substrate (components of the NAPL), and the limiting nutrient.

Numerical Solution

The flow and transport equations are solved in two space dimensions (vertical cross section or radial geometry) using a standard Galerkin finite element method

with linear triangular elements. A set-iterative scheme is used for computational efficiency. The sets of coupled flow, transport, and biodegradation equations, as well as multiple equations within sets, are decoupled and solved sequentially. Decoupling is accomplished by lagging, either by one iteration or one time step, the coupling terms which are phase density and interphase mass exchange. Iteration within and between equation sets is performed to account for nonlinearities and ensure solution accuracy. Numerical solutions of the flow, transport, and biodegradation equations have been independently verified with analytical solutions and intermodel comparisons. A detailed description of the model and example simulations are presented in Lang et al. (4).

Demonstration of SVE and BV Simulations

Hypothetical field-scale SVE and BV systems are presented to demonstrate model capabilities. The modeled scenario involves the remediation of a residual NAPL distributed within a layered soil system. Here the nonuniform initial NAPL distribution was generated with a multiphase flow model. NAPL contamination is present in both the unsaturated zone and capillary fringe.

SVE operations are examined by simulating relatively large pumping rates to an extraction well positioned in the center of the residual NAPL zone. Removal efficiency in the test simulations is shown to be sensitive to mass transfer rates, permeability contrasts, the initial NAPL distribution, and, to a lesser extent, pumping rate and well screen position.

A BV operation is also modeled by simulating small gas injection rates at a well located in the center of the contamination zone. Removal efficiency is shown to be sensitive to flow rate, interphase mass transfer, biodegradation rates, and NAPL distribution.

An example of the complex interplay between chemical and biological processes in BV systems is demonstrated in simulation results shown in Figure 1. Here contaminant removal is compared for BV systems run at a comparatively low flow (0.1 pore volumes/day) and high flow (1 pore volume/day). Total mass removed (Figure 1a) is greater at high flow. Due to nonequilibrium interphase partitioning occurring at the high flow, however, the difference in mass removed is less than the proportionate difference in flow rate. The rate of contaminant interphase partitioning also affects the quantity of mass removed by biodegradation (Figure 1b), which is far greater at high flow. At low flow, interphase partitioning of the contaminant is approximately at equilibrium, resulting in downgradient aqueous concentrations that are greater than an inhibitory threshold. Consequently, biodegradation in the low-flow scenario is restricted to the region upgradient of the NAPL contamination zone. Nonequilibrium partitioning at high flow rates produces downgradient aqueous concentrations below the inhibitory threshold, resulting in enhanced biodegradation. This greater degradation in the high-flow system produces a reduction in the contaminant mass in the gas phase arriving at a downgradient extraction point (Figure 1c).

Conclusions

A numerical model of SVE/BV systems has been developed that incorporates the complete compositional and biological processes representative of field conditions. Example simulations demonstrate the model capabilities and illustrate the complex interplay of chemical, physical, and biological processes occurring in SVE/BV systems.

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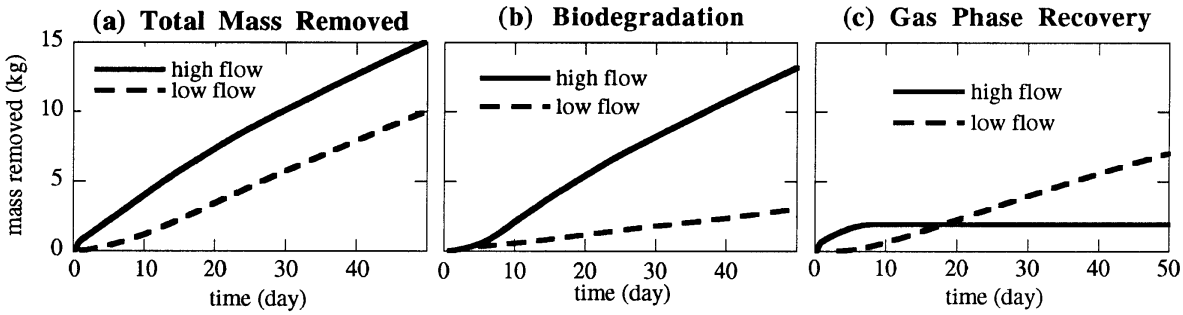

Figure 1. Contaminant removal versus time for hypothetical BV scenarios at comparatively low and high flow rates.

Aromatic Hydrocarbon Biotransformation Under Mixed Oxygen/Nitrate Electron Acceptor Conditions

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Introduction

Biodegradation of contaminants associated with sediments and ground water under mixed oxygen/nitrate electron acceptor conditions may prove to be more successful and feasible than remediation under strict aerobic or anaerobic conditions. In particular, the low level of oxygen may allow subsurface microorganisms to attack the aromatic rings of many organic compounds (using oxygenases), with nitrate serving as the electron acceptor to complete the degradation. Providing nitrate to the subsurface is less expensive than maintaining aerobic conditions, and, as nitrate is highly soluble, it is easier to maintain a residual concentration in ground water.

A laboratory investigation is being conducted to provide a better understanding of the effect of dual oxygen/nitrate electron acceptor conditions on the biodegradation of monocyclic and polycyclic aromatic hydrocarbon mixtures in aqueous solution. The specific objectives of the research are 1) to quantify the stoichiometry and kinetics of biodegradation of a mixture of aromatic hydrocarbons under microaerophilic conditions (defined as less than or equal to 2 mg/L O_2), and 2) to assess the relative efficacy of bioremediation under microaerophilic conditions compared with strict aerobic or denitrification conditions in the laboratory, using batch microcosms and aquifer sediment columns.

Microaerophilic Biodegradation by an Enrichment of Aquifer Bacteria

The microaerophilic biodegradation of a mixture of aromatic compounds was investigated by varying combined concentrations of oxygen and nitrate. Batch microcosms were prepared using a liquid enrichment of aquifer bacteria as inocula; a mixture of benzene, toluene, ethylbenzene, *m*-xylene, naphthalene, and phenanthrene as substrate; and oxygen and nitrate as electron acceptors.

The results of this study indicated that the level of oxygen had a significant affect on the extent of biodegradation of most of the aromatic hydrocarbons. Analysis of the consumption of electron acceptors indicated that both nitrate and oxygen acted as electron acceptors during biodegradation of the mixture of aromatic hydrocarbons. Denitrification may be inhibited by oxygen levels above 1 mg/L (1). In this study, toluene and naphthalene biodegradation was favored at microaerophilic oxygen levels between 1.5 and 2 mg/L. The data (measurements of oxygen and nitrate not shown here) suggested that oxygen and nitrate were used sequentially to biodegrade naphthalene and toluene, respectively (i.e., denitrification was inhibited until oxygen was depleted)(Table 1).

At lower levels of oxygen (0.5 to 1 mg/L), toluene and ethylbenzene biodegradation was favored. The mechanism for biodegradation of toluene and ethylbenzene at very low oxygen levels (less than or equal to 1 mg/L)

Table 1. Aromatic Hydrocarbons Degraded Under the Various Combinations of Oxygen and Nitrate Investigated (degradation is removal greater than or equal to 10 percent relative to killed controls)

	Oxygen (mg/L)					
	0	**0.5**	**1**	**1.5**	**2**	**8**
Nitrate (mg/L)						
10	T	T, E		T, N		B, T, E, *m*-X, N, P
50	T	T, E	T, E	T, N	T, N	B, T, E, *m*-X, N, P
150	T	T, E	T, E	T, N	T, N	B, T, E, *m*-X, N, P
400	T	T, E	T, E	T, N	T, N	B, T, E, *m*-X, N, P

B = benzene, T = toluene, E = ethylbenzene, *m*-X = *m*-xylene, N = naphthalene, and P = phenanthrene

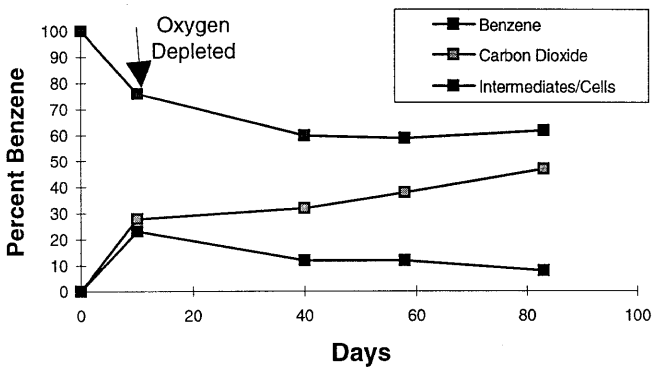

Figure 1. Conversion of benzene to intermediates, cells, and carbon dioxide.

may be quite different than at higher levels (greater than or equal to 1.5 mg/L). The data indicated that at low levels (less than or equal to 1 mg/L) of oxygen, nitrate played a role in biodegradation of both toluene and ethylbenzene. Evidence for simultaneous utilization of nitrate and oxygen has been documented (2). Oxygen levels below 1 mg/L may not inhibit denitrification and may actually be beneficial by increasing cell numbers (3, 4).

Benzene was recalcitrant under denitrifying and microaerophilic conditions. Extensive benzene mineralization, however, was observed under aerobic conditions (Figure 1). Although the majority of benzene biodegradation occurred in the presence of oxygen, partial transformation of the parent compound to intermediates and carbon dioxide was observed in the absence of oxygen.

Microaerophilic Biodegradation in the Presence of Sediments

A laboratory study of the stoichiometry of microaerophilic biodegradation in the presence of sediments is being conducted. Aquifer sediments may affect the stoichiometry of microaerophilic biodegradation by exerting an additional oxygen demand from natural organic matter or reduced metals, and by providing surfaces for microbial attachment that are not present in sediment-free microcosms. In this study, aquifer sediments were used as inocula in microcosms instead of a liquid enrichment of aquifer bacteria. Initial results indicate that toluene biodegradation under denitrifying conditions occurs after a significant lag time in microcosms containing sediment as inocula when compared with denitrification in liquid enrichment microcosms. This delay in denitrifying activity is likely due to the development of a sufficient denitrifying population. No biodegradation of aromatic compounds was observed under microaerophilic conditions (microaerophilic oxygen consumed by sediment demands). Biodegradation under aerobic conditions (7 mg/L), however, exceeded what was observed in the liquid enrichment microcosms. Studies of sediment microcosms and columns are ongoing.

Acknowledgment

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Nutrient Transport in a Sandy Beach

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Introduction

Bioremediation of beaches that are contaminated with crude oil is expected to be limited by the availability of nutrients. Addition of nutrients, such as nitrogen and phosphorous, has been shown to stimulate the rate of crude oil biodegradation in the beach environment (1, 2). For bioremediation to be effective, the nutrient concentration in contact with the oil-contaminated beach material must be high enough to allow hydrocarbon-degrading bacteria to grow at their maximum rates.

Oil often strands in the intertidal zone (3). When this occurs, washout will probably dominate the nutrient residence time in the bioremediation zone. The periodic flooding that occurs when the tide rises is one of several important nutrient transport mechanisms. Wave action can also affect nutrient transport in the intertidal region of beaches (4), as can the flow of fresh ground water from inland (5, 6). The objective of this research was to characterize the nutrient transport rates for a low-energy sandy beach. The results were used to determine the nutrient application rate that is required for effective bioremediation in this type of environment.

Methods

The tracer study was conducted on a long, uniform stretch of sandy beach south of Slaughter Beach, Delaware, near the southern end of Delaware Bay. The beach is composed largely of coarse sand with some gravel, which lies on top of an impermeable peat layer. The beach has a slope of approximately 11 percent in the upper intertidal zone.

Eight replicate plots (5 m x 10 m) were established in the upper intertidal zone. The tops of the plots were placed approximately at the spring high-tide line. The plots were divided into two blocks of four plots each. A water soluble tracer, either lithium or sodium nitrate, was applied to one plot in each block at each of four different stages in the tidal cycle (Figure 1): spring tide (full moon), falling midtide (i.e., as the tide proceeded from spring to neap), neap tide (last quarter moon), and rising midtide (i.e., as the tide proceeded from neap to spring). (Spring tide is the point in the lunar tidal cycle at which the difference between high and low tide is greatest. Neap tide is the point at which the difference is smallest.) Lithium nitrate was used as the tracer for the

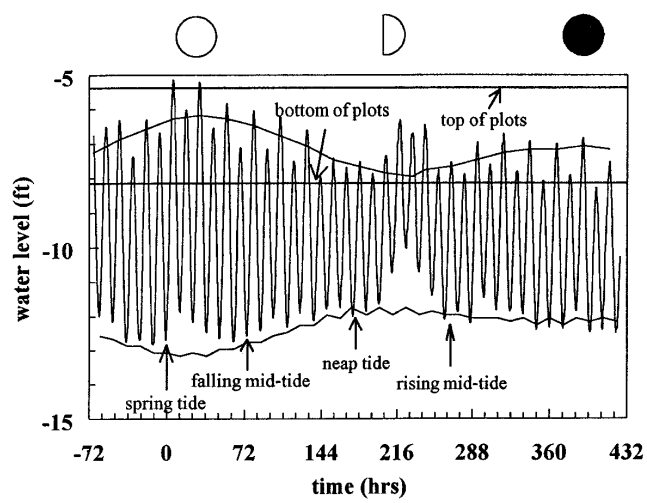

Figure 1. The actual and expected tidal elevations are shown, with the start times for the four tracer transport experiments. The elevations of the tops and bottoms of the plots are also shown. All elevations are relative to a benchmark that was placed behind the beach in a dune area well above the maximum high tide line.

spring- and neap-tide experiments, and sodium nitrate was used for both midtidal experiments. The tracer was dissolved in fresh water (the initial concentration was 20 g/L) and applied to the plots with a sprinkler system. Tracer was always applied at low tide, and the first samples were collected shortly afterward.

The spring- and neap-tide plots contained multiport wells that were used to collect water samples from discrete depths within the beach. These wells were placed along a transect through the middle of the plots at 2.5-m intervals, beginning 2.5 m above the top of the plots and continuing to 5 m below the bottom of the plots. The wells had sample ports every 6 in. The top port of each well was installed 3 in. below the beach surface. Sand samples were collected from randomly selected positions within five subsections that were marked off in each plot. The locations of these subsections corresponded to the positions of the wells in the spring and neap-tide plots.

Sand samples were collected as 10-in. cores using a 5-in. auger. Samples that were analyzed for lithium were kept as two separate 5-in. cores: an upper sample (0 to 5 in.) and a lower sample (5 to 10 in.). The cores taken for nitrate analysis, on the other hand, were composited into a single 10-in. (0 to 10 in.) sample. Lithium was extracted into 1 M ammonium acetate and analyzed by atomic absorption spectrophotometry (7). Nitrate, which was extracted and analyzed in the field, was analyzed by the cadmium reduction autoanalyzer method following extraction into 2 M potassium chloride (KCl) (8).

Results and Discussion

The average lithium concentrations in the two 5-in. core samples for the spring- and neap-tide tracer experiments are shown in Figure 2. The concentrations measured in samples collected from the five subsections of each plot were pooled to obtain the plot averages that are shown in this figure. The plot averages for each of the replicate plots are shown independently in this figure. The average lithium concentration was reduced to zero very rapidly following the spring-tide application (Figure 2A), but it was washed out more slowly after the neap-tide application (Figure 2B). Washout of nitrate following the two midcycle applications behaved similarly; nitrate was washed out quickly following the falling midtidal application, and it persisted for a relatively long time following the rising midtidal application (data not shown). Although the initial lithium concentrations were higher in the samples from the upper 5-in., it disappeared more rapidly in this region than it did from the 5- to 10-in. region.

It is clear from Figure 1 that one of the major differences among the four tracer experiments is the extent to which the plots were covered with water at high tide. Wave action also contributed to plot coverage, and the total

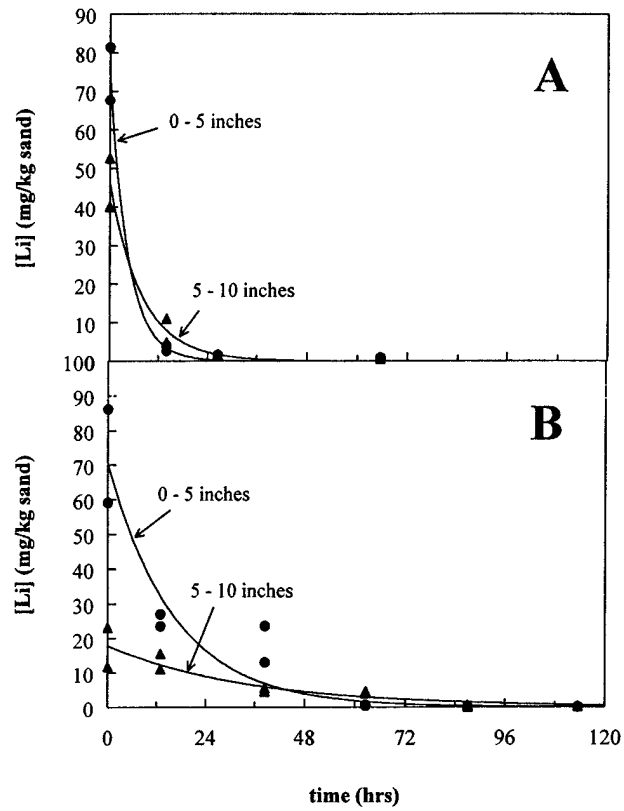

Figure 2. Lithium concentrations in sand samples collected in the bioremediation zone following tracer application at spring tide (A) and neap tide (B). The concentrations for the upper (0 to 5 in.) and lower (5 to 10 in.) samples are plotted separately for each experiment.

coverage is the sum of both effects. The cumulative effects of plot coverage on nutrient retention are shown in Figure 3, in which the ratio of the remaining sand nitrate concentration to its initial concentration is plotted as a function of the maximum extent of plot coverage that had occurred before collecting each sample (e.g., if

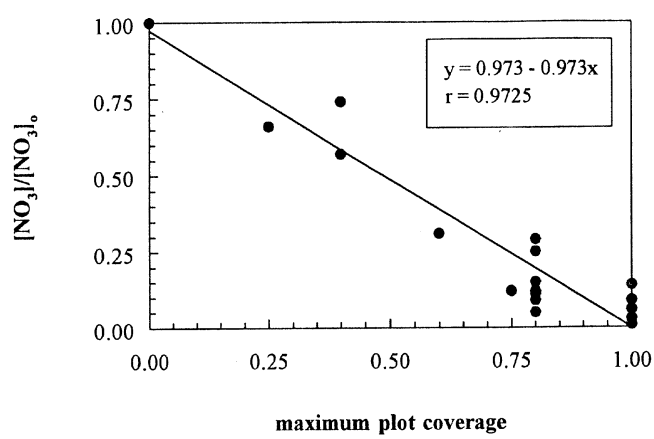

Figure 3. Relationship between the fraction of nitrate remaining and the maximum extent to which water had covered the plots before the samples were collected. Data from all four tracer experiments are included in this plot.

the first high tide following tracer application covered 75 percent of the plot with water and subsequent high tides covered only 50 percent, the maximum coverage for all of the data collected after the first high tide is reported as 75 percent). Data from all four tracer experiments, calculated as described for the lithium data plotted in Figure 2, were used to construct this plot. Figure 3 shows a strong correlation between the maximum extent of plot coverage and the remaining nitrate concentration, suggesting that nutrient retention in the bioremediation zone of a sandy beach can be predicted based solely on the extent of water coverage.

The simplest explanation for the results shown in Figure 3 is that the tracers become diluted by mixing with bulk seawater when the plots are covered by the rising tide, and they are washed away when the tide recedes. This explanation is consistent with a model for nutrient transport in a beach in Prince William Sound, Alaska (9). Pore-water data, however, show that the tracer movement is predominantly downward into the beach (Figure 4). These data are plot averages, calculated as described above, for all pore-water samples collected from each depth within the plot. Samples collected from wells outside of the plots were not used to compute these averages.

Figure 4 shows that nutrient is probably removed from the bioremediation zone by advective flow through the porous matrix of the beach, not by mixing with bulk seawater.

Conclusions

Effective bioremediation requires a sufficient supply of the growth-limiting substrate to be available to the bacteria responsible for biodegradation. For bioremediation of oil-contaminated beaches, nutrients such as nitrogen and phosphorus are expected to be most important (1, 2), and washout is expected to be the dominant nutrient removal mechanism. In many cases, it will be desirable to optimize nutrient application rates to minimize costs and to reduce the opportunity for eutrophication of adjacent bodies of water. Our data suggest a simple method for determining the frequency required for nutrient application.

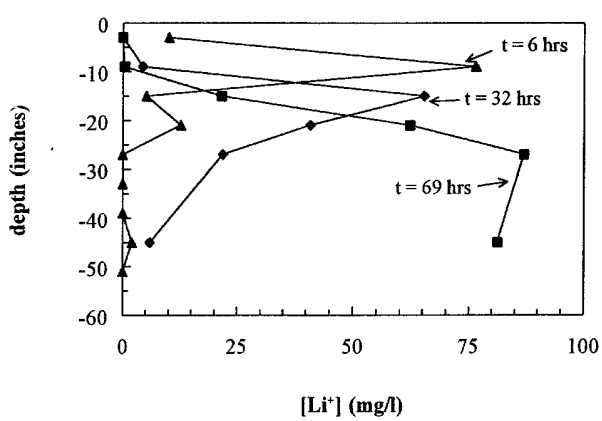

Figure 4. Average lithium concentrations in pore water collected at discrete depths below the beach surface for three time points during the spring tide tracer experiment. All samples were collected at high tide.

Nutrient retention in the bioremediation zone of the intertidal region of a sandy beach varies with the lunar tidal cycle. Our tracers were washed out of the bioremediation zone very quickly when they were applied during and shortly after the spring tide (when the high tide reached its maximum elevation), but they persisted through several tidal cycles when applied around neap tide, when the lowest high tides occurred. Our data suggest that the differences in nutrient retention time are related to the maximum extent to which our experimental plots were covered by water at high tide. Total coverage appears to be more important than coverage due to the tide alone. Therefore, wave activity and tidal elevation must both be considered to determine the appropriate fertilization frequency. When water-soluble fertilizers are used, visual inspection of the extent to which the contaminated area is covered by water during high tide is a reliable alternative to expensive and time-consuming chemical analyses for nutrient concentration.

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Bioremediation of Crude Oil Intentionally Released on the Shoreline of Fowler Beach, Delaware

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Introduction

A major factor contributing to the equivocal findings of past field studies (1-5) was that conclusions were usually based on comparisons between one large treatment plot and one large control plot. The problem with this type of experiment is that no replicate plots are established to provide a basis for estimating experimental error. The collection of numerous subsamples from one treatment plot and one control plot, termed pseudoreplication (6), is statistically invalid for drawing inferences on treatment effects because no experimental error can be computed. An experiment lacking replication is an uncontrolled experiment because it does not control for among-replicate variability inherent in the experimental material, introduced by the experimenter, or arising from chance occurrences. To eliminate uncontrollable and unknown environmental factors that could skew results in one direction, several replicate plots must be set up in random fashion on the beach surface. The experimental approach described herein was carefully designed to allow for valid and statistically authentic comparisons between treatments.

The goals of the study were 1) to obtain sufficient statistical and scientifically credible evidence to determine whether bioremediation with inorganic mineral nutrients or microbial inoculation enhances the removal of crude oil contaminating mixed sand and gravel beaches, and 2) to compute the rate at which such enhancement takes place.

Materials and Methods

The plan was to maximize the effectiveness of bioremediation by maintaining a certain level of nitrogen, in the form of nitrate (agricultural grade sodium nitrate), and phosphorus, in the form of tripolyphosphate (sodium tripolyphosphate), in contact with the degrading populations so that they would be able to grow at their maximal rates at all times. In a previous study (7), we had shown that the minimum nitrate-N concentration needed by oil degraders to grow on hydrocarbons at an accelerated rate under semicontinuous flow conditions was approximately 1.5 mg/L. It was also known that, if the incoming tide completely submerged the plot, the levels of nitrate in the interstitial pore-water diluted to undetectable limits (8). Thus, to maximize bioremediation during spring tides, we reasoned that nutrients would have to be applied every day. To achieve the target 1.5 mg/L interstitial pore-water concentrations, we assumed a 100-fold safety factor to account for dilution. The amount of nitrate-N needed under these circumstances was thus calculated to be about 55 g/m^2, applied once daily to each plot.

The approach used to assess treatment effects in the field study was a randomized complete block (RCB)

design with repeated measures. Five areas of beach were selected based on the homogeneity of geomorphology within each area. Each area ("block") was large enough to accommodate four experimental units or test plots. The blocks were situated in a row on the beach parallel to the shoreline. Three treatments were tested on oiled plots: no-nutrient control, water-soluble nutrients, and water-soluble nutrients supplemented with a natural microbial inoculum from the site. The inoculum was grown by isolating a mixed culture from the site and adding it to a 55-gal drum containing Delaware Bay seawater, the same Bonny Light crude oil, and the same nutrient mix used on the beach. A fourth treatment, an unoiled and untreated plot, served as a background control for microbiological characterization and baseline bioassays. The four treatments were randomized in each of the five blocks so that whatever inferences could be ascertained from the data would be applicable to the entire beach, not just the test plots.

Results and Discussion

The mean interstitial nitrate-N concentrations measured over the course of the 14-week investigation were: 0.8 ± 0.3 mg/L in the unamended control plots, 6.3 ± 2.7 mg/L in the nutrient-treated plots, and 3.5 ± 1.7 mg/L in the inoculum-treated plots. These results indicate that background nutrient levels on Fowler Beach were high enough to sustain nearly maximum oil degrader growth.

Figure 1 is a summary of the hopane-normalized alkane (Figure 1) and aromatic (Figure 2) oil components remaining after the first 8 weeks of the study. In regards to the alkanes, statistical analysis of variance (ANOVA) showed that the difference between both treated plots and the control plots were highly significant at Weeks 2, 4, and 8 ($p < 0.01$) but not at Week 6. Differences between the nutrient-treated and inoculum-treated plots were not significant at any time. Clearly, substantial natural biodegradation was taking place on the control

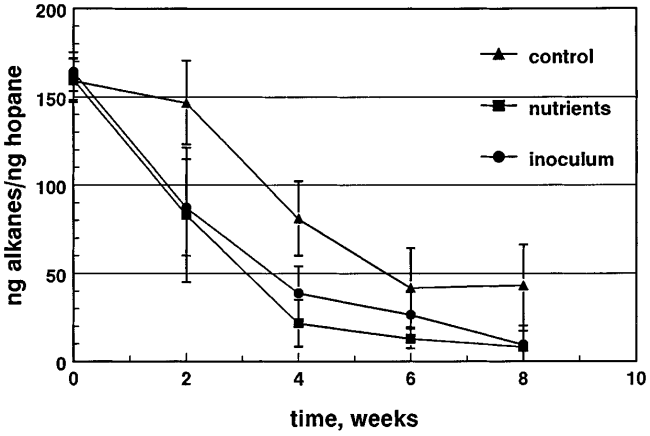

Figure 1. Decline in hopane-normalized total alkanes during the first 8 weeks.

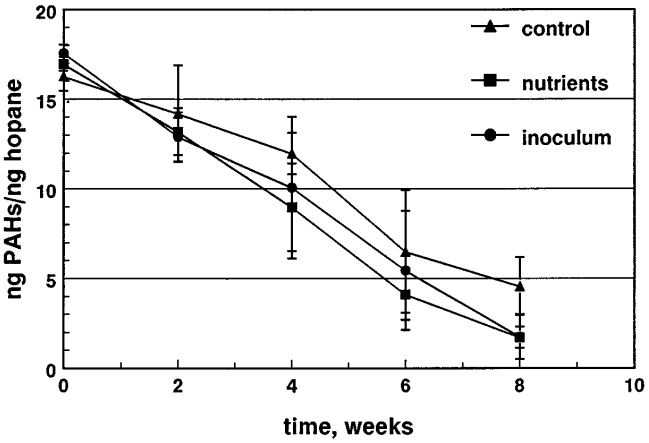

Figure 2. Decline in hopane-normalized total PAHs during the first 8 weeks.

plots without addition of nutrients. This observation is consistent with observations made on the background nutrient levels existent on Fowler Beach. Addition of nutrients significantly enhanced the natural biodegradation rates of the alkane fraction, but not to the extent expected on a less eutrophic beach.

With respect to the aromatic components, results of the ANOVA revealed no significant differences among any of the treatments at Weeks 0, 2, 4, and 6, although substantial biodegradation of the polycyclic aromatic hydrocarbons (PAHs) occurred on all plots. At Week 8, statistically significant differences between the treated and untreated plots were evident. Most of the disappearance occurred among the two- and three-ring PAHs and the lower alkyl-substituted homologues (data not shown). The four-ring PAHs began to show evidence of biodegradation during the eighth week of the study. These results suggest that biostimulation may not always be necessary to promote bioremediation if sufficient nutrients are naturally present at a spill site in high enough concentrations to effect natural cleanup. The evidence suggests that nutrient application to maintain a residual nitrate concentration in the interstitial waters at high enough levels to sustain maximum biodegradative metabolism resulted in a significant enhancement of alkane and, to a lesser extent, aromatic biodegradation over natural attenuation. Bioaugmentation (i.e., supplementation with a bacterial inoculum indigenous to the area), however, did not appear to result in further enhancement.

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Dynamics of Oil Degradation in Coastal Environments: Effect of Bioremediation Products and Some Environmental Parameters

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Introduction

Oil extraction, refining, and transshipment are often located in coastal regions, putting wetland systems at risk of exposure to spilled oil and oil products. The inaccessibility of wetlands and the fragile nature of those ecosystems preclude mechanical cleanup of oil, making bioremediation a preferred option. Moreover, the high level of indigenous microbial activity suggests a potential for biodegradation, especially if fertilizer additions can relieve environmental nutrient limitations.

Bioremediation strategies that have been proposed for oil spills in wetlands include fertilization, solubilization of oil, and bioaugmentation with oil-degrading bacteria. Although bioaugmentation has been demonstrated to be effective in engineered systems, the ability of introduced organisms to establish themselves in the complex web of microbial relationships that characterize wetlands is questionable. Moreover, supplies of oxygen and nutrients may be insufficient for concurrent degradation of oil and natural substrates. In this research, we propose a system for assessing the efficacy of bioremediation in wetlands and for testing the effectiveness of several bioremediation products. We also present data on the dynamics of the bacterial populations and the relative rates of degradation of natural substrates and oil in wetlands.

Methods and Materials

Sediment microcosms were constructed using glass columns (10-cm internal diameter, 20-cm length) fitted with fritted glass supports and filled with homogenized marsh sediments from Sapelo Island, Georgia. Seawater or artificial seawater was adjusted to a brackish salinity of 20‰ and exchanged on a tidal basis. An artificially weathered Alaska North Slope (ANS) crude oil was added at low tide to cover the sediment surface to a depth of 0.5 mm.

Bioremediation products consisted of bacterial preparations enriched in oil degraders, nutrients, surfactants, or combinations thereof. Products were applied in a manner consistent with the manufacturer's recommendations. In addition to commercial products, inorganic nutrients were added to microcosms to test the potential response of indigenous bacteria. Ground *Spartina alterniflora*, a salt marsh grass common to the coast of Georgia, was used as an alternate natural organic substrate. The grass (4.5 g) was added to the sediment surface in an amount equivalent to a 1-yr standing stock of aboveground biomass.

After 3 months, the residual hydrocarbons were extracted and analyzed by gas chromatography/mass spectrometry (GC/MS). Efficacy was assessed on the basis of reduced concentrations of specific components of oil, including straight-chained and branched alkanes and aromatics, expressed as ratios to conserved internal markers. Microbial populations were estimated using modified heterotrophic and oil most probable number (MPN) techniques (1). Heterotrophic bacteria were also quantified using standard plate counts.

Deoxyribonucleic acid (DNA) samples were extracted from each microcosm using a modification of the method of Tsa and Olson (2). Target DNA was filtered onto maximum-strength Nytran membranes. Sediment DNA was loaded onto the membranes in triplicate. The membranes were hybridized with a *Pseudomonas* 23S rRNA oligoprobe (Group I). Detection was carried out using Rad-Free Lumi-Phos 530 substrate sheet and exposure to x-ray film for 3 hr at room temperature. The signal was quantified using densitometry.

Results and Discussion

The alkane fraction of oil was degraded in the presence or absence of bioremediation products, as indicated by the absence of C13 and C14 compounds and the greatly diminished peaks for C13 through C33, including the branched alkanes, pristane, and phytane. Addition of inorganic nutrients to sediments containing only indigenous bacteria resulted in greater depletion of the alkane fraction than did additions of products composed of surfactants or bacterial enrichments. Moreover, in the presence of surfactants, the extent and range of degradation was less than that in the control treatment, suggesting inhibition of microbial activity.

The aromatic fractions of the oil (Figure 1) were degraded to a lesser extent in all treatments than were the alkane fractions (data not shown), although the rank order of the treatments was the same. Both the range of compounds degraded and the extent to which they were degraded were significantly greater in the treatment containing nutrients than in the presence of the commercial products.

The abundance of oil-degrading bacteria (Figure 2) was not consistent with the extent of oil degradation observed. In the absence of bioremediation products, the number of oil degraders (1.51×10^3 bacteria/g sediment) after 3 months of experimentation was not appreciably different from that observed in the initial Sapelo Island sediment (1.38×10^3). Even at such low numbers, however, the microbial community was capable of degrading the alkane fraction of oil, especially when fertilized with inorganic nutrients. No enhanced degradation resulted from the addition of a product purported to be enriched in oil-degrading bacteria, regardless of the relatively high numbers of total heterotrophs and potential oil-degrading bacteria that the treatment yielded.

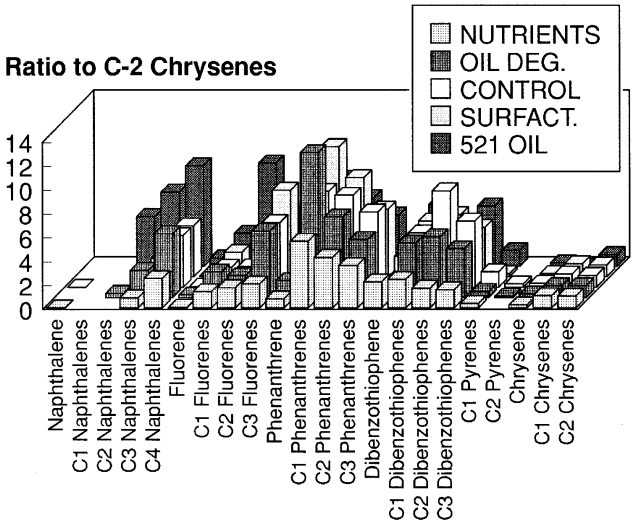

Figure 1. Residual aromatics in surface layer of microcosms after 3 months in the presence of bioremediation agents.

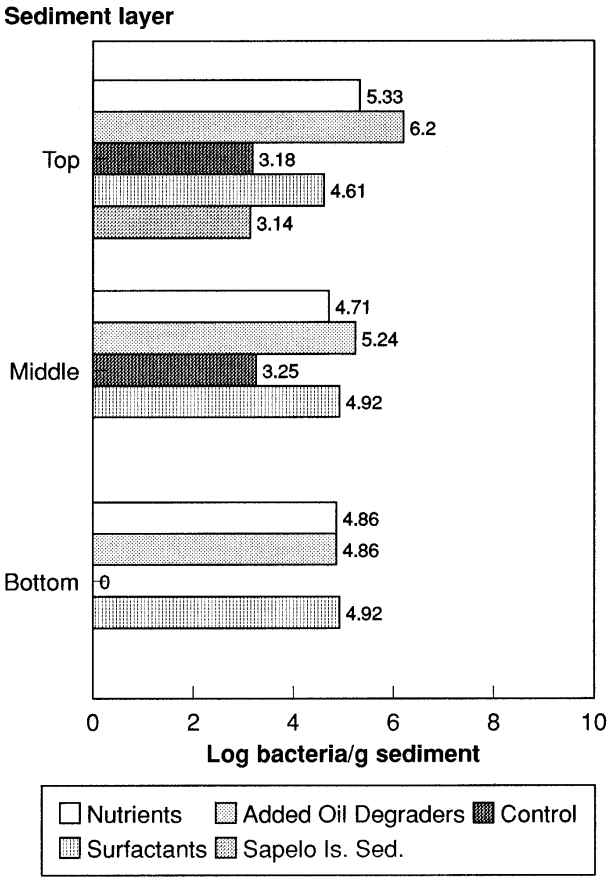

Figure 2. Distribution of oil degraders in wetlands microcosms after 3 months of exposure to bioremediation agents.

The inhibition of oil degradation by the addition of surfactant was consistent with the microbial numbers, which show that, in the presence of the surfactant, the abundance of oil degraders was less than in the treatments containing nutrients or added oil degraders. The heterotrophic bacteria, however, were not affected by the addition of surfactants, as indicated by the numbers in this treatment being comparable with the numbers obtained in the presence of added nutrients (data not shown). The surfactant product could have been used as a carbon or nutrient source by the heterotrophic bacteria, thus increasing their numbers. Furthermore, the surfactant-based product may be inhibitory to the indigenous oil degraders, which were only enhanced in the presence of inorganic nutrients.

Enumeration of oil-degrading bacteria gives no information about the source of the active bacteria because the oil-degrading bacteria may have been of sediment or product origin. DNA hybridizations with 16S rRNA oligoprobes indicated that *Pseudomonas* Group I was significantly reduced in the presence of the microbial product. This suggests that addition of the bacterial preparation suppressed some indigenous populations, including perhaps the indigenous oil-degrading bacteria. None of the other treatments produced numbers of

Pseudomonads that were significantly different from those observed in the initial sediment; data on other sediment microbial groups will be forthcoming.

Under nutrient-amended conditions, both alkane and aromatic fractions were degraded relative to the 521 oil; however, additions of *Spartina alterniflora* detritus decreased the extent of degradation for both fractions. The results for aromatic constituents are shown in Figure 3. Given that microbial activities in wetlands ecosystems are usually limited by oxygen and nutrients, oil degradation may compete with the degradation of natural organic substrates for these substances. In addition, the hemicellulose fraction of lignocellulose constitutes a readily degradable carbon source that may compete with the utilization of petroleum hydrocarbons by the indigenous microbial community.

In the treatments containing no oil, the number of oil degraders was constant over the 3-month period (approximately 6.31 x 10^5 bacteria/g of sediment), which indicates that addition of nutrients only does not increase the population of degraders. The two treatments containing oil produced an increase of oil degraders during the second month of experiment (Figure 4), which may correspond to the initial, rapid degradation of the alkane fraction. By the third month, the oil degraders had decreased, possibly reflecting the slower rate of degradation of aromatic compounds.

The numbers of heterotrophic bacteria were higher in the presence of *Spartina* and oil together than in any of the other treatments. Although the numbers of oil degraders in both treatments containing oil were very similar, the extent of oil degradation was significantly different. The diminished oil degradation in the presence of oil and *Spartina* suggests that the combination of substrates may antagonize the activity of indigenous oil degraders, either directly or by competition for nutrients and/or oxygen. Given the abundance of *Spartina alterniflora* in coastal regions of the United States, the interactions between oil-degrading and other heterotrophic bacteria and the impact of natural substrates should not be overlooked in the design of remediation strategies.

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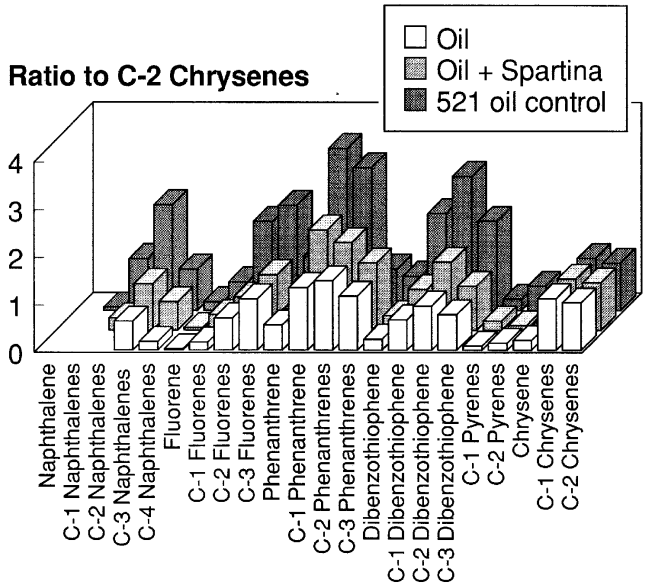

Figure 3. Residual aromatics in surface layer of microcosms after 3 months in the presence of a natural organic substrate.

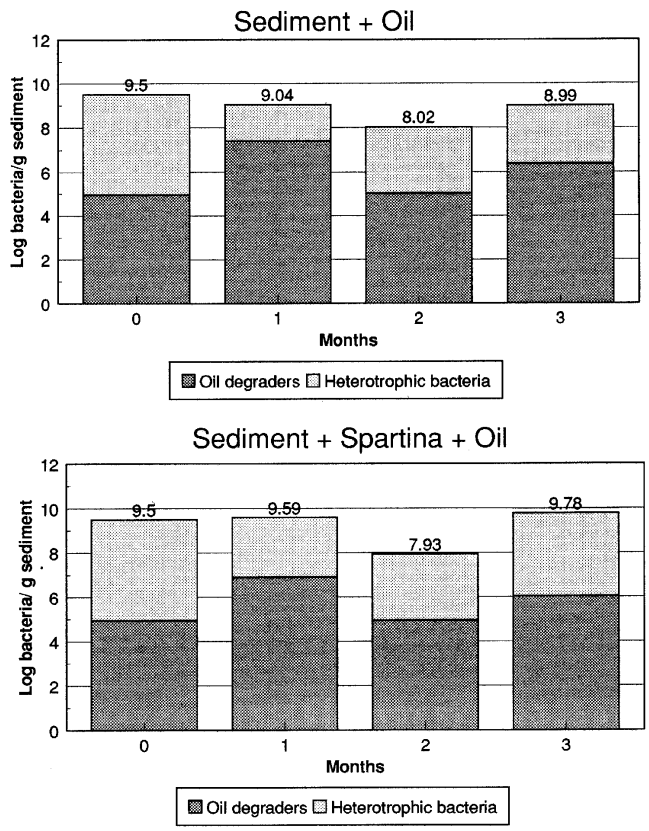

Figure 4. Heterotrophic bacteria and oil degraders in surface layer of microcosms after 3 months of exposure to oil.

Progress Toward Verification of Intrinsic Cobioremediation of Chlorinated Aliphatics

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A plume consisting of chlorinated aliphatic and aromatic hydrocarbons mixed with JP-4 (benzene, toluene, ethylbenzene, and the xylenes; BTEX) from a former Air Force fire-fighting training area shows evidence of continual natural bioremediation. This site is being characterized and monitored by the National Center for Integrated Bioremediation Research and Development (NCIBRD) at Wurtsmith Air Force Base, a decommissioned installation located in lower northeast Michigan.

Wurtsmith is bounded by the Au Sable River to the south and west and by Van Etten Lake to the north and east. The base sits on a 20-m bed of homogeneous glacial alluvial sand and gravel aquifer underlain by a thick clay aquitard. The average ground-water depth in the study area is 6 m. The hydraulic conductivity has been reported to be 3e-3 cm/sec (v = 2.6 m/day) at the site, which has resulted in a narrow 50 m x 300+ m plume. The plume is monitored on a quarterly basis through the use of dedicated bladder pumps installed in 37 monitoring wells at the site. Local ground-water elevations are continually recorded by a datalogging network of recorder wells.

The site has been characterized through 2 years of quarterly sampling of the well and pieziometer network, as well as direct analysis of continuous cores (gathered by resident Geoprobe sampling equipment) across the plume. This information is supplemented by a weekly monitoring of the vertical temperature profile of the site and periodic soil gas profiles.

In general, the site has a large amount of residual fuel/solvent residing near the interface of the water column and capillary fringe, extending at least 125 m from the source. Soil gas measurements in the vadose zone near the source indicate that the interstices contain approximately 65 percent methane, 30 percent carbon dioxide, ppb level hydrogen sulfide and nitrogen, and virtually no oxygen. The ground water beneath the free product has almost no dissolved oxygen and has depressed redox potential, increased electrolytic conductivity, depressed pH levels, and increased concentrations of reduced iron. BTEX levels steadily decrease over the length of the plume. While perchloroethylene (PCE) and trichloroethylene (TCE) levels are significant (greater than 1,500 mg/kg) in the solids, only trace levels are found in the ground water. As the dissolved plume moves downgradient, the predominant chlorinated species are cis-1,2-dichloroethylene and vinyl chloride. On the fringe of the contamination, BTEX metabolites such as m,p-toluic acid and salicylic acid have been identified.

The disappearance of TCE, PCE, and BTEX and the appearance of bacterial metabolites of these compounds over the length of the plume suggest that these contaminants are being bioattenuated within the same plume. Changes in redox potential, temperature, and pH support this assumption. It remains to be seen whether or not these processes are interrelated, and are perhaps influenced by bacterially mediated iron or manganese reduction.

Phytoremediation of Petroleum-Contaminated Soil: Laboratory, Greenhouse, and Field Studies

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Common environmental problems associated with the pumping and refining of crude oil are the disposal of petroleum sludge and pipeline leaks. Contaminants are often treated by incorporation into the soil. If the soil is frequently tilled and fertilized, soil microorganisms will be stimulated and organic contaminants biodegraded. Unfortunately, the biodegradation rate of more recalcitrant and potentially toxic contaminants, such as the polycyclic aromatic hydrocarbons (PAHs), is rapid at first but declines quickly. Biodegradation of these compounds is limited by their strong adsorption potential and low solubility.

Recent research suggests that vegetation may play an important role in the biodegradation of toxic organic chemicals in soil. The establishment of vegetation on hazardous waste sites may be an economical, effective, low-maintenance approach to waste remediation and stabilization. The use of plants for remediation may be especially appropriate for soils contaminated by organic chemicals to depths of less than 2 m. The beneficial effects of vegetation on the biodegradation of hazardous organics are two-fold: organic contaminants may be taken up by the plant and accumulated, metabolized, or volatilized; and the rhizosphere microflora may accelerate biodegradation of the contaminants.

Completed greenhouse studies indicate that vegetative remediation is a feasible method for cleanup of surface soil contaminated with petroleum products. A field demonstration is necessary, however, to exhibit this new technology to the industrial community. In this project, several petroleum-contaminated field sites have been chosen in collaboration with three industrial partners. These sites have been thoroughly characterized for chemical properties, physical properties, and initial contaminant concentrations. A variety of plant species have been established on the sites, including warm and cool season grasses and legumes. Soil analyses for the target compounds over time indicate that the interaction between plants and rhizosphere microflora significantly enhances remediation of the contaminated soils. Continued monitoring will allow us to assess the efficiency and applicability of this remediation approach.

Section 3
Performance Evaluation

In an effort to evaluate the performance of various bioremediation technologies, researchers assess the extent and rate of cleanup for particular bioremediation methods. They also study the environmental fate and effects of compounds and their byproducts because remediation efforts at a contaminated site can produce intermediate compounds that can themselves be hazardous. Thus, another important aspect of performance evaluation projects involves assessing the risk of potential health effects and identifying bioremediation approaches that best protect public health.

To this end, EPA's National Health and Environmental Effects Research Laboratory (NHEERL) developed an integrated program to address 1) the toxicity of known hazardous waste site contaminants, their natural breakdown products, and their bioremediation products; 2) the development of methods to screen microorganisms for potential adverse health effects; 3) the potential for adverse effects when chemical/chemical chemical/microorganism interactions occur; and 4) the development of methods to better extrapolate toxicological bioassay results to the understanding of potential human toxicity.

Two performance evaluation papers were presented at the symposium studying the toxicity of hazardous waste mixtures before and after bioremediation. One group of microorganisms *(Pseudomonas aeruginosa* and *Phanerochaete chrysosporium)* were found to be unable to significantly degrade or eliminate the mutagenic activity of a mixture of several aromatic chemicals.

Detoxification of Model Compounds and Complex Waste Mixtures Using Indigenous and Enriched Microbial Cultures

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Introduction

Biological degradation of organic compounds may be considered an economical tool for remediating hazardous waste contaminated environments. While some environments may be too severely contaminated for initial in situ treatment to be effective, most contaminated media will use some form of biological degradation in the final treatment phases. Traditional studies monitor the success of bioremediation by the loss of a parent compound or class of chemicals. Prior studies, however, have observed both an increase and decrease in toxicity as a result of degradation (1-3). This study uses both genotoxicity bioassays and chemical analysis to monitor the efficacy of biological degradation of model compounds and a complex chemical mixture in soils.

Methods

A Weswood sandy loam soil was allotted in 7-kg portions into 40 stainless steel boxes. Four soil treatment categories included: 1) no amendment; 2) amendment with three model chemicals, i.e., benzo(a)pyrene (BAP), 2,4,6-trinitrotoluene (TNT), and pentachlorophenol (PCP); 3) amendment with a wood preserving bottom sediment waste (WPW); and 4) amendment with both the model chemicals and WPW. Additionally, each of the four soil treatment categories were divided into three-box subunits and inoculated with either an indigenous culture, a *Pseudomonas aeruginosa* culture, or a *Phanerochaete chrysosporium* culture. Thus, a total of 40 boxes were prepared, including the soil with no chemical amendment and the soil with indigenous, bacteria, or fungi inocula (NI, NB, NF); a wood preserving waste amended soil with each of the three inocula (WI, WB, WF); soil amended with the model chemicals and each of the microbial inocula (MI, MB, MF); soil amended with both the model chemicals and wood preserving waste and each inolula (CI, CB, CF); and sterilized soil receiving each of the chemical amendments (St-N, St-W, St-M, St-C). The soil boxes were stored in a greenhouse under constant temperature and humidity, and monitored to maintain moisture content. Samples were collected on Days 0, 90, 180, and 360 posttreatment. The solvent was extracted with methylene chloride and methanol, then evaluated for genotoxicity in the *Salmonella*/microsome assay. Benzo(a)pyrene and pentachlorophenol were quantified using gas chromatography (GC), whereas TNT was quantified using high-performance liquid chromatography (HPLC).

Results

The data presented in Table 1 describe the quantity of solvent-extractable organics recovered from the soil treatments using methylene chloride and methanol. The data indicate that smaller quantities of organics were recovered from the treatments with indigenous organisms on 0 and 30-day sampling periods. The soil treatment with the wood preserving waste, model chemicals (CI), and indigenous organisms was not appreciably changed during the 360-day incubation. The waste and bacteria treatment (WB) yielded an average of 32 mg/g solvent-extractable organics on Day 0 and 20 mg/g on Day 360. Overall, none of the treatments amended with wood-preserving waste displayed an appreciable reduction over the initial 360 days of treatment.

The extract of the unamended Weswood soil induced an average negative mutagenic response at Days 0 and 90. In general, treatments with the model chemicals, wood preserving waste, or combined treatments induced an average positive mutagenic response at all time points. With metabolic activation, the methylene chloride extract of the MB treatment induced an average of 167 net TA98 revertants on Day 0 and 122 net revertants on Day 90. A slight increase in the mutagenic response was observed in the majority of extracts of samples collected on Day 180. The methylene chloride extracts of the waste amended soils (WI, WB, and WF) collected on Day 180 induced responses with activation that ranged from 46 net revertants (WI) to 63 net revertants per mg

Table 1. Solvent Extractable Organics Recovered From Soil Treatments (mg residue/g soil)

	Time (days after treatment)					
Treatment[a]	**0**	**30**	**60**	**90**	**180**	**360**
WI	19.80 ± 3.2[b]	23.18 ± 1.6	29.45 ± 2.2	24.18 ± 0.8	20.28 ± 1.8	18.25 ± 0.8
WB	32.25 ± 1.1	27.25 ± 2.5	26.10 ± 2.8	23.81 ± 1.2	25.24 ± 3.8	19.78 ± 1.6
WF	21.04 ± 0.9	28.35 ± 2.0	30.84 ± 4.6	23.79 ± 2.0	21.19 ± 1.7	18.14 ± 1.0
CI	19.17 ± 3.5	29.26 ± 1.7	28.16 ± 3.2	26.67 ± 2.7	24.06 ± 0.7	20.21 ± 0.4
CB	26.45 ± 1.6	25.42 ± 0.9	26.35 ± 1.0	23.54 ± 0.4	23.97 ± 1.8	19.62 ± 1.1
CF	23.36 ± 2.0	31.51 ± 0.6	29.90 ± 1.9	26.17 ± 0.2	21.71 ± 2.6	19.57 ± 0.9
StC	27.05	30.31	32.88	36.60	26.95	28.78

[a] WI = wood preserving waste with indigenous bacteria.
WB = wood preserving waste with *P. aeruginosa*.
WF = wood preserving waste with *P. chrysosporium*.
CI = combined model chemicals and wood preserving waste with indigenous bacteria.
CB = combined model chemicals and wood preserving waste with *P. aeruginosa*.
CF = combined model chemicals and wood preserving waste with *P. chrysosporium*.
StC = sterilized soil with combined model chemicals and wood preserving waste.
[b] Values are the mean of three replicates ± standard deviation.

of residue (WB). The methylene chloride extracts of the combined waste and model chemical soils (CI, CB, and CF) collected on Day 180 induced responses that ranged from 71 net revertants (CB) to 76 net revertants per mg of residue (CF and CI). Most of the methanol extracts induced a stronger response than was observed in the methylene chloride extract. At least one solvent-extractable fraction from each of the soils collected on Day 180 induced a positive mutagenic response.

Discussion

Approximately 1 year after the application of both wood preserving waste and model chemicals to a Weswood soil, the level of solvent-extractable organics was not appreciably reduced. Although chemical analysis suggested reductions in the model chemicals in some of the treatments, contaminant concentrations were all detectable after 360 days. The data do suggest that the presence of oils and hydrophobic chemicals in the waste amended soil may have limited the availability of chemicals for microbial degradation. Changes in mutagenicity over the initial year of the study indicate that the bioavailability of chemicals was increased in some treatments. These results indicate that 360 days of treatment was insufficient to eliminate the mutagenic activity of soils amended with either the model chemicals or the wood preserving waste. Data collection will continue for an additional 720 days to monitor the chemical and toxicological changes associated with each treatment.

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Assessing the Genotoxicity of Complex Waste Mixtures

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Introduction

Some chemicals associated with environmental spills and hazardous waste sites can induce permanent genetic alterations in all organisms. These changes, called mutations, can have deleterious effects on individuals and their descendants. In human populations, mutations are known to increase the incidence of cancer and genetic diseases and may play a role in numerous other diseases. In nonhuman biota, mutations may alter the balance of an ecosystem or change the virulence of pathogens.

In spite of 20 years of research concerning environmental mutagenesis, current knowledge concerning the identity and effects of most environmental mutagens (including genotoxic carcinogens) is limited, and data on their effects on other living organisms and the ecosystem are practically nonexistent. Because their mode of action theoretically does not depend on a threshold limit, genotoxicants are a class of toxic substances that should be examined when found in even low concentrations in the environment.

The major objective of this paper is to review the U.S. Environmental Protection Agency's (EPA's) research in the area of genetic toxicology as it relates to bioremediation. Readers can refer to a completed manuscript (1) for more in-depth information, examples of studies and data, and a discussion of the regulatory context.

Research Targeted by the Risk Assessment Paradigm

The complexity of environmental situations complicates the monitoring, evaluation, and risk assessment of hazardous waste sites and spills. The composition of pollutant mixtures may consist of a few or thousands of individual compounds, and remediation efforts produce additional components that add to the complexity of evaluating risk. EPA research targets efforts that enhance the risk assessment process. Research, therefore, can be placed into the four categories associated with the risk assessment process: hazard identification, exposure assessment, dose-response assessment, and risk assessment modeling and calculation methods.

Hazard Identification Research

Hazard identification research strengthens the risk assessment process by developing methods that are more reliable and more cost effective, and, when possible, give some sense of relative toxicity when identifying toxic agents in the environment. This research involves the development of new assay methods (e.g., the spiral mutagenicity assay), the enhancement of existing assays (e.g., the modification of the prophage assay), the integration of bioassay and analytical chemistry methods, and the modification of methods for use with complex environmental mixtures rather than with single environmental chemicals.

Exposure Assessment Research

Generally, a hazardous substance must come into contact with a specific biological component (e.g., deoxyribonucleic acid) before a toxic response can occur. Measuring or estimating the potential level of contact between a toxicant and its reactive target (or a surrogate for that target) is exposure assessment. Exposure assessment is done on a population and not an individual basis. Quantitative toxicological assays and analytical chemistry methods have been developed to determine relative amounts of toxicity potential existing in different environmental (e.g., treated versus untreated) sites, to determine the relative bioavailability of environmental toxicants and their metabolites, and to follow the change in environmental concentrations of toxicants over time. In collaboration with other EPA laboratories, the National Health and Environmental Effects Research Laboratory (NHEERL) has demonstrated the usefulness of bioassays to enhance and calibrate exposure assessment methods.

Dose-Response Assessment

Dose-response assessments quantitate the potency of an environmental agent(s) for a specific outcome. Although the NHEERL bioremediation program does not support whole animal carcinogenicity and mutagenicity studies, the Environmental Carcinogenesis Division (ECD) of NHEERL does develop, in support of other programs, information relevant for bioremediation hazard and risk assessments. In addition, ECD has developed a statistical software system for analyzing, in a quantitative manner, short-term mutagenicity tests.

Risk Assessment Modeling Research

When information is limited or only a general characterization is required, a qualitative assessment can be made; however, there is often a need to be as quantitative as possible in risk assessments. ECD, therefore, not only develops quantitative data but also examines mechanisms and developing models to help scientists understand to what extent the toxic effect is relevant to human populations. Mechanistic research, for example, may demonstrate that rodents respond to a toxin in a manner not relevant to humans, alleviating the need for a quantitative human risk assessment. Likewise, mechanistic research may demonstrate whether or not synergism should be considered when evaluating a multichemical site.

The purpose of the presentation given at this conference is to catalog NHEERL bioremediation research, to show its relevance, and to provide examples of how the information obtained will be useful in future assessments.

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Section 4
Pilot-Scale Research

By studying bioremediation processes under actual site conditions on a small scale, researchers can gather critical information on issues such as operation, control, and management of residuals and emissions before moving to full-scale research. This is a critical intermediate step in which the success of laboratory experiments are further tested in an expanded but controlled setting.

Pilot-scale evaluations covered many different tools for bioremediation, including biofilters, compost piles, and slurry bioreactors. One paper studied the optimization of biofilters for use in removing volatile organic contaminants from the air, while another sought to establish the optimal operating conditions for compost media. Finally, a third paper found that many organic contaminants become concentrated in the foam produced in slurry bioreactors and suggested that this effect could be utilized to isolate contaminants from the rest of the slurry.

Two papers focused on the use of combinations of aerobic and anaerobic conditions to degrade recalcitrant chlorinated wastes. Work was presented on biofiltration with gel beads that provide an oxic environment at their surface and an anoxic environment at their centers. The authors found that such filtration was highly effective at degrading trichloroethylene (TCE). A system was evaluated for land treatment that involved switching between anaerobic and aerobic conditions.

Research continued on the integration of physical and biological processes to clean up organic wastes. One paper showed how wood preserving wastes can be first removed from their soil matrix by washing and distillation processes and then degraded in a bioreactor.

Two presentations covered research into optimizing the biodegradation of TCE. The first was the result of the study of a variety of parameters that might affect the success of field-scale TCE treatment. The next characterized several potentially useful bacteria isolated from a biofiltration device.

Two papers investigated the use of microorganisms to clean up inorganic wastes. One technique mentioned involves the biological reduction of sulfates into sulfides. This technique helps to remove metal cations from solution. The other technique involves the biological reduction of metal cations into less harmful oxidation states. The authors of the final paper tested the ability of microorganisms to reduce chromium (VI).

Poster presentations described several pilot-scale research projects. The poster projects within this category involved the testing of a compost bioreactor, a biofilm-electrode reactor, and a laminar-type flow reactor.

In Situ Bioremediation of Trichloroethylene With Burkholderia Cepacia *PR1: Analysis of Parameters for Establishing a Treatment Zone*

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Introduction

The use of chloroethenes, including trichloroethylene (TCE), has led to an extensive contamination of groundwater resources in the United States. In situ bioremediation of this contaminant is being pursued with the aerobic microorganism *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) G4 (1). Mutant strains G4 $PR1_{23}$ and $PR1_{31}$ constitutively produce a toluene *ortho*-monooxygenase (TOM) that mineralizes TCE. The gene for this enzyme is located on the degradative plasmid TOM of *B. cepacia* (2). The constitutive expression of this gene is the result of secondary transposition following *Tn5* insertion mutagenesis, which also confers kanamycin (km) resistance to these bacteria. $PR1_{23}$ contains a single entire *Tn5* in the chromosome and an IS50R of *Tn5* in the plasmid. $PR1_{31}$ bears an entire *Tn5* and an IS50R in the TOM_{31c} plasmid. The IS50 elements are at nearly the same locations and are thought to be responsible for the constitutive expression of *tom*.

This project involves ground-water flow control with sealed sheet piling to funnel ground water through a narrow gate area in which treatment technologies can be applied. Parameters for the establishment of a biological treatment zone with PR1 have been investigated in the laboratory. Transport and survival characteristics of the bacterium have been examined in ground water and sediment from a targeted release site (the aquifer under the Canadian Armed Forces Base, Borden, Ontario). Monitoring techniques have been developed for tracking PR1 populations in the treatment zone and determining the extent of dispersal and survival beyond the treatment zone.

The functional integration of non-native bacteria in natural microbial communities and maintenance of bacterial populations above normal environmental background concentrations provides a challenge for both microbial ecologists and applied microbiologists. Monitoring of population dynamics and trophic interactions is critical for successful bioaugmentation applications. Risk assessment associated with uncontained biotechnological introductions also requires careful monitoring of the survival and dispersal of released microorganisms and altered genes. Although releases of non-native or recombinant bacteria have not been reported to result in adverse environmental effects to date, there is a responsibility to ensure that released microorganisms will be constrained by the selective pressures of the target environment.

Tracking

Selective media provide a first step in tracking the organism. Phenol, o-cresol, and phthalic acid combined with kanamycin (km) were tested for growth of PR1 and isolates from the Borden aquifer. Growth of PR1 was optimal on 20 mM phthalate medium. In aquifer sediment slurries, numbers of native bacteria isolated on this medium range from 0 to less than 5×10^5 colony forming units (CFU) and direct epifluorescence microscopy counts of bacterivorous protists are less than 8×10^2 mL^{-1} in unamended incubations.

A monoclonal antibody (mab) specific to G4 lipopolysaccharide (LPS) (3) has been used both in direct immunofluorescent counts and to confirm CFU of PR1 on phthalate plates. Details of the production and testing of this mab can be found in Winkler et al. (3).

Polymerase chain reaction (PCR) primers targeting the *Tn5* insertion junctions have been developed and tested. The primers were designed based on the

assumption that the insertion points would be unique for PR1. The PCR products resulting from this primer set are of slightly different size for $PR1_{23}$ and $PR1_{31}$, reflecting the slightly shifted location of the IS50R in the plasmid in these two strains. Attempts to extract indigenous bacterial deoxyribonucleic acid (DNA) from Borden aquifer sediments and to amplify product from these extractions with the PR1 primers have failed. We have demonstrated that PCR amplification with these primers will work with whole, intact cells added directly to the PCR reaction. The current limit of detection for positive amplification from cell suspensions (equivalent to pore water samples) is 1×10^3 cells/mL^{-1}, but from sediment slurries the detection limit drops an order of magnitude. These results indicate that this assay will be most useful for large volumes of pore water samples collected on Sterivex filters (Millipore Corp.) for extraction of DNA (4).

Potential host range for the TOM plasmid from $PR1_{31}$ in 24 random isolates from Borden aquifer sediments (R2A medium; 5) was assessed by direct filter matings. Overnight cultures of $PR1_{31}$ grown in lactate medium and aquifer isolates in R2A medium were pelleted and resuspended in R2A medium to an optical density of 1.0 @ 600 nm. One milliliter of donor and recipient were mixed and filtered on a 0.2 µm pore-size polycarbonate filter. Filters were placed on R2A plates and incubated overnight at 30°C. Filters were then removed and vortexed in R2A broth and suspensions plated on selective media predetermined by antibiotic screening. Out of 24 isolates, 42 percent were positive for TOM_{31c} transfer by PCR detection, and 80 percent of these were positive for *tom* activity as indicated by a positive transformation of trifluoromethyl phenol (TFMP) (1) and mineralization of TCE in standing cell assays. These data indicate a wide potential host range within the target environment for TOM, highlighting the need to ensure tracking capability for both the organism to be introduced and its associated genetic elements. These native bacterial strains bearing functional TOM plasmids may also be better adapted for bioremediation application in the target environment than PR1.

Analysis of PR1 Transport in Borden Aquifer Material

Transport characteristics of PR1 in Borden sand are important in determining the retardation and dispersal of cells within a treatment zone and in the downstream aquifer. Cell retardation is important to allow contaminated water to flow past the inoculated populations of PR1. A series of experiments was carried out at three scales: 3.8-cm, 10-cm, and 40-cm length columns packed with sterile and nonsterile Borden sand and commercially available silica sand. Artificial ground water (AGW) was used as the solute. All columns were packed under standing water to minimize air entrapment and tamped with a glass rod to attain a uniform bulk density of 1.8 g/cm^{-3} and a porosity of 0.4. Ground-water velocity was set to the approximate velocity of water in the target site. Bacterial suspensions were pulsed through for 1 void volume, and the effluents were collected in sterile vials with a chromatography fraction collector. Numbers of PR1 were determined by plating on selective media. Chloride (Cl) ion as a conservative tracer was measured with Ag/AgCl electrodes calibrated against Cl ion analysis by ion chromatography.

Both the Cl tracer and bacterial breakthrough curves displayed similar patterns between columns, indicting good replication. All bacterial breakthrough curves exhibited a notable pulse of bacteria corresponding to the breakthrough of the Cl tracer. Reduced peak concentrations of bacteria relative to breakthrough concentrations of Cl (greater than 99 percent) indicate irreversible sorption of PR1 onto the geologic media. Well-defined tailing was also observed over the duration of the experiments, indicating reversible sorption of PR1 to the geologic media. Peak heights and tailing were three orders of magnitude lower in Borden material than silica sand, possibly due to clays and iron coatings on Borden sands. Breakthrough of PR1 was not affected by sterilization of the sediment or the addition of a cotransported bacterium. These results were integrated with predation loss rates of PR1 added to Borden sediment in slurry microcosms to develop a predictive model with both physical and biological parameters for the transport and fate of the organism within the Borden aquifer.

Survival of PR1 in Aquifer Microcosms

Much of the target environment consists of anaerobic saturated sediments. Survival of the organism beyond an oxygenated target zone will likely depend on its ability to withstand conditions of little or no oxygen. Plate counts of a suspension of cells with Nitrogen (N_2) gas flushed through the head space indicate little effect on $PR1_{31}$ viability through 6 days. After this point, culturable numbers drop precipitously but maintained a low population level through 25 days of anaerobic conditions.

No PR1-specific viruses could be isolated from the target environment. Samples of aquifer sediment were incubated with growing PR1 cells as an enrichment, and supernatants were tested on PR1 overlay plates to look for cleared viral plaques.

Survival of PR1 above 1×10^7 cells/mL^{-1} is of interest in establishing needed inoculation densities for effective bioremediation (6). Survival below this concentration and long-term integration of the organism into the native microbial community was of interest for risk assessment. A series of experiments has been conducted in ground water, sediment slurries, and flow columns to examine population dynamics of PR1 and the bacterivorous protists from the Borden aquifer that are the primary vector for loss of PR1. Use of a monoclonal

antibody (mab) to PR1 LPS (3) allows enumeration of PR1 after PR1 numbers have been reduced to the background level of phthalate utilizers in the system. The loss of PR1 cells and corresponding tracking data by mab indicates that with increasing inoculation density, PR1 populations are sustained for longer periods prior to rapid loss. This delay in the loss of cell numbers can be attributed to exceeding the maximum response of the bacterivorous protists in the system.

Data on the time of sustained PR1 populations and the loss rate of PR1 cells after this delay were compiled for all sediment slurry incubations. The delay in rapid loss of PR1 was incorporated into a regression analysis of time of sustained PR1 populations above 1×10^7 as a function of inoculation density. A half-life estimation by regression analysis incorporates loss rate data after the period of sustained population numbers as a function of inoculation density. This former analysis provides an estimated lifetime for a pulse of PR1 into the treatment zone. The latter analysis provides an extinction coefficient for cells in the downstream aquifer.

An aquifer sediment column has been established to test the response of PR1 within the flow regime of the target system. A commercial spring water (GMW) was used as the diluent. A chromatography column was fitted with cut gas chromatography (GC) vials closed with Teflon septa to provide sampling ports. Teflon tubing and fittings were used throughout the setup. Tygon tubing connected to a constant temperature recirculating bath was used to jacket the column and maintain 15°C. Flow was controlled at the column outflow to the flow rate in the target environment (2 cm/day^{-1}). Cells and TCE were added by syringe pump. An overflow ensured constant supply of diluent, cell suspension, or TCE solution to the top of the sediment. Pore-water samples were taken by syringe (2:300 µL each), and used for plate counts, direct counts of bacteria and protists, and TCE analysis (alternate sampling periods).

With an inoculum of 1×10^8 cells/mL^{-1} for 1 void volume, a population of greater than 10^7 CFU/mL^{-1} pore water was maintained for 5 days at in the top portion of the column. Column data expressed as depth profiles showing a roughly linear decrease in $PR1_{31}$ numbers with distance traveled through the column from Days 1 through 5, and the combined effect of predation and elution decreased numbers in the upper portion of the column at Days 8 and 10. By Day 15, the pulse has been eliminated at the upper and lower portions of the column, leaving residual cells in the central portion. Bacterivorous protists increased in proportion to the numbers of PR1 added. These organisms form resistant cysts on sediment surfaces when food is not available. Decreases observed over time for protist numbers are likely due mainly to encystment of these organisms after depletion of $PR1_{31}$. This results in a reservoir of bacterivores capable of responding to subsequent additions of bacteria.

Integration of PR1 Into Stable Microbial Consortia

Persistence of a non-native bacterium introduced into an environment is dependent on the ability of that organism to find refuges from predation and compete with native bacteria. One such refuge may be in biofilms. To examine the ability of PR1 to integrate into biofilms, PR1 was introduced into existing biofilms developed from Borden aquifer material and into a defined microbial community (including protists) grown with input of TCE and dibutyl phthalate. PR1 was found not to stick to pre-existing biofilms of Borden aquifer origin, but successfully integrated into the degradative community growing in the presence of TCE and dibutyl phthalate. The addition of these substances apparently provided a competitive advantage to PR1 in this system. One year post-inoculation, PR1 was located in biofilms from this system by fluorescent monoclonal antibody and scanning confocal laser microscopy. PR1 was found throughout the biofilms as scattered cells and microcolonies. This information suggests that in the presence of TCE it may be possible to maintain and grow PR1 within a treatment zone in the target aquifer.

Modeling of Transport and Fate of PR1 in Borden Aquifer

Using the transport data and loss rates due to predation, preliminary modeling exercises have been conducted. These initial approximations assume grazing losses will be the same for pore water and attached bacteria, and do not address excystment/encystment processes of the protists, but do incorporate protist grazing as a dynamic response to variable bacterial density. In all simulations, predation reduces the bacterial concentrations in the effluent from a modeled transport column. With an increased predation constant, peak breakthrough numbers are reduced, but tailing is affected more significantly. Tailing, which can be attributed to the process of reversible sorption, is of concern for transport of bacteria to greater distances in geologic media. Thus, reductions in tailing by the protist response reduces the concern of offsite migration of the introduced microorganism.

Conclusions

Data collected from these laboratory studies indicate that biological interactions, particularly with bacterivorous protists, will limit the survival of PR1 introduced into the system and may provide for a natural containment of the bacterium. Maintenance of PR1 numbers high enough for mineralization activity will likely require repeated additions of sufficient cells to exceed protist

maximum response. Preliminary data on TCE additions suggest that TCE may impede protist activity until it is mineralized, providing a gradient of protist response in proportion to TCE removal. Ultimately, the utility of laboratory analyses will be gauged against data from the field release.

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Characterization of Trichloroethylene-Degrading Bacteria From an Aerobic Biofilter

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Introduction

The microbial community colonizing a vapor-phase biofilter was examined to determine the population(s) capable of trichloroethylene (TCE) degradation. The community had been exposed to low levels of TCE continuously for 24 months and maintained degradation in the absence of a canonical co-metabolite. Although low levels of autotrophic ammonia-oxidizing bacteria were present, nitrapyrin inhibitor studies suggested that alternative bacteria were responsible for TCE oxidation. In addition, replacement of ammonia with nitrate did not affect TCE degradation. Incubation of biofilter biomass in a toluene or benzene atmosphere resulted in a turbid culture within 2 to 3 days. In light of these observations, aromatic hydrocarbon-oxidizing bacteria were pursued as putative candidates mediating TCE degradation.

A significant fraction of the culturable heterotrophs (greater than 80 percent) were capable of growth on toluene or benzene. This study describes the naturally occurring TCE-degradative populations that became established over time in the biofilter. Individual isolates were tested for TCE-degradative capacity under several growth conditions. The pure cultures tested were all capable of co-metabolic TCE degradation. These organisms had persisted in the biofilter regardless of conditions that would exert a negative selective pressure due to generation of the TCE-derived epoxide during aerobic TCE degradation. Several isolates were selected for further study. Initial sampling of the biofilter yielded three isolates: *Rhodococcus* sp. TA1, *Pseudomonas putida* TA2, and *Nocardia* sp. AR1. Both the *Rhodococcus* and the *P. putida* could be repeatedly isolated from the biofilter. Two other organisms, *P. putida* DC1 and *Burkholderia cepacia* GR3, were isolated more recently. This consortium appears relatively resistant to the toxic effects of TCE oxidation at the concentrations used in the biofilter.

Background

Halogenated aliphatic compounds are a major class of industrially important chemicals that have become significant environmental contaminants with mutagenic and carcinogenic potential. A widespread ground-water contaminant, TCE can undergo co-metabolic oxidation by a variety of physiologically diverse bacteria (1). Co-metabolic TCE degradation by aromatic hydrocarbon utilizing bacteria was originally reported by Nelson et al. (2). Since that time, efforts to employ these organisms to ameliorate TCE contamination problems in situ and in reactors have been conducted. This study examines the toluene-degrading bacteria surviving in a vapor-phase biofilter. Although only a mineral salts medium was supplied to the biofilter, toluene oxidizers survived and TCE degradation was maintained at a level of 20 percent of a 21-ppmv gas stream.

Experimental Methods

Biofilter and Sampling

The biofilter consisted of ceramic plates in a stainless steel casing. The initial inoculum was a municipal sludge sample that was acclimated to a volatile organic compound (VOC) mixture (benzene, toluene, ethylbenzene, and TCE) for a period of 3 months. At this point, all VOCs except TCE were removed. The biofilter was operated at a gas-flow rate of 520 mL/min, and had an empty-bed residence time of 1.9 min. TCE inlet concentration was 21 ppmv. A mineral salts solution was applied to the biofilter at a flow rate of 357 mL/day. Biofilter sampling was conducted by opening the biofilter and scraping biomass off the ceramic matrix. VOC-degrading bacteria

were isolated by incubation of biofilter material in a mineral salts medium, with the appropriate VOC supplied in the vapor phase.

TCE Mineralization Assays

Degradation experiments using ^{14}C-TCE were conducted in 20.0 mL vials, with teflon-lined silicone septa closures allowing injection into the vial. An inner vial containing 0.4 N NaOH served as a CO_2 trap. Sterile control vials were subtracted from experimental values when determining conversion of TCE to CO_2 or soluble products. All data represent a mean value of triplicate vials.

Biochemical and Genetic Characterization of Biofilter Bacteria

Aromatic hydrocarbon utilizing bacteria were isolated as follows. Biofilter biomass was inoculated into mineral salts medium, and the medium was exposed to toluene or benzene vapor as a sole carbon source for 48 hr. Cultures were then plated onto mineral salts medium and grown in a toluene or benzene atmosphere for 10 days. Organisms that appeared were picked and streaked onto mineral salts plates and grown again with the carbon source in the vapor phase. The isolates were then checked for purity and TCE mineralization capability. Isolates were sent to Microbial ID, Inc. (Newark, Delaware), for fatty acid methyl ester (FAME) analysis. 16S rDNA sequencing was carried out by amplifying the 27 to 321 base pair region of the 16S rDNA gene by polymerase chain reaction (PCR). The primers used are forward ’5AGAGTTTGATCCTGGCTCAG-3’ (positions 27-46) and reverse ’5AGTCTGGACCGTGTCTCAGT-3’ (positions 321-301). Both forward and reverse DNA strands were sequenced. Gene probes for characterized toluene/TCE co-metabolic oxygenases were obtained from other researchers. The *todABC* probe was obtained from Dr. D.T. Gibson, and the *tbu* probe was obtained from Dr. A. Byrne and Dr. R.H. Olsen (3, 4).

Results and Discussion

The predominant bacteria in the biofilter were shown to be degrading TCE by toluene/benzene oxygenase co-metabolic route. The biofilter community had not been exposed to these compounds for over 24 months, yet these organisms persisted and were shown to be the key population mineralizing TCE. These isolates are of interest because they arose spontaneously from a naturally occurring population and were maintained in the continual presence of TCE. It might be expected that TCE-oxidizing organisms would be selected against in such a system (5). Mixed cultures mineralized ^{14}C-TCE (data not shown) and exhibited very little diversity when plated out onto a mineral salts medium and grown with toluene or benzene vapor as a sole carbon source. These plates generally had only one or two colony morphotypes shown to be a *P. putida* (designated TA2) and a *Rhodococcus* (designated TA1). A *Nocardia sp.* (designated AR1) was isolated during an early sampling time but was not reisolated. Two additional organisms, *B. cepacia* GR3 and *P. putida* DC1, were more recently isolated.

The predominant toluene degrader isolated from the biofilter was TA2. Growth on glucose, succinate, or tryptophan completely inhibited TCE mineralization by TA2. A gene probe for the alpha subunit of the *tbu* toluene monooxygenase strongly hybridizes with TA2 (Figure 1).

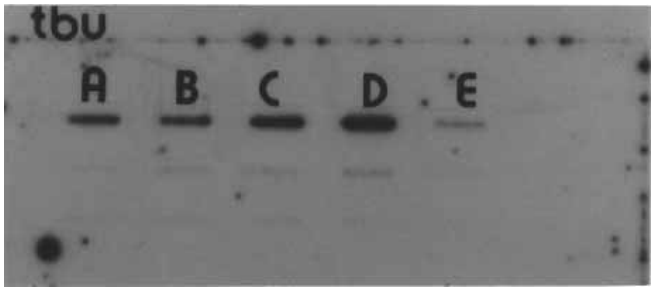

Figure 1. Slot blot analysis of biofilter DNA extracts probed with the *tbu* monooxygenase alpha component: A) *P. putida* DC1, B) *B. cepacia* GR3, C) *P. putida* TA2, D) *Nocardia* sp. AR1, and E) *Rhodococcus* sp. TA1.

This probe will also hybridize to two other toluene monooxygenases, *tmo* and *tom* (data from our laboratory and unpublished information from M.S. Shields, University of West Florida, and R.H. Olsen, University of Michigan; therefore, the mode of toluene oxidation is not definitively established. Slot blot analysis of biofilter isolates probed with *tbu* is shown in Figure 1.

Rhodococcus sp.TA1 did not hybridize strongly to either the *tbu* or *tod* toluene oxygenase probes, indicating the uniqueness of its toluene oxygenase. In addition to TA1 and TA2, three other TCE-co-metabolizing organisms from the reactor were investigated, and their properties are listed in Table 1. All organisms are being evaluated to determine basal levels of TCE catabolic activity as toluene is depleted. The effect of acclimation to alternative substrates on TCE degradation is also being examined.

Table 1. Properties and Growth Substrates of TCE-Co-metabolizing Biofilter Isolates

Organism	Oxidase	Indole[a]	Benzene	Ethylbenzene	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol
TA1	–	–	+ + +	+ + +	+ + +	+ + +	+ + +	+
TA2	+	+	+ + +	+ + +	–	+	+	–
AR1	–	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
DC1	+	–	+ + +	+ + +	_	–	–	–
GR3	+	+	+ + +	+ + +	–	+	–	–

[a] Conversion of indole to indigo.

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Anaerobic/Aerobic Degradation of Aliphatic Chlorinated Hydrocarbons in an Encapsulated Biomass Biofilter

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Introduction

During the past decade, public awareness and concern about the quantity and diversity of persistent (recalcitrant to degradation) synthetic chemicals produced by industry have increased. Release of these chemicals into the environment is inevitable, and hence there is a strong need to control, direct, and improve the processes for degradation of these chemicals. Large differences in the rates and mechanisms of biodegradation of various compounds under oxic and anoxic conditions exist. Consequently, sequential anoxic and oxic conditions, enabling cooperation of anaerobic and aerobic bacteria, are desirable for rapid complete mineralization of many polyhalogenated compounds (1-6).

Encapsulation of Biomass in Hydrogels

The goal of this research is to use hydrogel-encapsulated bacteria for simultaneous creation of oxic and anoxic zones inside the hydrogel bead. Further, the oxic and anoxic zones created inside the hydrogel bead can be successfully used to mineralize chlorinated compounds such as trichloroethylene (TCE) and perchloroethylene (PCE). Hydrogel beads with encapsulated bacteria can be used to mineralize chlorinated compounds present in air, ground water, and soil, and can also be used to promote ecological competitiveness of laboratory-grown cultures that are specially adapted for biodegradation of specific environmental pollutants.

Preparation of New Gel Material (7)

The gelation procedure used is as follows: Silica sol, obtained commercially as LUDOX colloidal silica SM grade, is mixed with 1 to 3 percent sodium alginate in the following proportion: silica sol (90 wt percent to 99 wt percent) : 1 to 3 percent sodium alginate solution (1 wt percent to 10 wt percent). The mixture, after adjustment of pH between 7 and 8 by 5N HCl, is mixed with active aerobic and anaerobic biomass cells, with the initial cell wt percent varying from 2.0 to 10.0. The mixture is stirred, then poured into a petri dish to a depth of 5 mm. Calcium chloride solution (0.1 molar) is poured on top of the mixture in the petri dish. The silica sol and sodium alginate mixture immediately gel due to the diffusion of calcium forming calcium alginate on the outer surface, and the gel is allowed to cure from 10 min to 24 hr. During the curing process, the pH of the silica sol decreases, thereby forming silica gel with pockets of calcium alginate inside the silica gel. The survival of active cells is maximized by using a combination of silica sol and sodium alginate. Once the silica sol has formed silica gel, stainless-steel wire mesh cylinders 2.5 mm in diameter and 5.0 mm long (open at both ends) are pushed into the gel layer, thereby enclosing the gel inside the wire mesh. The use of the stainless steel mesh gives the silica gel/calcium alginate bead structural strength so that the beads can be packed in a bed without compaction.

Experimental Studies Conducted

A 40-mL bioreactor (1.9 cm inner diameter) consisting of a jacketed cylinder was constructed from borosilicate glass. The reactor was packed randomly with the gel beads. Air was passed at a controlled rate through the bioreactor, and nutrient solution was trickled down from the top of the bioreactor counter current to the air flow. The air was contaminated with chlorinated pollutants, such as TCE or PCE, using a syringe pump that injected the liquid contaminant into the air line through a septum. The concentration of the contaminant in the air stream

was varied within the following range: toluene 0 to 100 ppmv; TCE 0 to 25 ppmv; and PCE 0 to 25 ppmv. The reactor temperature was maintained at 25°C by circulating water from a constant temperature bath through the jacket of the bioreactor. Nutrient solution was trickled down the bioreactor at a flowrate of 1 liter per day, and the nutrient composition was as follows: KH_2PO_4 (85 mg/L), K_2HPO_4 (217.5 mg/L), $Na_2HPO_4 \cdot 2H_2O$ (334 mg/L), NH_4Cl (25 mg/L), $MgSO_4 \cdot 7H_2O$ (22.5 mg/L), $CaCl_2$ (27.5 mg/L), $FeCl_3 \cdot 6H_2O$ (0.25 mg/L), $MnSO_4 \cdot H_2O$ (0.0399 mg/L), H_3BO_3 (0.0572 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.0428 mg/L), $(NH_4)_6Mo_7O_{24}$ (0.0347 mg/L), $FeCl_3 \cdot$EDTA (0.1 mg/L), yeast extract (0.15 mg/L), and formate (50 mg/L). Results obtained are shown in Table 1.

Table 1. Percent degradation of TCE in the bioreactor at various air flowrates. Inlet TCE concentration was 25 ppmv, and nutrient flowrate was 1 liter/day.

Air Flowrate (mL/min)	Percent Degradation of TCE
35	100.0
40	67.2
50	40.7
60	22.1
65	10.8

Carbon and chlorine balances were made by monitoring the increase in carbon dioxide in the exit air, and increase in chloride ion concentration in the exit nutrients was analyzed by an ion chromatograph. The chlorine balance was developed at steady-state conditions within an error band of 15 percent of the calculated increase in chloride ion concentration.

The proposed degradation pathway was shown to be a partial dehalogenation in the anoxic zone followed by oxic biodegradation of the anoxic degradation products in the outer aerobic zone of the gel bead. The anoxic zone was created due to oxygen consumption in the aerobic zone by the oxic degradation of the partially dehalogenated products as they diffused out from the anoxic zone.

A mathematical model was developed to describe the diffusion of TCE and oxygen, and consumption of oxygen due to aerobic degradation of the dehalogenated products. At the outer surface of the gel bead (denoted by dimensionless position of 1.0) the oxygen concentration is about 8 mg/L due to presence of air outside the bead. As oxygen diffuses inside the gel bead, it is consumed due to aerobic degradation of the dehalogenated products diffusing outwards. At some point in the interior of the gel bead, oxygen is completely consumed producing an anoxic zone in the interior portion of the gel bead. It is in this anoxic zone that dehalogenation of TCE occurs. The formate in the nutrient medium is rapidly absorbed by the gel bead and provides the organic carbon source needed for partial dehalogenation of TCE in the anoxic zone by anaerobic microbiota. Other potential carbon sources for anaerobic microorganisms are acetate and other carboxylic acids.

Experiments also were conducted with perchloroethylene (PCE) at an inlet concentration of 25 ppmv. Results obtained are shown in Table 2. Chloride ion balances were obtained at steady-state to prove that complete mineralization of PCE had occurred. Each experiment had to be conducted for over 5 days to achieve a stable exit concentration of chloride ion in the exit nutrients. No other by products were observed in the exit gas phase at the above operating conditions.

Table 2. Percent degradation of PCE in the bioreactor at various air flowrates. Inlet PCE concentration was 25 ppmv, and nutrient flowrate was 1 liter/day.

Air Flowrate (mL/min)	Percent Degradation of PCE
10	100.0
15	86.7
20	72.4
30	41.8
50	12.8

Conclusions

The hydrogel-encapsulated biomass reactor is capable of biodegrading trichloroethylene (TCE) and perchloroethylene (PCE) through an anaerobic/aerobic degradation mechanism. Experimental results indicate that the degradation of TCE and PCE is complete, and the empty-bed gas phase residence time for complete removal is less than a few minutes. Further studies are ongoing to quantitate the transport parameters and apply the process for treatment of TCE or PCE in ground water.

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Operation and Optimization of Granular Air Biofilters

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Introduction

Since enactment of the 1990 amendments to the Clean Air Act, the control and removal of volatile organic compounds (VOCs) from contaminated air streams have become major public concerns (1). Consequently, considerable interest has evolved in developing more economical technologies for cleaning contaminated air streams, especially large, dilute air streams. Biofiltration has emerged as a practical air pollution control (APC) technology for VOC removal (2-4). In fact, biofiltration can be a cost-effective alternative to the more traditional technologies, such as carbon absorption and incineration, for removal of low levels of VOCs in large air streams (5, 6). Such cost-effectiveness stems from a combination of low energy requirements and microbial oxidation of the VOCs at ambient conditions.

Our biofiltration research has focused on expanding the range of application of biofiltration technology to the treatment of high VOC loadings at consistently high removal efficiencies. The preliminary period of our research was dedicated to the pilot-scale comparison of three different types of biological attachment media: a patented peat mixture and two synthetic inorganic media, one channelized and the other pelletized. The biofilters containing the latter two media were operated as trickle bed air biofilters (TBABs), called such because the media received a steady application of water. After 18 months of testing, the pelletized medium (Celite 6-mm R-635 Bio-Catalyst Carrier) was demonstrated to be significantly better than the other two for handling high VOC loadings (7-9). Subsequent work to evaluate the performance and behavior of biofilters using the R-635 pelletized medium produced two significant findings: first, that an increase in the biofilter operating temperature permits a significantly higher practical VOC loading (i.e., a significantly smaller required media volume), and second, that biofilter performance decreases substantially with the buildup of back pressure due to the accumulation of biomass within the media bed (10, 11).

Working exclusively with this pelletized medium, our continued research focused on the development of strategies for long-term operation with high VOC loadings at sustained high-removal efficiencies. This research effort demonstrated that this objective could be achieved using a biomass removal and control strategy employing periodic backwashing of the media with water. Backwashing (the upflow washing of the fluidized media with water) gently removes excess biomass from the media, circumventing the problems noted earlier for this medium. A second finding of this research was that NO_3-N as the sole nitrogen source was superior to NH_3-N. The use of NO_3-N resulted in lower volatile suspended solids (VSS): chemical oxygen demand (COD) and VSS:N ratios. In other words, for a given set of operating conditions, less biomass is produced and less nitrogen is consumed. Finally, it was also observed that both the recovery of the VOC removal efficiency with time after backwashing (unsteady state) and the VOC removal efficiency with depth (at near steady state) were superior when using NO_3-N.

This paper discusses the continuing research being performed for the development of biofiltration as an efficient, reliable, and cost-effective VOC APC technology. The objectives of this effort were to investigate the removal efficiencies of TBABs under high toluene loadings and low residence times, and to evaluate the associated development and control of excess biomass with time. The biofilter operational period between backwashings was evaluated to determine its effect on the stability of biofilter performance. Backwashing variables, including backwashing frequency and backwashing duration, were evaluated.

Methodology

The biofilter apparatus used in this study consisted of four independent, parallel biofilter trains, each containing 3.75 ft of pelletized Celite 6-mm R-635 biological attachment medium. A detailed schematic, equipment description, and typical system operation are given elsewhere (8). Each biofilter had a circular cross section with a 5.75-in. internal diameter (ID). The air feed was mass-flow controlled, and the VOC (liquid toluene) was metered by syringe pumps into the air feed stream. Each biofilter was fed a buffered nutrient feed solution containing all necessary macro- and micronutrients with a sodium bicarbonate buffer, described elsewhere (8). For each biofilter, the sole nitrogen nutrient source was NO_3-N. The flow directions of the air and nutrients were downward. All biofilters were insulated and independently temperature controlled at 32.2°C.

Results

Each biofilter was loaded with clean, sterilized pellets and seeded with backwashing water from a similar, previous run. For each biofilter, a significant dip in performance occurred from Days 17 to 26 due to an error in preparing the nutrient solution that resulted in feeding insufficient NO_3-N. After the target VOC loading was achieved, at a COD:NO_3-N ratio of 50:1, it was maintained for the duration of the run.

Three different backwashing strategies were tested on all biofilters sequentially. After restart following backwashing, effluent samples were collected to determine the recovery of the VOC removal efficiency with time. On days when no backwashing occurred, samples were collected along the length of the bed to determine the VOC removal efficiency with respect to depth.

Biofilter A

This biofilter was started up at 50 ppmv toluene influent concentration, 1.33 min empty bed residence time (EBRT), and 21 mmol NO_3-N per day. On Day 17, the biofilter was backwashed for the first time. Detailed schematic and backwashing descriptions are given elsewhere (12). The procedure used was to recycle 70 L of 32.2°C tap water through the bed, bottom to top, at a rate of 57 L/min to induce full media fluidization at a bed expansion of about 40 percent. At the end of the backwashing period, the media was flushed at the same rate with another 50 L of clean, 32.2°C tap water. For this first backwashing strategy, the period and frequency were 1 hr twice per week. The target influent VOC concentration (500 ppmv toluene) and loading (6.2 kg COD/m^3 day) were reached on Day 53. On Day 129, the second backwashing strategy was started using a period and frequency of 2 hr twice per week. On Day 171, the third and final backwashing strategy was started using a period and frequency of 1 hr every 2 days. The performance of Biofilter A is shown in Figure 1.

Biofilter B

This biofilter was started up at 50 ppmv toluene influent concentration, 0.67 min EBRT, and 21 mmol NO_3-N per day. On Day 17, the biofilter was backwashed for the first time using the first backwashing strategy of 1 hr twice per week. The target influent VOC concentration (250 ppmv toluene) and loading (6.2 kg COD/m^3 day) were reached on Day 35. On Day 129, the second backwashing strategy of 2 hr twice per week was begun, and on Day 171, the third strategy of 1 hr every 2 days was begun. The performance of Biofilter B is shown in Figure 2.

Biofilter C

This biofilter was started up at 50 ppmv toluene influent concentration, 1.0 min EBRT, and 21 mmol NO_3-N per day. On Day 17, the biofilter was backwashed for the first time using the first backwashing strategy of 1 hr twice per week. The target influent VOC concentration (250 ppmv toluene) and loading (4.1 kg COD/m^3 day)

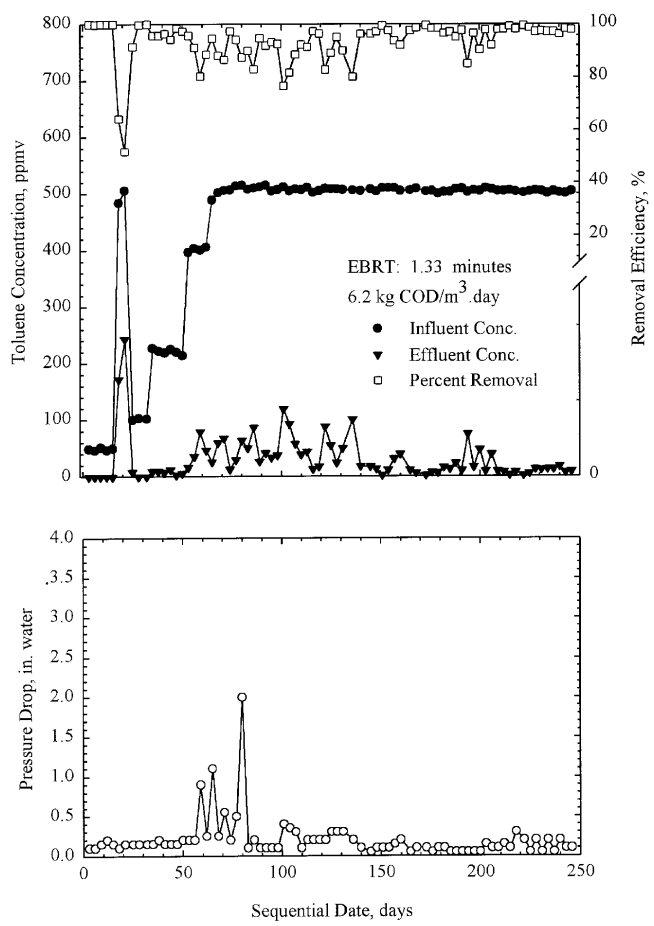

Figure 1. Performance of Biofilter A with backwashing.

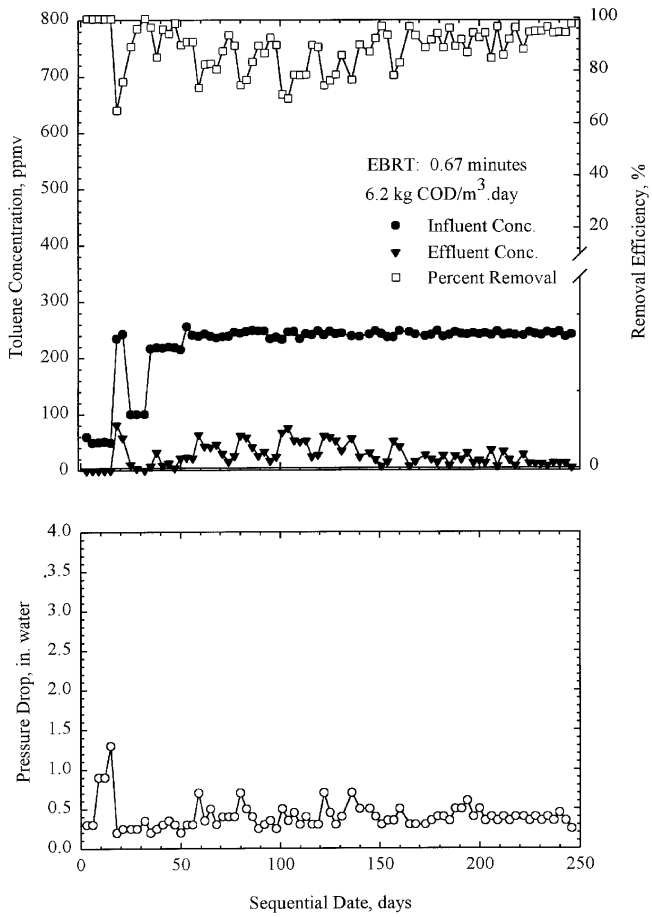

Figure 2. Performance of Biofilter B with backwashing.

were reached on Day 53. On Day 129, the second backwashing strategy of 2 hr twice per week was begun, and on Day 171, the third strategy of 1 hr every 2 days was begun. The performance of Biofilter C is shown in Figure 3.

Biofilter D

This biofilter was started up at 50 ppmv toluene influent concentration, 2.0 min EBRT, and 21 mmol NO_3-N per day. On Day 17, the biofilter was backwashed for the first time using the first backwashing strategy of 1 hr twice per week. The target influent VOC concentration (500 ppmv toluene) and loading (4.1 kg COD/m^3 day) were reached on Day 53. On Day 129, the second backwashing strategy of 2 hr twice per week was begun, and on Day 171, the third strategy of 1 hr every 2 days was begun. The performance of Biofilter D is shown in Figure 4.

Conclusions

The performances of the four biofilters with respect to the three backwashing strategies were similar, although clearly affected by both the loading and the residence time. The effectiveness of the three strategies increased from the first through the third strategy. This shows that both backwashing duration and frequency are very important parameters for control of the biofilters' VOC removal efficiency. The third and best strategy, however, actually had less total backwashing time per week. At the higher loading, the greater than 90 percent removal efficiencies of both biofilters were unexpectedly high for the third backwashing strategy but below the sustained 99.9 percent achieved by the lower loaded biofilters. It can also be seen that for a given loading, the performance at the lower EBRTs is more sensitive to the backwashing strategy employed. Both of these effects were anticipated; what was not anticipated was that this pelletized medium would perform so well for any backwashing strategy at a loading of 6.2 kg COD/m^3 day. These findings, as well as biofilter recovery of performance after backwashing, will be presented.

Acknowledgment

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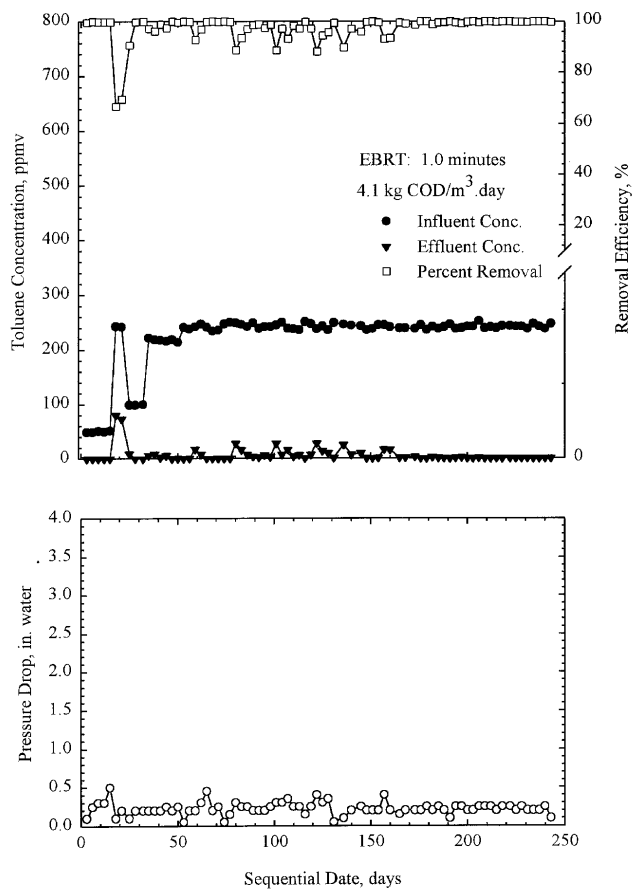

Figure 3. Performance of Biofilter C with backwashing.

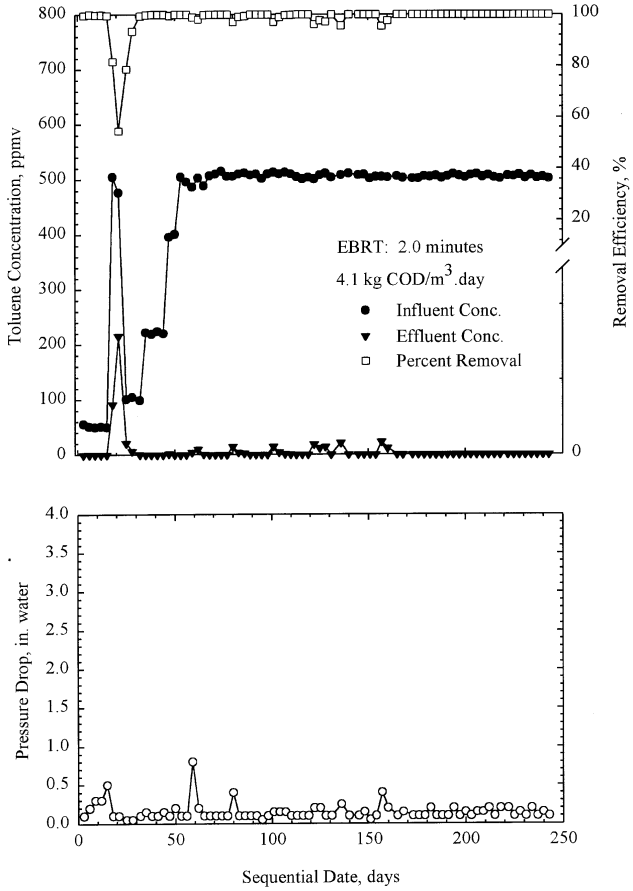

Figure 4. Performance of Biofilter D with backwashing.

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Abiotic Fate Mechanisms in Soil Slurry Bioreactors

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Introduction

Soil slurry treatment of contaminated soil has been shown to offer a viable technology for soil bioremediation. This technology, however, has not sufficiently progressed to be a durable, reliable, and cost-effective treatment option (1).

The use of aggressive mixing energy to provide conditions for improved contact between soil contamination and microorganisms capable of degrading the contamination is the hallmark of slurry treatment technology. A more complete description of pollutant mass transfer during the treatment phase is required that includes treatment fate mechanisms attributable to biotic and abiotic processes. Losses attributable to abiotic means can be overlooked in field application of the technology, because limited questions can be successfully addressed at field scale. Discussions with U.S. Environmental Protection Agency (EPA) regional personnel and inspection of active field-scale soil slurry bioreactor operations have identified operational problems, such as foaming, that could result in possible abiotic loss (2).

Field bioslurry operations have adopted various approaches to reduce foaming: 1) addition of defoaming agents, 2) reduction of the rotational speed of the agitator, and 3) reduction of gas flow through the bioreactor system. The foaming phenomenon is generally considered a nuisance, rather than a potential beneficial removal mechanism. Where pollutants have a specific gravity less than water once desorbed from the slurried soil, the pollutants would rise to the surface, as in a flotation process. One of our working hypotheses was that foam formation could be related to this pollutant release process. If this analysis has merit, it is possible that the operational strategy used in the field is counterproductive, because a separated contaminant phase is re-entrained with partially cleaned soil material.

We have conducted two bench-scale slurry bioreactor treatability studies at the EPA Testing & Evaluation Facility in Cincinnati, Ohio, which were designed to assess operating factors leading to foam formation, and to identify the most advantageous means to deal with foaming. The initial study was previously presented as a general treatability study for treatment of creosote contamination in a soil (3). During this previous study, foaming became a major problem for operation. Use of a defoamer controlled foaming conditions, as did reduction of the mixer rotational speed and gas flow in the more extreme cases. Subsequent studies devoted specifically to investigating the causes and conditions of foaming and using a different batch of soil from the same site as the earlier study showed little foaming at the beginning of the study.

Methodology and Experimental Designs

Foam Study (First Study)

A soil from St. Louis Park, Minnesota, contaminated with polynuclear aromatic hydrocarbons (PAHs) was used to assess the importance of foaming conditions to the performance of bench-scale slurry reactors. The design of the bench-scale experimental bioslurry reactor has been reported (3). Operational slurry volume was 6 L, representing 75 percent of the total reactor volume.

To evaluate the conditions and causes of foam formation, a subsequent study was designed. This investigation used the monitoring conditions specified for the treatability study, and was conducted using six bench-scale slurry reactors. Each reactor was loaded at 30 percent solids, with an initial volume

of 6 L. The study design employed two reactors that were permitted to develop foam, two reactors in which foam formation was suppressed through the use of a defoamer, and two reactors in which formaldehyde was used to suppress biological activity. The study duration was 1 week, with foam sampling based on the cumulative production throughout the study period.

Foam and Scale Study

Experimental variables selected for the foam and scale study were 30 percent soil solids loading, two treatment conditions of condensed foam (removed), and foam retention within the reactors. Each condition was replicated with a single replicated control based on the foam retention condition. The foam-retained conditions were maintained through the use of a dispersant (Westvaco, Reax 100M). The soil was classified to a minus 3/16 in. dimension; no provisions were developed for preliminary dispersion of the soil solids or sand exclusion. Scale was collected from the reactor walls after the mobile soil solids were removed. The scale was strongly fixed to the reactor wall, and some effort was required to chip the scale away from the reactor.

General Reactor Operation Condition

The control reactors were operated under abiotic conditions to serve as bioinactive control reactors. Formaldehyde was used as a biocide in these reactors and maintained at 2 percent residual concentration.

The following monitoring and operating conditions were held constant for the reactors:

- Dissolved oxygen: greater than 2 mg/L
- pH: range of 6 to 9
- Ambient temperature: recorded daily
- Treatment duration: 10 weeks
- Nutrients: C:N:P ratio = 100:10:1
- Antifoam: as needed to control foam

Results

In a previous treatability study, a high solids control reactor showed the greatest amount of foaming (3). The amount of foaming was surprising because foam formation was expected to be related to the formation of biosurfactants by the microbiota. Addition of formaldehyde to the control reactor was the only other explanation for the foam formation observed. Other reactors had foaming problems, but this reactor was very noticeable by contrast. Higher solids loading was also observed to contribute to foam formation.

In contrast to the earlier studies in which foaming was observed, the foaming study showed little foam formation. A small amount of foaming occurred on the first day of operation. Figure 1 shows the increased concentration of the t-PAH analytes in the foam, which is five times greater than the concentration found in the initial suspended soil slurry. A second attempt to evaluate foam formation is shown in Figure 2. These data show a decrease in the foam concentration factor, and are probably more realistic than the first foam study results. The second study was also designed to evaluate the deposition of t-PAH analytes as part of a scale formed in the reactor. Figures 3 and 4 show the results of t-PAH deposition in the scale under conditions in which the foam was condensed (removed from the reactor) and retained within the reactor. From inspection of the results, it is clear that the higher molecular weight components of the t-PAHs were deposited in the scale in quantities 10 to 90 percent above the initial suspended slurry concentration. A mass balance analysis will be presented that puts the importance of these abiotic fate mechanisms into perspective.

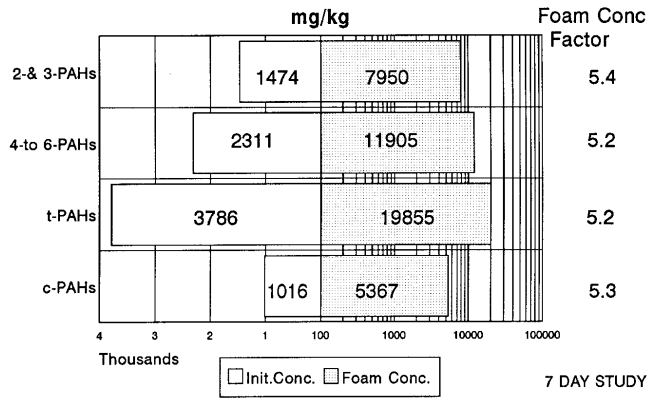

Figure 1. Foam composition and concentration factor (Study 1).

Differences in physical characteristics of the soil and operation of the bioslurry reactor between the two studies may have contributed to decreased foam formation in the foam study. Although soil for the same site was used for both the treatability study and foam study, the batch of soil used for the foam study was coarser (less than 1/4 in.) which may have resulted in lower PAH

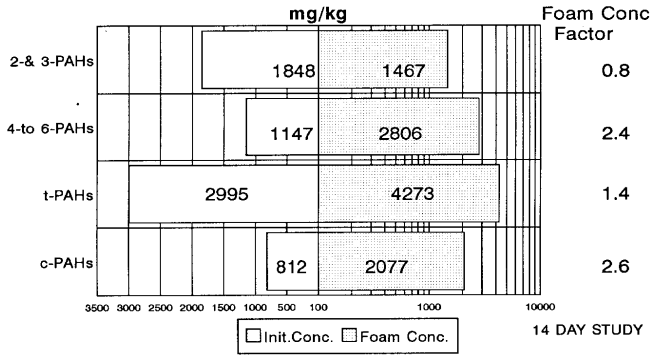

Figure 2. Foam composition and concentration factor (Study 2).

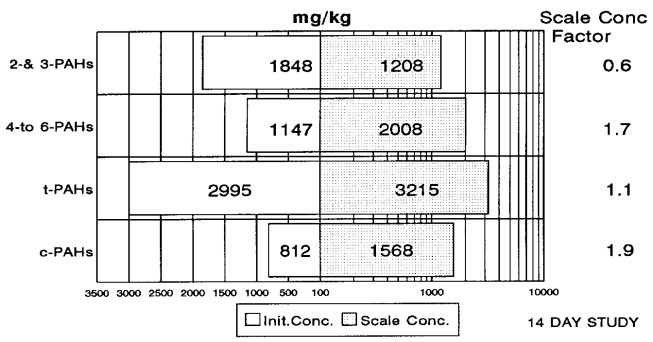

Figure 3. Scale concentrations for foam condensed reactors (Study 2).

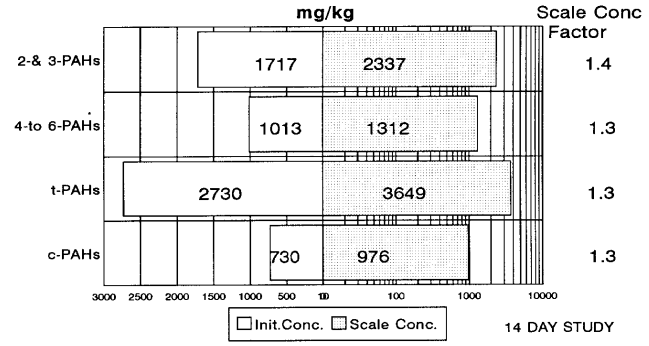

Figure 4. Scale concentrations for foam retained reactors (Study 2).

concentrations in the foaming study and to decreased foam formation. Furthermore, the air flow rate for reactors in the foam study (1 ft^3/min) was approximately 20 percent of that used in the treatability study (5 ft^3/min), which also may have contributed to decreased foam formation.

Conclusions

Foam formation continues to be an unpredictable and poorly understood event associated with slurry treatment. The results of our studies are based on the bench-scale reactor and may exaggerate the abiotic fate mechanisms due to high surface-to-volume ratio considerations. The concentration effects associated with foam formation indicate that foam removal may be desirable to optimize slurry reactor performance. Future studies will endeavor to evaluate foam separation as part of the slurry process.

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Design and Testing of an Experimental In-Vessel Composting System

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Introduction

The goal of this compost research is to evaluate the potential use of compost systems in remediation of soils contaminated with hazardous chemicals. We have developed bench-scale composters to evaluate factors controlling compost treatment at large scale. We are currently studying the ability of compost microorganisms to biodegrade polynuclear aromatic hydrocarbons (PAHs) in in-vessel reactors located at the U.S. Environmental Protection Agency's Test & Evaluation (T&E) Facility in Cincinnati, Ohio.

Composting differs from other ex situ soil treatment systems in that bulking agents are added to the compost mixture to increase porosity and serve as sources of easily assimilated carbon for biomass growth. Aerobic metabolism generates heat, resulting in significant temperature increases that bring about changes in the microbial ecology of the compost mixture.

Optimal conditions for composting may vary depending on many factors, but generally aerobic conditions with 45° to 55°C (mesophilic temperature range), 40 to 60 percent moisture, and a carbon-to-nitrogen ratio of 20:1 to 30:1 have been considered best. Mesophilic composting in the range of 35°C to 50°C might prove to be the most effective at destroying certain wastes. Maintaining temperature below 50°C, however, may not always be cost effective if cooling requires too much energy.

In an active compost pile, temperature can easily exceed 55°C, and temperatures above 70°C have been reported. When the temperature exceeds 55°C, called the thermophilic stage, most bacteria are killed. Organisms capable of sporulation, such as some bacteria (2) and fungi (3, 4), will sporulate and remain dormant until aerobic activity slows; the temperature falls back into the mesophilic range when they re-emerge.

Reactor Design

Ten 55-gal, insulated stainless steel compost reactors have been fabricated to provide the closely monitored and controlled conditions required for treatability studies. These fully enclosed, computer-monitored, bench-scale reactors hold about 1/4 yd^3 total compost mixture.

The reactor units stand upright with air flowing vertically up through the compost mixture for 23 hours per day. Enclosed units permit on-line analysis of oxygen, carbon dioxide, and methane at inlet and exit locations. A data-logging system accumulates data and transmits them to the PC-based central data system that monitors and controls each reactor. XAD traps in the exit line of each composter permit trapping of volatile organic compounds (VOCs) for analysis.

The bottom of each reactor contains a conical collection system for periodic sampling of any leachate leaving the reaction mixture. The space above the leachate collection system holds 2 in. of gravel. Mass balance studies on soil contaminants are possible by direct sampling of the reaction mixture at different depths through bung holes in the lid, together with capture of VOCs and leachate leaving the reactor.

Periodic determination of compost moisture content in each reactor unit permits adjustment of total moisture content in the compost matrix to 40 to 50 percent. Moisture condensers inside compost units promote retention of moisture within the reactor. Otherwise, with typical air flows, each unit could lose significant amounts of water daily. If moisture falls below 40 percent, a water distribution system inside the reactor may be used to add water to the reaction mixture without opening the reactor.

The cylindrical reactor design permits mixing of reactor contents by rolling each unit on a drum roller at desired intervals. Mixing can be used to break up anaerobic pockets and to avoid packing of the compost mixture. All

reactors are mixed simultaneously by placing them on rollers over a modified conveyor belt that forces the reactors to turn in unison. Baffles inside the reactors promote mixing during rolling.

Insulation between the reactor core and outer shell reduces heat loss from the reactor during aerobic activity. Heating coils provide the option of warming the reactor to accelerate composting during startup. Each composter houses five thermocouples connected to a central computer for on-line temperature measurements. Thermocouples reside at four equally spaced locations within the compost mixture, and a fifth thermocouple tracks ambient temperature outside the reactor. If the mean temperature of the middle two reactor thermocouples exceeds a predetermined high value, the computer switches that unit to high air flow (60 L/min) to cool the reaction mixture. After the high-temperature unit cools to a specified low temperature, the computer switches the unit back to low air flow (5 L/min) to reduce further heat loss from the reaction mixture.

Methods

Current studies focus on defining acceptable operating conditions and process characteristics to establish suitable parameters for treatment effectiveness. Parameters of interest include aeration, moisture dynamics, heat production, and physical and chemical properties of the compost mixture. Growth of microorganisms and disappearance of parent compounds serve as indicators of parameter suitability.

A 24-day treatability study, using field soil from the Reilly Tar Pit Superfund site near Minneapolis, Minnesota, was conducted to evaluate performance of the compost reactor system. The soil was contaminated with creosote and contained 22 PAHs that were measured during the study.

The study design included five replicated treatment conditions involving different ratios of corn cobs to soil and different air flow rates in 10 reactors. Soil/bulking agent compositions evaluated in this study were 50:50 (four reactors) and 30:70 (two reactors) ratios of corn cobs to soil (50 percent soil and 70 percent soil, respectively). Selected air flow rates were 5 and 10 L/min.

Results and Discussion

Temperatures in reactors with 50 percent soil and moisture content of about 50 percent or less climbed to the upper mesophilic and lower thermophilic ranges. Temperatures in reactors with moisture content above 53 percent failed to increase much above 30°C. This might indicate that higher moisture content restricted air flow through the compost mixture, resulting in insufficient aerobic activity to attain high temperatures. Reactors with air flows of 5 and 10 L/min exhibited similar temperatures within the compost mixture.

Reactors with 70 percent soil in the compost remained relatively cool throughout the entire run, never reaching the mid to upper mesophilic temperature range. These reactors tended to maintain higher moisture content throughout the study. Fewer corn cobs to absorb excess moisture in the mixture may have resulted in flooding of the pore space, blocking of air flow through the mixture, and reduced drying.

Total heterotrophic populations increased from a range of 10^7-10^8 to 10^9-7.6×10^{10} (60- to 300-fold increases) in reactors during the first 24 hr of composting. Heterotroph counts ranging from 1.6×10^9 to 1.4×10^{10} remained after 24 days in reactors with 50 percent soil, but had returned to around 2×10^8 in reactors with 70 percent soil.

Small PAHs (two to three rings) were reduced by averages of 50 and 30 percent in compost mixtures of 50 and 70 percent soil, respectively, after 24 days. Large PAHs (four to six rings) were not decreased under any treatment condition after 24 days. Continued evaluation of the compost mixture will provide more information on the long-term ability of composting to destroy large PAHs.

Future investigations will include application to pentachlorophenol and other soil contaminants yet to be specified. Evaluation of pollutant mass balance and biotransformation products is an important aspect of future research.

To judge the abilities of microorganisms to degrade hazardous wastes in soil under various composting conditions, emphasis will be placed on diagnosing population changes throughout treatment and identifying microbial species responsible for biodegradation of contaminants. Early microbiological studies have focused on enumerating total microorganisms and determining the presence of PAH degraders. Future studies will focus on characterizing changes in biological activity during the four stages of composting, and on identifying the microbial species responsible for significant biodegradation of PAHs during each composting stage. Reappearance of fungi and other mesophiles (e.g., *Actinomycetes*) during the cooling stage is also of interest.

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Integrated Systems To Remediate Soil Contaminated With Wood Treating Wastes

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Introduction

Approximately 15 percent of Superfund Records of Decision (RODs) are directed towards sites contaminated with wood treating wastes (1). Several types of pollutants characterize these sites, including pentachlorophenol (PCP), creosote, polycyclic aromatic hydrocarbons (PAHs), other hydrocarbons, and heavy metals such as copper, chromium, arsenic, and zinc (2). A process (Figure 1) that integrates soil washing with sequential anaerobic and aerobic biotreatment is being developed to cost-effectively remediate soil contaminated with these wood treating wastes. Soil washing facilitates degradation by mobilizing the target compounds and expanding the range of feasible remediation technologies (3). To reduce costs and the volume of PCP-bearing liquid, the soil wash liquid is concentrated via distillation, and the recovered ethanol and water is recycled to the first soil washing unit. The remainder of the wash solution is initially bioremediated in an anaerobic environment. Mineralization of the target compounds is completed aerobically (4). Process development began by independently evaluating soil washing and target compound bioremediation. PCP-contaminated soils were the initial focus, but this work is currently being extended to include soils contaminated with both PCP and PAHs. Based on preliminary results, the integrated process will meet the target cleanup level in 73 to 55 percent of the RODs directed towards PCP remediation, resulting in soil with a residual PCP level of 8 to 13 mg/kg, respectively (2).

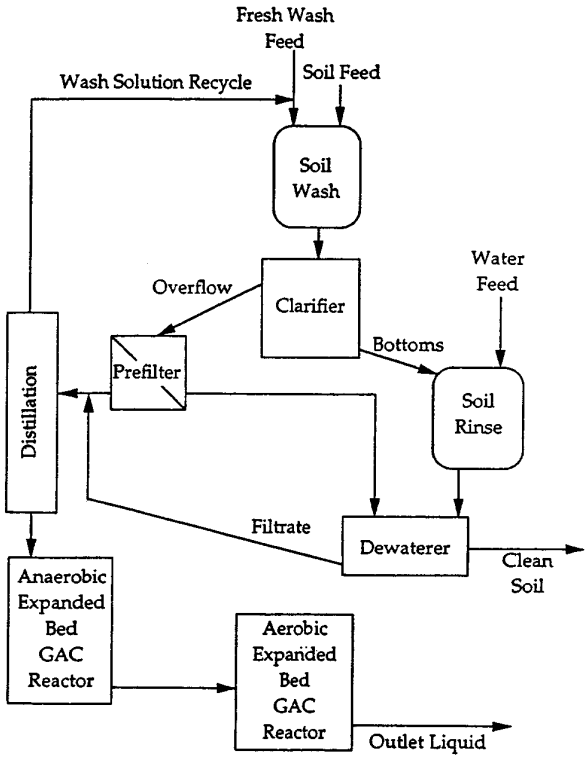

Figure 1. Integrated soil treatment process.

Soil Washing/Solvent Extraction Studies

An equimass (50 percent) mixture of ethanol and water (5) was found to be the optimal solution to remove PCP from a variety of spiked soils in a bench-scale soil washing process. This soil washing method removes PCP at levels comparable with those achieved through the analytical techniques of sonication and soxhlet extraction. Starting with initial spike levels of 85 to 100 mg/kg, 70 to 100 percent of the PCP added to the soil was removed by washing, depending on soil particle size, contamination age, and soil washing format. PCP is extracted from soil in a 30-min contact time. The availability of residual PCP on soils of 20 x 40, 100 x 140, and greater than 200 U.S. meshsize has been evaluated through a serial procedure: soils were

washed with 50 percent ethanol solution, rinsed with water, and finally treated by soxhlet or sonication extraction using methanol/methylene chloride. Less than 4 percent of the residual PCP (less than 0.6 mg/kg) was removed from the soil by the final sonication or soxhlet extraction, demonstrating the limited availability of the residual PCP. The solvent washing of soil with mixtures of water and ethanol is also being investigated for PAH-contaminated soils, using four compounds on the U.S. Environmental Protection Agency's list of priority pollutants as model compounds: naphthalene, acenaphthene, pyrene, and benzo(b)fluoranthene (6). A more ethanol-rich mixture may be required to effectively mobilize PAHs from soil.

A sequential soil washing train is being optimized for PCP-contaminated soils in which ex situ soil washing is performed with the 50 percent ethanol solution in three batch-wash stages. After washing the soil for 30 min in each stage, the washed soil is recovered from the soil-solvent slurry via vacuum filtration of the slurry, and a fresh batch of solvent is added to the soil in the next stage. Preliminary design data indicate that a series of three 20-mL solvent washes will clean 5 g of soil (1:12 soil:solvent ratio) as effectively as a single extraction of 100 mL cleans 1 g of soil (1:100 soil:solvent ratio). Additional optimization will further decrease the soil:solvent ratio.

Biological Treatment Studies

In the integrated process, the distillate bottoms will be fed to an anaerobic fluidized-bed granular activated carbon (GAC) reactor. Two of these reactors were constructed and operated for over 40 months, evaluating variables such as PCP loading and reactor empty bed contact time (EBCT) (7). The reactor volume is 10 L with a 1-L recycle loop. Based on this evaluation, the following optimal operating variables were identified: EBCT, 2.3 hr; ethanol loading, 33.3 g/day (loading rate 6.3 g chemical oxygen demand/L day); and PCP loading, 4.8 g/day (loading rate 0.55 g/L day). When the GAC reactor operated at an EBCT of 2.3 hr, on a molar basis, greater than 99.97 percent of the influent PCP was dechlorinated to monochlorophenol (MCP). In addition, data from the extraction of the reactor GAC during the operating period indicated negligible accumulation of PCP on the surface of the GAC. An aerobic fluidized-bed GAC reactor will polish the effluent from the anaerobic GAC reactor to attain complete mineralization of PCP. Operation of the aerobic reactor has recently been initiated.

An additional anaerobic fluidized-bed GAC reactor has been constructed to evaluate the biotreatment of chemically synthesized solutions of the four PAHs and PCP in ethanol. Greater than 99 percent transformation of the influent PCP concentration of 100 mg/L has been achieved in the reactor, while operating the reactor with an EBCT of 9.3 hr. The reactor effluent data represent greater than 99 percent removal for naphthalene, acenaphthene, and pyrene, and greater than 90 percent removal for benzo(b)fluoranthene in the reactor.

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Biological Treatment of Contaminated Soils Using Redox Control

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Introduction

Land treatment is a well-understood, cost-effective means of conducting aerobic biological treatment of soils contaminated with aerobically biodegradable compounds, such as petroleum. Common contaminants in soil also include highly chlorinated organics that are not readily biodegraded aerobically, such as pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), and 1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl)ethane (DDT). These compounds may, however, be efficiently degraded using a sequential anaerobic/aerobic treatment strategy. A cost-effective process to treat soils contaminated with these highly chlorinated contaminants is needed. A modified type of prepared-bed land treatment that incorporates variable redox states (i.e., anaerobic and aerobic phases) is being evaluated in this project. The first pilot-scale study, using PCP-contaminated soil from the American Wood Products site in Lake City, Florida, is in progress at the U.S. Environmental Protection Agency's Test and Evaluation (T&E) Facility in Cincinnati, Ohio.

Methodology

The pilot-scale study is being conducted in soil pans that simulate a prepared-bed land treatment unit with permeate collection. Each Plexiglas enclosure contains four soil pans suspended in a controlled-temperature water bath maintained at 20 ± 2°C. Each soil pan (13 in. x 13 in.) is loaded with contaminated soil to a depth of 8 in. over a graded gravel underdrain system, which is separated from the soil layer by a coarse mesh stainless steel screen. During the anaerobic phase, permeate recycle is optional from the underdrain to the top of the pan at flowrates of 5 to 20 mL/min. Anaerobic conditions are maintained in the soil by flooding the pans with clean creek water from the site. Aerobic conditions are produced and maintained in the soil by draining the water and tilling the soil in a manner consistent with landfarming techniques.

Initial Anaerobic Phase

Based on a review of the literature, which is dominated by studies evaluating spiked soils, three variables were selected for study in the initial anaerobic phase: 1) permeate recycle vs. no recycle; 2) addition of a supplemental organic source (ethanol or anaerobic sewage sludge); and 3) soil PCP contamination level. The experimental design was a three-factor analysis of variance with replication. The amounts of soil, ethanol, anaerobic digestor sludge (32.8 g dry solids/L, 60 percent volatile solids), and site water initially added to each of the 24 pans are summarized in Table 1. In situ oxidation-reduction potential (ORP) probes were placed in one pan representative of each of the 12 treatments. Four probes were buried in each pan, two in opposite corners approximately 1 in. from the soil/gravel interface and two in the remaining corners approximately 1 in. from the soil/water interface near the top of the reactor.

Recycle flow rates were maintained at 8 to 10 mL/min. The pH of the water flooding the pans was measured in situ each week. The soil in the pans was sampled and analyzed for PCP and its less chlorinated phenolic metabolites, hydrocarbons, and percent moisture on a monthly basis. Hydrocarbons were present in the site soil in significant quantities (1,000 to 2,500 mg/kg dry soil) because diesel fuel serves as a carrier for PCP in wood treating operations. The water flooding the pans was analyzed for PCP and less chlorinated phenolic metabolites each time a soil sample was collected.

Aerobic Phase

After 6 months, all of the pans except four of the sludge-amended pans (2D, 3A, 4B, and 5C) were converted to aerobic conditions. After the free water was drained from the soil, the soil was tilled three times a week for 4 weeks, until it dried to less than 10 percent total moisture. When the conversion phase was completed, some of the pans were continuously supplied with air at a low flow rate (in addition to weekly tilling of all pans) and/or amended with poultry manure (see Table 1). Each month, the soil in the pans is sampled and analyzed for PCP and its less chlorinated phenolic metabolites, as well as hydrocarbons. Moisture content and water addition volumes are measured weekly to maintain moisture content in a constant range.

Results

Anaerobic conditions were established in the soil pans after the first week. The measured ORPs ranged between -150 to -500 mV (versus Ag/AgCl reference electrode). No apparent correlation was found with respect to probe depth, soil type, or treatments. The soil sample PCP concentration data showed no significant amount of PCP removal in any of the treatments after 6 months. Changes in PCP concentration in the flood water of several of the pans were noted, however. After 2 months, the PCP concentration in the flood water of the soil pans containing sludge dropped from 15 to 55 mg/L to less than 0.5 mg/L. After 4 months, the PCP concentration also dropped to less than 0.5 mg/L in the two replicate pans with low-PCP soil treated without recycle or supplemental organic source. The less chlorinated phenolic metabolites were not detected as intermediates in the flood water from any of these pans. No degradation of hydrocarbons was noted in any of the pans in the initial anaerobic phase, but degradation has occurred in the aerobic phase. The presence of hydrocarbons may have interfered with the bioavailability of PCP in the initial anaerobic phase. To test this hypothesis, aerobically treated soil will be reconverted to anaerobic conditions once the hydrocarbon concentration has been reduced. Another possibility is that appropriate anaerobic PCP degraders are not present in sufficient quantities in the soil pans. A bench-scale study using soil from the same source as the pilot-scale study has been initiated to investigate the effect of amendment with PCP-acclimated culture.

Conclusions

Adaptation of the pilot-scale land-treatment units to anaerobic operation has been evaluated. Flooding the soil with water successfully creates a low redox (anaerobic) state. The in situ ORP probes constructed for the project work well. Monthly sampling intervals and the analytical techniques used adequately characterize system behavior. The tilling strategy used in the conversion from anaerobic to aerobic operation was successful. The presence of significant amounts of hydrocarbon co-contamination may have affected PCP degradation, suggesting that a more appropriate treatment sequence may be aerobic-anaerobic-aerobic. This observation reinforces the importance of technology evaluation with soils characteristic of those found at actual sites.

Table 1. Soil Pan Operation Summary

	Initial Anaerobic Phase Treatments[b]						Aerobic Phase Treatments[c]	
	Soil (kg as is)							
Pan[a]	Low[d]	High[e]	Ethanol (mL)	Sludge (L)	Water (L)	Recycle (mL/min)	Air (mL/min)	Manure (g/pan)
6B	36	-	-	-	16	-	-	-
1C	36	-	-	-	16	-	10	750
2B	36	-	-	-	16	8-10	-	750
6C[f]	36	-	-	-	16	8-10	10	-
1A[f]	36	-	17.2	-	16	-	-	-
3B	36	-	17.2	-	16	-	10	750
3C	36	-	17.2	-	16	8-10	-	750
2C[f]	36	-	17.2	-	16	8-10	10	-
4C	36	-	-	5.16	11	8-10[g]	-	-
5C[f]	36	-	-	5.16	11	-	Not converted	
4B[f]	36	-	-	5.16	11	8-10[g]	Not converted	
6D	36	-	-	5.16	11	-	10	-
2A	-	35	-	-	16	-	-	-
1D[f]	-	35	-	-	16	-	10	750
5A	-	35	-	-	16	8-10	-	750
4D[f]	-	35	-	-	16	8-10	10	-
3D[f]	-	35	17.2	-	16	-	-	-
5B	-	35	17.2	-	18[h]	-	10	750
6A	-	35	17.2	-	16	8-10	-	750
5D[f]	-	35	17.2	-	16	8-10	10	-
1B	-	35	-	4.88	12	-	-	-
2D[f]	-	35	-	4.88	12	-	Not converted	
3A[f]	-	35	-	4.88	10.5[h]	8-10[g]	Not converted	
4A		35	-	4.88	12	8-10[g]	10	

[a] Pan location for treatments (Pans A-D in Boxes 1-6) randomly assigned for statistical purposes.
[b] Initial anaerobic phase from August 11, 1994, to March 2, 1995.
[c] Aerobic phase from March 2, 1995, to July 7, 1995.
[d] Soil from 5 ft depth at site containing approximately 250 mg PCP per kg dry soil.
[e] Soil from 12 ft depth at site containing approximately 650 mg PCP per kg dry soil.
[f] In situ ORP probes added to pan during initial anaerobic phase.
[g] Recycle was set at 9 mL/min initially but was discontinued after the first week due to extremely low flow (less than 1 mL/min) through sludge-amended soils.
[h] Amount of water required to maintain a constant depth of 2 in. above soil surface varied somewhat.

Development of a Sulfate-Reducing Bioprocess To Remove Heavy Metals From Contaminated Water and Soil

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Introduction

Acid mine drainage is characterized by low pH (1.5 to 3.5) and high concentrations of sulfate and dissolved heavy metals. Bacterial sulfate reduction has been identified as a potentially cost-effective process for removing metals from mine drainage (1, 2). Sulfate-reducing bacteria convert sulfate to sulfide using an organic carbon source as the electron donor. The sulfide precipitates the various metals present in the wastewater, yielding a very low concentration of dissolved metals in the effluent. In this study, acetic acid was used as the carbon source for two reasons: it is relatively inexpensive, and being an acid, it can effectively leach out metals from contaminated soils such as mine tailings.

Reactor Selection

To effectively treat metal-contaminated wastewater, a reactor must establish an anaerobic environment to support sulfate reduction, resulting in metal precipitation as metal sulfides, and must provide an efficient clarifier to remove metal precipitates from the effluent. Because sludge (metal precipitates and biomass solids) would accumulate and eventually clog the reactor, ease of sludge removal or cleaning is an important consideration in selecting a reactor. Two reactors were evaluated: an upflow anaerobic filter packed with plastic Pall rings and an anaerobic sludge blanket reactor. To clean the reactor, sludge can be removed from the bottom of the sludge blanket reactor. The same technique can be used for the filter; however, it may be more difficult due to packing material. The cleaning of the filter, therefore, was an additional aspect of research.

Reactor Operation and Performance

Two filters (A and B) and one sludge blanket reactor were operated at a temperature of 30°C and at a pH of 7.2, optimal pH for sulfate-reducing microorganisms. The feed concentration of metals (shown in Table 1) used in this study were among the highest concentrations observed at mines in Montana and Colorado. After an initial acclimation, Filter A was fed the metals listed in Table 1, while Filter B and the sludge blanket reactor were fed iron at a concentration equal to the sum of the molar concentrations of all the metals fed to Reactor A. Table 1 shows characteristic effluent

Table 1. Influent and Effluent Concentrations (mg/L) for Filter A

		Effluent Concentration	
Influent Constituent	**Influent Concentration**	**Filtered**	**Total**
Iron	840	0.09	0.497
Zinc	650	0.14	0.310
Manganese	280	5.4	5.60
Copper	130	0.02	0.022
Cadmium	2.3	0.02	0.019
Lead	2.1	0.005	0.005
Arsenic	1.5	0.01	0.01
Acetate	3000	11.0	—
Sulfate	5000	800	—
Total sulfide	0	31	—

concentrations for Filter A, operating at a hydraulic detention time of 5 days.

During this period, the level of sludge rose above the packed bed in Filter A. To investigate whether sludge withdrawal from the bottom would control the sludge height in the filter, 1 L of sludge was removed. Sludge withdrawal lowered the sludge level and the filter continued to operate efficiently, with less than 1 percent change in effluent conditions. A similar situation in Filter B was also corrected in the same fashion. Sludge withdrawal from the bottom can, therefore, control the accumulation of sludge and prevent clogging of the filters.

The sludge blanket reactor did not perform very well as a clarifier. Although the soluble iron concentration in the effluent was less than 0.25 mg/L, the total concentration was as high as 25 mg/L and varied between 18 and 22 mg/L. This reactor had a high concentration of total suspended solids in the effluent compared with Reactors A and B. It was concluded that this type of reactor was not effective in clarification and was unable to meet the requirements. Therefore, the operation of the sludge blanket reactor was discontinued.

Conclusions

Compared with an anaerobic sludge blanket reactor, an upflow anaerobic filter packed with Pall rings was found to be a very efficient reactor for the treatment of water contaminated with heavy metals. The filter, unlike the sludge blanket reactor, worked very well as a clarifier, and all metals except manganese were reduced to a concentration close to drinking-level standards. Sludge withdrawal from the bottom of the filter can be used to remove accumulating sludge, and, therefore, the filter can be operated continuously. Ongoing work will evaluate the performance of the filters as a function of hydraulic retention time, lower temperatures, and pH. Sludge removal frequency will also be optimized.

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Development of Techniques for the Bioremediation of Chromium-Contaminated Soil and Ground Water

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The potential for biotic Cr(VI) reduction in samples from a Cr(VI)-contaminated aquifer (Elizabeth City, North Carolina) was evaluated by inoculating aquifer material into anaerobically prepared mineral salts medium that did not contain chemical reductant. In inoculated microcosms, the Cr(VI) concentration decreased after 5 days incubation at 25°C, and almost all of the Cr(VI) was gone after 25 days (Figure 1). Little or no change in Cr(VI)

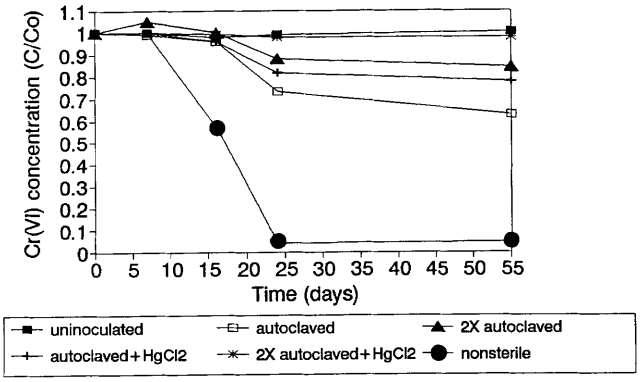

Figure 1. Biological reduction of Cr(VI) with aquifer material.

concentration was observed in uninoculated controls, or in sterile controls prepared by autoclaving, boiling, or the addition of $HgCl_2$ or chloramphenicol. Hydrogen reduced the lag time before Cr(VI) reduction occurred but did not markedly affect the rate of Cr(VI) reduction (Figure 2). The addition of other exogenous electron donors such as glucose, acetate, formate, or benzoate did not affect the rate or lag time associated with Cr(VI) reduction in microcosms compared with controls that lacked an exogenous electron donor. The addition of phenol, lactate, and ethanol to microcosms inhibited Cr(VI) reduction. Subsequent addition of Cr(VI) to microcosms with benzoate as the electron donor decreased the lag time and increased the rate of Cr(VI) reduced compared with that observed initially.

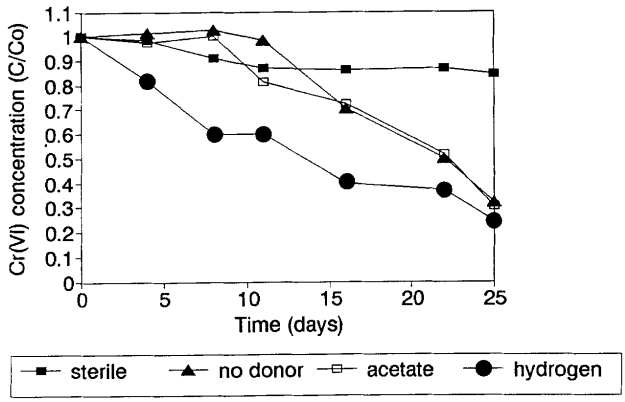

Figure 2. Effect of electron donors on Cr(VI) reduction with aquifer material.

The effect of sodium sulfate, sodium nitrate, amorphous ferric hydroxide (each at 10 mM) on Cr(VI) reduction was tested with benzoate-amended microcosms. The presence of sulfate and nitrate inhibited the reduction of Cr(VI) compared with microcosms that did not receive any of the three additional electron acceptors. Sulfide levels remained unchanged during the course of the experiments. In bottles with nitrate, nitrite accumulated after 8 days and decreased after 16 days. Ferric-hydroxide-supplemented microcosms reduced Cr(VI) to a much greater extent than unsupplemented control cultures; ferrous iron production coincided with Cr(VI) reduction.

Two facultative bacteria that can reduce Cr(VI) were isolated from Elizabeth City aquifer material, and one bacteria was isolated from an aquifer underlying a landfill in Norman, Oklahoma, using a mineral salts medium with 5 mM benzoate, 500 μM Cr(VI). All three isolates are gram-negative, motile rods that grow singly, in pairs, and in branched chains. On agar medium, the isolates formed shiny, smooth, pink colonies and produced a diffusible green pigment. Upon initial isolation, Cr(VI) was rapidly reduced (Figure 3); however, the rate and

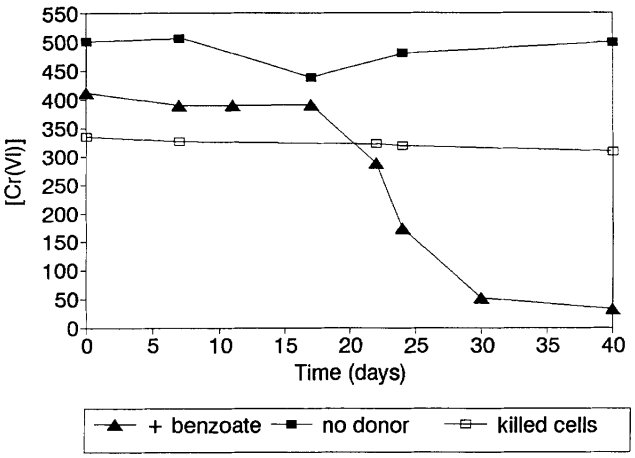

Figure 3. Cr(VI) reduction by strain NLB.

extent of Cr(VI) reduction decreased with repeated transfer of the culture in benzoate-Cr(VI) medium. The use of Cr(VI) was dependent on the presence of an electron donor and an active inoculum. In addition to benzoate, other substrates supported Cr(VI) reduction (Table 1). Increases in cell numbers were observed when the electron donor and Cr(VI) were both present. In the absence of Cr(VI) or electron donor, little or no increase in cell number was observed: less than 6 x 10^5 cells/mL.

Four observations supported the conclusion that the decrease in Cr(VI) concentration was a biologically mediated reduction process: 1) Cr(VI) concentrations decreased faster and to a greater extent in nonsterile versus sterile microcosms; 2) phenol, ethanol, and lactate inhibited Cr(VI) reduction in microcosms; 3) repeated additions of Cr(VI) to microcosms decreased the lag time and stimulated the rate of Cr(VI) reduction; 4) bacteria were isolated and capable of using Cr(VI) as an electron acceptor. Iron hydroxide stimulated Cr(VI) reduction in microcosms, most likely by an indirect mechanism involving the production of ferrous iron. The extent and rate of Cr(VI) reduction by aquifer microcosms was not affected when exogenous electron donors, with the exception of hydrogen, were added. This indicates that the aquifer material had sufficient levels of endogenous electron donors to support Cr(VI) reduction.

Table 1. Electron Donors That Support Cr(VI) Reduction by Strain NLB

Additions	Cr(VI) Reduced (μM)	Increase in Cell Numbers ([cells/mL] x 10^6)
Fumarate	414	0
Purine/Pyrimidine mix[a]	337	31.8
p-Toluic acid	184	30.1
Lactate	169	62.8
Phenoxyacetate	166	56.3
Malate	80	3.8
Benzoate	68	142.9
Phenol	59	19.3
Ethanol	32	7.8
No addition	0	0

[a] Mix contains 0.5 mM each: adenine, guanine, thymine, uracil.

Bioremediation of Chlorinated Pesticide-Contaminated Sites Using Compost

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Many sites throughout the United States are contaminated with chlorinated pesticides. Of particular interest to this project are those sites contaminated with chlordane and toxaphene. One objective is to determine the feasibility of using compost as a culture medium for mediating the biodegradation of these pesticides. A second objective is to determine major pathways of chlordane and toxaphene biodegradation that lead to mineralization. These objectives are particularly challenging because chlordane and toxaphene each consist of several chlorinated cyclic hydrocarbons that individually may follow different biodegradation pathways, or may be only partially dechlorinated.

Biodegradation of chlordane and toxaphene and other chlorinated pesticides is expected to require an organic co-substrate as a carbon source for the growth of acclimated microorganisms that enzymatically are capable of dechlorinating the pesticides through reductive or oxidative reactions. Co-substrates considered for use in field applications include milk solids, sugar, blood meal, sewage solids, methane, or, in the current project, compost.

The test program includes the development and operation of a pilot-scale compost reactor that contains a mixture of 10 percent municipal-sludge compost, 10 percent spent-mushroom compost, 40 percent grass, and 40 percent alfalfa hay to provide an environment suitable for the culture of chlordane- and toxaphene-degrading microorganisms. This compost is used to amend various contaminated-soil matrices followed by analysis of the fate of the pesticide. Residual pesticides are monitored using gas chromatography, thin layer chromatography, and mass spectroscopy. Test parameters include soil type, compost-soil ratio, moisture level, oxidation-reduction potential, pH, presence of sulfates and nitrates, and the effect of supplemental soluble organic co-substrates.

Reductive Electrolytic Dechlorination

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Reductive dehalogenation is the only known mechanism to biologically degrade some highly chlorinated organic compounds, including pentachlorophenol (PCP) (1), and occurs primarily in anaerobic environments (2). A biofilm-electrode reactor (BER) was constructed to evaluate PCP dechlorination as a function of ethanol concentration and the presence of an electrical current. The BER was operated as follows: current, 20 mA; hydraulic retention time, 0.38 days; PCP feed, 5 mg/L; ethanol feed range, 0 to 100 mg/L. The best observed dechlorination occurred when 5 mg/L PCP and 25 mg/L ethanol were fed to the reactor. The effluent under these conditions contained 0.013 mg/L PCP, 0.26 percent of the feed concentration. At lower ethanol levels, PCP was not as effectively dechlorinated. The trichlorophenols (TCP) and dichlorophenols (DCP) displayed a two- to threefold increase in effluent concentration as the substrate ethanol was decreased, particularly at concentrations less than 10 mg/L. The monochlorophenols (MCP), however, reached a maximum of 0.014 mM at ethanol concentrations of 10 to 25 mg/L. The total dechlorination decreased significantly when the ethanol was removed from the feed, indicating that the ethanol stabilized dechlorination.

After characterizing the ethanol requirements in the system, the role of the current in the dehalogenation of the PCP was evaluated by turning off the current. Electrical current was shown to play a necessary role in dechlorination, although it is unknown whether this role was the result of the hydrogen generation or the low reducing potential surface formed on the cathode of the anode-cathode cell. Two trials of reactor operation without current were conducted. Each current removal trial caused a reduction in PCP dehalogenation, demonstrated by the successive appearance of the higher chlorinated phenols in the effluent. The two trials displayed very different temporal behavior, however. The first removal resulted in a quick rise in effluent PCP concentrations, increasing one order of magnitude in a few hours. Following the second current removal, a much slower appearance of chlorinated phenols was observed; the effluent PCP concentration increased one order of magnitude in approximately 6 days. During each trial, after the current was reapplied, the system recovered. Each trial showed a recovery pattern similar to the failure preceding it, the first trial showing a quick recovery and the second trial showing a much slower recovery. The causes of the different behaviors have not been characterized. We are presently evaluating the role of electrical current by varying the current while keeping the ethanol concentration constant at 10 mg/L.

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Biological Ex Situ Treatment of PAH-Contaminated Soil

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The goal of this project is to evaluate the potential of biological ex situ soil treatment systems (biopiles) to remediate soils contaminated with hazardous chemicals. A laminar-type flow pilot-scale reactor with a volume of 3 yd^3 has been constructed at the U.S. Environmental Protection Agency's Test & Evaluation (T&E) Facility in Cincinnati, Ohio. Laminar-type flow from one side of the reactor to the other may provide even aeration to all areas of the reactor while avoiding the use of pipes inside the reactor. This design greatly facilitates loading and unloading of the reactor and is readily scalable to larger systems.

Passing smoke through the reactor for visual observation of flow indicated uniform, laminar-type flow through the empty reactor. Further testing involved filling the reactor with vermiculite or a synthetic soil, flushing with argon, and then passing air through the reactor to evaluate air flow through this uniform solid matrix. Oxygen probes, located at 27 positions within the reactor, indicated rapid and uniform air saturation of the system. Analysis of gas flow through an empty reactor and through uniform matrices allowed evaluation of reactor performance without confounding effects of soil inhomogeneities that may lead to nonuniform aeration of the reactor space.

The reactor uses pulsed air flow through the pile to permit maximum distribution of air within the soil. Air is driven into the soil during pulse action, then allowed to diffuse in all directions during the rest interval.

Soil contaminated with polynuclear aromatic hydrocarbons (PAHs) from the Reilly Tar Pit Superfund site in St. Louis Park, Minnesota, has been brought to the T&E Facility for research on soil aeration and effectiveness of this ex situ reactor design for biological treatment of contaminated soils. Micronutrients were adjusted to 100:20:1 phosphorus:carbon:nitrogen, and 0.5 percent by weight cow manure was added to the soil. A 10-week treatability study is under way to evaluate disappearance of parent PAHs and microorganism population changes in this reactor system.

Effectiveness of Gas-Phase Bioremediation Stimulating Agents (BSAs) for Unsaturated Zone In Situ Bioremediation

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Background

Successful in situ bioremediation in the unsaturated zone requires that water, oxygen, nutrients, primary substrate, and perhaps co-metabolites be available to the microorganism via physical transport mechanisms. Any of these substances may be called a bioremediation stimulating agent (BSA), given that a shortage of any one may adversely affect the performance of an in situ bioremediation system. Other potential BSAs include substances (e.g., surfactants) that are not ordinarily required for microbial growth but that may enhance substrate or nutrient bioavailability.

Much work has focused on engineering approaches to deliver BSAs at field scale. Little research has been conducted, however, to evaluate which in situ delivery approaches are best for transporting BSAs to microorganisms. Given the complexity of two-phase (gas/water) or three-phase (gas/water/nonaqueous-phase liquid) fluid and contaminant transport in the unsaturated zone, considerable uncertainty exists about the ultimate distribution of BSAs in contaminated soils. Further, microbial growth processes affect fluid and contaminant transport not only through biochemical reactions but also through a spatial-temporal influence on fluid permeability. (Plugging of pore spaces by microorganisms can reduce wetting fluid permeability by greater than 99 percent.) The present study will identify in situ BSA delivery strategies that are most likely to achieve a uniform BSA spatial distribution and, therefore, most likely to improve bioremediation field performance. As a byproduct of this work, the project aims to identify and measure the fundamental physical and microbial processes that affect bioremediation performance enhancement through BSA delivery methods.

Because of strong capillary forces that affect the distribution and movement of wetting fluids in unsaturated soils, gas-phase BSAs are more likely to achieve uniform in situ spatial distribution. It is, in fact, well known that movement of water in the unsaturated zone often occurs in discrete fingers that occupy a small fraction of the total pore space. Relatively little is known, however, about the characteristics of in situ gas-phase BSA transport, including physical factors that may lead to complex and undesirable flow patterns (e.g., interactions of water saturation and air permeability), chemical transport factors that may limit gas-phase BSA spatial distribution (e.g., BSA solubility), and dynamic microbial factors that may affect BSA transport in the field (e.g., microbial BSA utilization rates and plugging that leads to heterogeneous effects on air/water permeability). Because of the promise of gas-phase BSAs and the significant unknowns regarding their effectiveness, this project will focus on effective gas-phase addition of nutrients, co-metabolites, oxygen, and moisture.

Objectives

The objectives of this work are to:

- Evaluate the effectiveness of field systems for gas-phase delivery of BSAs to the unsaturated zone for enhancing in situ bioremediation performance. These BSAs include nutrients (organophosphates), co-metabolites, surfactants or solvents, and water vapor.
- Identify and measure the physical and microbial factors affecting the bioavailability of gas-phase BSAs in the unsaturated zone, including uneven spatial distribution of BSAs at the pore- and core-scales and complex changes in unsaturated zone air permeability caused by microbial-growth dynamics.

- Develop visible light tomography (VLT) systems that allow visualization of in situ unsaturated zone physical and microbial processes for controlled evaluation of alternative BSAs and delivery systems.
- Use the VLT systems to evaluate alternative BSAs for remediation of aged contaminated soils in controlled but realistic environments.

Accomplishments

We have designed and constructed two different laboratory systems for observing dynamic fluid distribution in the unsaturated zone under simulated BSA delivery. A three-dimensional column system has been designed to collect data on fluid migration through discrete fingers in disturbed and undisturbed soil cores, and will be used to measure the limitations on BSA delivery caused by fingering under a variety of soil conditions and fluid application rates. This work will be completed by December 1995. The columns are 30 cm in diameter and comprised of stackable 10-cm sections separated by 1-mm spacers. The columns rest on a base that allows manipulation of the bottom pressure boundary condition, and the side boundary condition is manipulated through 1-mm gaps between rings. Water is applied uniformly to the top surface of the soil columns via a carefully designed air-atomizing nozzle. After the fluid flow is developed, a dye mixture marks the locations of any preferential flow pathways. The pathways will be exposed at the surfaces of each 10-cm ring, and the complete three-dimensional character of each pathway will be recorded. Different color dyes will be used to investigate the persistence of individual fingers when fluid application is cycled, allowing the soil to drain to varying water contents between application.

A two-dimensional, vertical, thin-slab visible light tomography (VLT) system has been designed to visualize and measure the interactions between gas-phase BSA and liquid-phase flow in a controlled environment. The system will also serve as a bioremediation simulator to measure the effectiveness of various gas-phase BSAs, and to visualize dynamic microbial-growth processes under simulated in situ bioremediation conditions (with and without BSA addition). The vertically oriented chamber dimensions are 1 m x 2 m x 1 cm. The top boundary will be either open or closed to the atmosphere, and will be capable of having controlled amounts of liquid added uniformly over the slab length. Side boundaries will be either closed or open to the atmosphere, thereby providing the ability to control gas-phase BSA injection or extraction (simulating the operation of BSA injection or extraction wells). The bottom boundary will be either open to the atmosphere or, via a manifold, will simulate water table conditions.

The advantage of the thin-slab system is the ability to visualize the complex flow and microbial processes occurring in the unsaturated zone under simulated in situ conditions. A bank of high frequency fluorescent lamps will illuminate the system from the back. Because light transmission is related to water saturation, the water distribution can be easily visualized without the use of dyes. Fluorescent gases will be investigated for visualization of the gas movement, as will color-marked gas- and liquid-phase pH indicator solutions. Data will be recorded via a CCD camera and a data acquisition system so that actual fluid flow and microbial processes can be recorded and visualized. The CCD camera system will collect data at a spatial resolution on the order of the pore scale (approximately 0.5 to 1.0 mm).

Section 5
Process Research

Process research involves isolating and identifying microorganisms that carry out biodegradation processes and the environmental factors affecting these processes. It also deals with the development of techniques for modeling and monitoring biodegradation. Through this research, scientists establish the building blocks of new biosystems for treatment of pollutants in surface waters, sediments, soils, and subsurface materials. Thorough evaluation is critical at this level of research because a firm scientific foundation can facilitate the scaling up of a promising technology. Process research is being conducted on many environmental pollutants.

Two research projects quantified the extent of biodegradation of organic compounds using carbon isotopes. The methods employed in this research can be used both in the laboratory, where target compounds are isotopically labeled, and in the field, where contaminants contain different concentrations of isotopes than the surrounding environment. The methods were applied in the measurement of petroleum degradation and the demethylation of organometallic compounds.

Another project determined the kinetic rate constants of anaerobic degradation of quinoline by methanogenic bacteria. This system is potentially of great practical significance because quinoline is found in wastes at wood preserving plants.

Several papers focused on the use of microorganisms to degrade alkyl halides and polychlorinated aromatics. Research included the characterization of a bacterial enzyme (toluene 2-monooxygenase) that can degrade trichloroethylene and of a strain of *Pseudomonas cepacia G4* that expresses the enzyme constitutively. Further research covered the effects of varying environmental conditions on the bioremediation of chlorinated compounds.

Other process research projects studied heavy metal inhibition of the bioremediation of polychlorinated aromatics and the effects of different primary substrates on the reduction of 2,4-dinitrotoluene. Primary substrates are used to sustain the growth of microorganisms when the target contaminant cannot act as a food source.

Several poster presentations at the symposium involved process research. The presentations covered methods for monitoring bioremediation, the use of surfactants in sediments, and characterization of an anaerobic dehalogenating microorganism. Another presentation dealt with the potential of *Mycobacterium* to mineralize polycyclic aromatic hydrocarbons.

Monitoring Crude Oil Mineralization in Salt Marshes: Use of Stable Carbon Isotope Ratios

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Introduction

The ability to monitor mineralization of hydrocarbons is of prime importance in a successful remediation strategy. Hydrocarbon mineralization must be ensured, because hydrocarbons can be sorbed, transformed, or buried, or otherwise be undetected but still pose threats to the existing system ecology. One successful technique has been monitoring changes in oil composition relative to a stable, nondegradable compound (1, 2). Two disadvantages to this method exist, however: 1) its inability to demonstrate mineralization instead of transformation, and 2) its inability to measure absolute oil degradation, because only "resolved" compounds are quantified.

A promising new technique for the detection and quantification of hydrocarbon mineralization is the use of stable carbon isotope ratios (3). Carbon dioxide gas ratios vary from of ^{12}C to ^{13}C depending on the source of the gas. Crude oils are more depleted in ^{13}C, and thus the mineralization of oil produces CO_2 with lower δ-^{13}C values. Oil has a δ-^{13}C value of -29 to -32 (0/00) depending on the source of the oil. Salt marshes are predominantly colonized by C3 plants, and CO_2 evolved from these soils has δ-^{13}C ratios of -14.4 to -17.7 (0/00) (4). If biodegradation is occurring in a contaminated salt marsh, the δ-^{13}C value of the produced CO_2 should decrease due to the presence of ^{13}C-depleted CO_2 from the crude oil. If this occurs, it would be possible to qualify and quantify hydrocarbon degradation by measuring total CO_2 production and changes in the ^{13}C signature of CO_2 produced from the marsh.

Theoretical

The rate of CO_2 produced from each carbon source can be easily computed using three equations describing CO_2 production and the δ-^{13}C signature:

$$R_o + R_i = R_t \qquad \text{(Eq. 1)}$$

$$\frac{R_o}{R_t} + \frac{R_i}{R_t} = 1 \qquad \text{(Eq. 2)}$$

$$\frac{R_o}{R_t}(S_o) + \frac{R_i}{R_t}(S_i) = S_t \qquad \text{(Eq. 3)}$$

where R_o and R_i are the rates of CO_2 production from the crude oil and indigenous carbon sources respectively, and R_t is the total rate of CO_2 production. S_o and S_i are the δ-^{13}C signatures of the crude oil and indigenous carbon, and S_t is the measured δ-^{13}C signature of the produced CO_2. S_o,S_i, S_t, and R_t are experimentally determined. R_o and R_m can then be determined from Equations 2 and 3. This assumes that CO_2 is generated from only these two carbon pools and that there is no addition of atmospheric CO_2.

Results and Discussion

Kinetic Experiments

Microcosm studies showed rapid and nearly complete (greater than 90 percent reduction in the hopane ratio) degradation of parent alkanes in the fertilized treatments under completely mixed, aerated conditions. In the unfertilized treatment, less than a 10 percent reduction was observed in the hopane ratio of the alkanes (Figure 1A). Polycyclic aromatic hydrocarbon (PAH) degradation

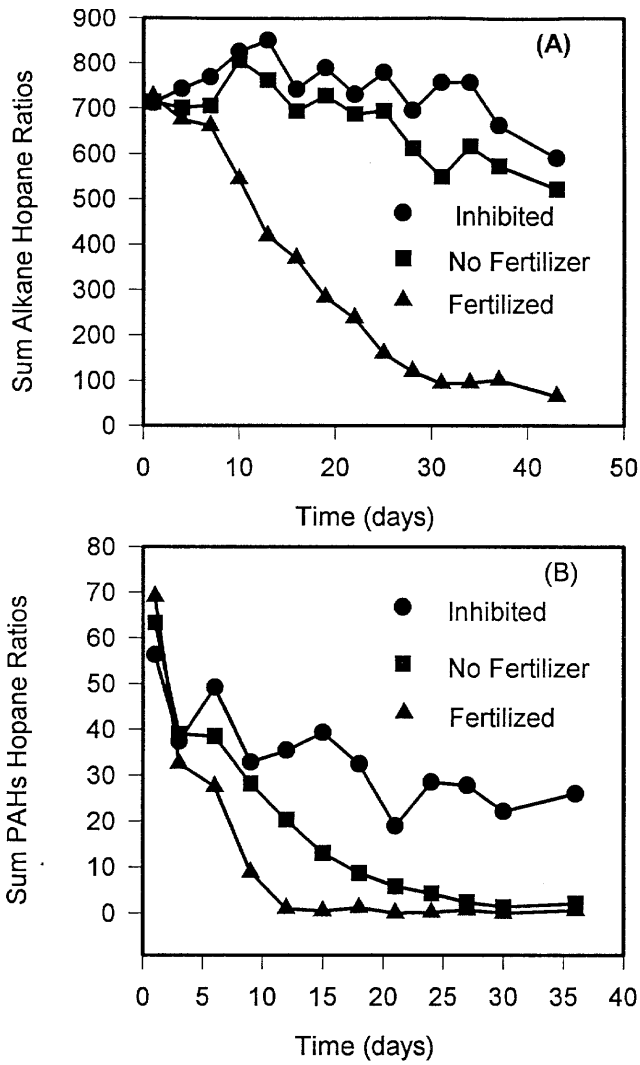

Figure 1. (A) Total (C15-C44) alkane-hopane ratio versus time in aerated microcosms. (B) Total PAH (phenanthrene, C1, C2; naphthalene, C1, C2) hopane ration versus time.

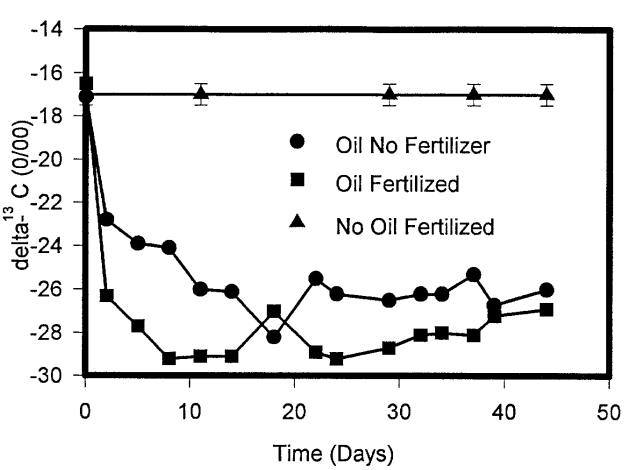

Figure 2. Delta-^{13}C ratios versus time in aerated microcosms.

was evident in both unfertilized and fertilized treatments (Figure 1B).

A definite decrease in the δ-^{13}C signature of evolved CO_2 was observed in both treatments contaminated with oil (Figure 2). The measured δ-^{13}C signature of CO_2 evolved from noncontaminated marsh soil is $17.1^{0/00} \pm$ 0.2. The fertilized treatment approaches the δ-^{13}C ratio of pure oil ($29.1^{0/00}$) and varies between -27 and $-29^{0/00}$. The nonfertilized treatment varies between -25 and -270/00.

CO_2 production was correspondingly greater in the fertilized treatment. The average rate of total CO_2 production for the nonoiled-fertilized, oiled-unfertilized, and oiled-fertilized microcosms was 0.174, 0.396, 1.86 mg CO_2-C/day-gram soil, respectively. The large increase in CO_2 production between the oiled and the nonoiled treatments suggests that the increase in CO_2 production is from the mineralization of the crude oil and not from indigenous carbon sources.

The amount of CO_2-C produced from crude oil can be computed, using the δ-^{13}C signatures, CO_2 production data, and the isotope dilution equations. The predictive ability of these equations is supported by the similarity between the pseudo first-order rate constants in the reduction of hopane ratios and the calculated CO_2 production from the crude oil carbon pool, 0.082 and 0.087 day^{-1}, respectively.

Field Experiment

A small-scale field experiment was conducted to verify the ability to detect δ-^{13}C signature changes in CO_2 evolved in situ from oil-contaminated soils. Significant decreases in the δ-^{13}C signature of evolved CO_2 was detected 5 weeks after oiling in fertilized and unfertilized treatments. The alkane- and PAH-hopane ratios decreased for all treatments. The C1 and C2-phenan-

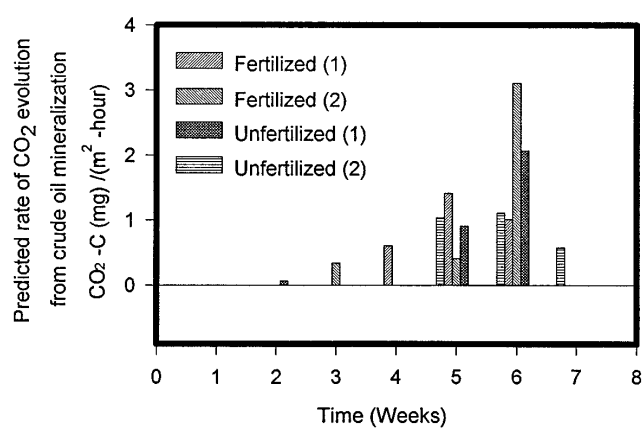

Figure 3. Rates of CO^2-C mineralized from crude oil in fertilized and unfertilized salt marsh soils (calculated from isotope dilution equations).

threne, however, appear to be stable over the period of this experiment.

The CO_2 production rates and the δ-^{13}C ratios measured were used to calculate the CO_2 produced from crude oil (Figure 3). No mineralization of crude oil was detected until Week 2, and the majority of mineralization appears to begin at Week 5. The fertilized treatments appeared to show higher mineralization rates before the unfertilized and to mineralize at a more even prolonged rate. The unfertilized treatments have a more intense rate of mineralization but for only one sampling date. Amendments of fertilizer inconclusively increased degradation, as evidenced by hopane ratios of specific oil components.

The importance of the δ-^{13}C data is the data's ability to calculate mineralization rates directly. They measure the final product, while monitoring hopane ratios only measures the disappearance of the parent compound, not mineralization. These experiments support the ability to use δ-^{13}C ratios in conjunction with CO_2 production to qualitatively and quantitatively monitor crude oil degradation.

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Mercury and Arsenic Biotransformation

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This presentation will cover our recent findings with regard to bacterial processes affecting 1) methylmercury demethylation and 2) the dissimilatory reduction of arsenic (As) (V).

Methylmercury Oxidative Degradation Potentials in Contaminated and Pristine Sediments of the Carson River, Nevada

Sediments from mercury-contaminated and uncontaminated reaches of the Carson River, Nevada, were assayed for sulfate-reduction, methanogenesis, denitrification, and monomethylmercury (MeHg) degradation. Demethylation of ^{14}C-MeHg was detected at all sites, as indicated by the formation of $^{14}CO_2$ and $^{14}CH_4$. Oxidative demethylation was indicated by the formation of $^{14}CO_2$ and was present at significant levels in all samples. Oxidized/Reduced demethylation product (ORDP) ratios (e.g., $^{14}CO_2/^{14}CH_4$) generally ranged from 4.0 in surface layers to as low as 0.5 at depth. Production of $^{14}CO_2$ was most pronounced at sediments surfaces that were zones of active denitrification and sulfate-reduction, but was also significant within zones of methanogenesis. In a core taken from an uncontaminated site having more oxidized, coarse-grained sediments, sulfate-reduction and methanogenic activities were very low, and $^{14}CO_2$ accounted for 98 percent of the product formed from ^{14}C-MeHg. No relationship was apparent between the degree of mercury contamination of the sediments and the occurrence of oxidative demethylation. Sediments from Fort Churchill, the most contaminated site, however, were most active in terms of demethylation potentials. Inhibition of sulfate reduction with molybdate resulted in significantly depressed ORDP ratios, but overall demethylation rates were comparable between inhibited and uninhibited samples. Addition of sulfate to sediment slurries stimulated production of $^{14}CO_2$ from ^{14}C-MeHg, while 2-bromoethane- sulfonic acid blocked production of $^{14}CH_4$. These results reveal the importance of sulfate-reducing and methanogenic bacteria in oxidative demethylation of MeHg in anoxic environments.

The Dissimilatory Reduction of As(V) to As(III) in Anoxic Sediments and as an Electron Acceptor for Growth of Strain SES-3

Anoxic sediment slurries amended with millimolar levels of As(V) achieved a complete reduction of this oxyanion to As(III) upon incubation. As reduction was enhanced when slurries were provided with the electron donors H_2, lactate, or glucose, although no effect was achieved with acetate or succinate. Aerobically incubated slurries did not reduce As(V), nor did formalin-killed or autoclaved controls. Even though acetate did not stimulate As reduction, the oxidation of 2-^{14}C-acetate to $^{14}CO_2$ in anoxic slurries could be coupled with the abundance of As(V). The selenium (Se) (VI) respiring anaerobe strain SES-3 was found to be capable of achieving growth by carrying out the dissimilatory reduction of As(V) to As(III). Although growth parameters were meager (e.g., Y_m = 0.53 g cells/mole lactate; maximal cell density = 9.2 x 10^7 cells/mL), the ability to reduce As(V) to As(III) was constitutive and occurred rapidly in either selenate- or nitrate-grown cells. These results suggest that the reduction of As(V) to As(III) in nature may be achieved by bacteria-like strain SES-3 carrying out dissimilatory As(V) reduction.

Monod Degradation Kinetics of Quinoline in Natural and Microbially Enriched Methanogenic Microcosms

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Introduction

The methanogenic biodegradation of nitrogen-containing heterocyclic compounds found in wastes from petroleum refineries, coke operations, coal tar production, and wood preservation has not been studied in detail. Quinoline, the largest single component in creosote (1), is first oxidized to 2(1H)-quinolinone, which is then degraded to CH_4 and CO_2. In this study, the Monod no-growth kinetic constants for the oxidation of quinoline and the Monod kinetic constants for the methanogenesis of 2(1H)-quinolinone were determined under natural and microbially enriched methanogenic conditions using nonlinear regression analysis (2). In microcosms simulating natural aquifer conditions, it was necessary to model the oxidation and subsequent methanogenesis independently (1). In microbially enriched microcosms, the two must be coupled to include the biomass increase from the methanogenesis of 2(1H)-quinolinone as below:

$$\frac{dQ}{dt} = -\frac{k_n X_a Q}{K_n + Q}$$

$$\frac{dQ_n}{dt} = \frac{k_n X_a Q}{K_n + Q} - \frac{\mu_{max} X_a Q_n}{Y(K_s + Q_n)}$$

$$\frac{dX}{dt} = \frac{\mu_{max} X_a Q_n}{K_s + Q_n}$$

where

Q = quinoline, $mg \cdot L^{-1}$
k_n = no-growth oxidation constant, day^{-1}
X_a = active biomass, $mg \cdot L^{-1}$
K_n = one-half saturation no-growth coefficient, $mg \cdot L^{-1}$
μ_{max} = maximum specific growth rate, $mg \cdot L^{-1} \cdot day^{-1}$
Q_n = 2(1H)-quinolinone, $mg \cdot L^{-1}$
K_s = one-half saturation coefficient, $mg \cdot L^{-1}$
Y = growth yield, mg biomass·mg substrate^{-1}

Materials and Methods

The study site is located adjacent to an abandoned wood preserving plant within the city limits of Pensacola, Florida (1). The wood preserving process consisted of steam pressure treatment of pine poles with creosote and/or pentachlorophenol. For more than 80 years, large but unknown quantities of waste waters (consisting of extracted moisture from the poles, cellular debris, creosote, pentachlorophenol, and diesel fuel from the treatment processes) were discharged to nearby surface impoundments. These impoundments were unlined and in direct hydraulic contact with the underlying sand-and-gravel aquifer. Contamination of the ground water resulted from the accretion of wastes from these impoundments. Methanogenesis in the aquifer was simulated using microcosms containing approximately 3 kg of freshly collected anaerobic aquifer material in a 4-L glass serum bottle with 2.5 L of prereduced anaerobically sterilized mineral salts solution. Approximately 40 mg/L of quinoline was added, simulating a concentration similar to that found in the aquifer (1). The microcosms were prepared, incubated, and sampled in an anaerobic glove box containing an O_2-free atmosphere maintained at 22°C to 24°C. Microbially enriched microcosms were prepared as above but were batch fed for three cycles by removing 50 percent of the liquid volume and replacing that volume with fresh mineral salts containing enough quinoline to bring the final concentration back to 40 mg/L. After the last feeding cycle, the liquid culture was removed from the sediment, resulting in liquid-only culture which was batch fed for six more cycles.

Substrate concentrations were determined at approximately 4-day intervals by high-performance liquid chromatography. Total biomass concentrations were determined at approximately 20-day intervals by total protein using the Coomassie brilliant blue staining procedure of Gälli (3).

Results and Conclusions

The oxidation of quinoline, a reaction that is endergonic (4), is modeled using derived Monod no-growth kinetic constants. This oxidation is uncoupled with the degradation of 2(1H)-quinolinone in microcosms simulating natural conditions, as shown by the complete and stoichiometric oxidation to 2(1H)-quinolinone before the onset of methanogenesis. In microbially enriched microcosms, however, the oxidation of quinoline is linked to the degradation of 2(1H)-quinolinone; the increase in biomass from methanogenesis must be included in the equations describing the oxidation of quinoline (Figure 1). The kinetic values derived from the microbially enriched, all-liquid microcosm experiments were not significantly different from those values from sand-filled natural microcosms (Table 1). The Monod kinetic constants for both the oxidation and subsequent methanogenesis are representative of values describing substrate utilization in an oligotrophic and somewhat hostile environment (4).

It is still unclear, however, what number of microbial populations are involved and to what extent each of the populations influences the steps in the biodegradation of quinoline. This uncertainty can be seen by the high concentration of biomass capable of the oxidation of quinoline in natural microcosms, suggesting that the ability to oxidize quinoline is not unique to just this consortium but may be common to many of the individual members of the creosote-degrading consortia. The enrichment procedure has altered the microbial population of the natural microcosms by potentially removing all of the microorganisms that can oxidize quinoline but are not directly involved in the methanogenesis of 2(1H)-quinolinone. These results suggest that as long as the culture is derived from the contaminated aquifer, enrichment does not alter the kinetics of quinoline oxidation and subsequent methanogenesis of 2(1H)-quinolinone. The size of the various active microbial populations, however, must be known before fate-and-transport modeling can be attempted.

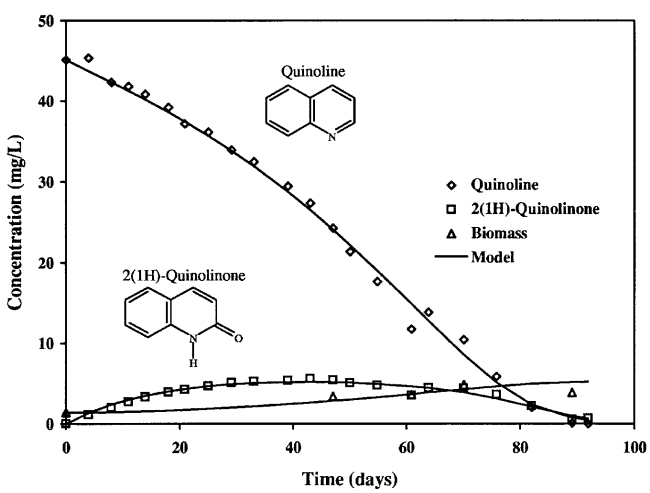

Figure 1. Quinoline oxidation and 2(1H)-quinolinone methanogenesis in microbially enriched laboratory microcosms.

Table 1. Monod Kinetic Constants ±95 Percent Confidence Interval for Parameters Determined by Nonlinear Regression for Both Natural and Microbially Enriched Microcosms

Kinetic Constant	Natural Microcosms	Microbially Enriched Microcosms
k_n, day^{-1}	0.31 ± 0.06	0.29 ± 0.02
K_n, mg·L^{-1}	2.0 ± 1.4	7.58 ± 4.0
μ_{max}, day^{-1}	0.09 ± 0.06	0.14 ± 0.07
K_s, mg·L^{-1}	11.4 ± 0.6	33.1 ± 11.5
Y, mg·mg^{-1}	0.03	0.07
Starting biomass, mg·L^{-1} Oxidation	17.3	1.39
Starting biomass, mg·L^{-1} Methanogenesis	0.003	1.39

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Stimulating the Biotransformation of Polychlorinated Biphenyls

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Introduction

The discovery that polychlorinated biphenyls (PCBs) can be reductively dechlorinated by microorganisms under anaerobic conditions has stimulated interest in the development of a sequential anaerobic/aerobic biotreatment process for their destruction. While the aerobic degradation of PCBs is generally limited to congeners with four or fewer chlorines, the anaerobic process can dechlorinate more highly substituted congeners, producing products that are aerobically degradable. Indeed, all products from the anaerobic dechlorination of Aroclor 1254 (1) have been shown to be aerobically degradable by one or more strains of aerobic bacteria (2). Also, the high proportion of monochlorinated biphenyls that can accumulate as a result of anaerobic PCB dechlorination may serve to induce PCB-degrading enzymes in aerobic microorganisms (3). More highly chlorinated congeners can be aerobically cometabolized but are not inducing substrates (4).

A greater understanding of the factors controlling the anaerobic dechlorination of PCBs is necessary before a successful sequential anaerobic/aerobic biotreatment process can be developed for PCBs. In particular, it is important to determine how to stimulate more rapid and complete dechlorination in areas where the natural rate and/or extent of dechlorination is limited. The general approach we have taken is to identify the most probable site-specific factors limiting in situ PCB dechlorination, then to apply treatments to alleviate the limitation(s). During the past year of this project, we have focused on enhancing the dechlorination of PCBs present in River Raisin (Michigan) and Silver Lake (Massachusetts) sediments.

River Raisin Sediment Experiment

In a previous project, we found that little in situ dechlorination of the PCBs present in River Raisin sediment collected near Monroe, Michigan, had occurred. PCB-dechlorinating microorganisms were found to exist in the sediment, however. The sediment supported dechlorination in laboratory assays when spiked with additional PCBs and inoculated with PCB-dechlorinating microorganisms (meaning inhibitory compounds were not present), and the PCBs already present in the sediments were bioavailable because they were dechlorinated under the conditions of our treatability assay. In fact, individual congeners in the contaminated sediment decreased 30 to 70 percent in 24 weeks at rates nearly identical to rates for the same congeners freshly spiked into noncontaminated sediments.

The treatability assays were conducted using air-dried River Raisin sediments. The sediments were slurried with an equal weight of non-PCB-contaminated sediments and reduced anaerobic mineral medium (RAMM). The slurry was then inoculated with microorganisms eluted from Hudson River sediment to ensure that PCB-dechlorinating microorganisms were present, and with 2',3,4-trichlorobiphenyl (34-2-CB) in a small volume of acetone. The 34-2-CB was added because the addition of a single PCB congener (or other halogenated aromatic compound) can sometimes "prime" the dechlorination of PCBs already present in a contaminated sediment (5). Non-PCB-contaminated Red Cedar River sediments were added to provide a source of unidentified nutrients. The RAMM included essential mineral salts and a chemical reductant (Na_2S) to lower the initial redox potential. We have conducted separate experiments with slurries made from both air-dried and always wet River Raisin sediments to help determine what aspect of our treatability assay fosters the dechlorination of the PCBs present in these sediments.

Materials and Methods

Air-Dried River Raisin Sediments

With slurries made from the air-dried sediments, the factors considered were 1) addition of 34-2-CB, 2) addition of the mineral salts in RAMM, 3) addition of Na_2S, and 4) addition of the non-PCB-contaminated sediment.

The River Raisin sediment was added to Balch tubes (1 g per tube). An additional 1 g of non-PCB-contaminated Red Cedar River sediment was added to the appropriate treatments. Inocula for each treatment were prepared by eluting PCB-dechlorinating microorganisms from Hudson River sediments with a medium appropriate to the treatment (i.e., with or without mineral salts, with or without reductant), and 7 mL of an inoculum was added to each tube using an anaerobic technique. 34-2-CB in a small volume of acetone was added to one treatment, while the rest received the same volume of acetone. The tubes were sealed with Teflon-lined rubber stoppers and aluminum crimps. Autoclaved treatments served as controls. A tube was sacrificed for each sample. Triplicate samples were taken at 8-week intervals, extracted, and analyzed for PCBs using capillary gas chromatography with electron capture detection.

Wet River Raisin Sediments

The same four factors described above plus the necessity of inoculating with Hudson River microorganisms were considered in an experiment with River Raisin sediments that had been kept wet since the time of collection. Portions of the sediment were mixed with the appropriate medium (i.e., with or without salts, reductant, or inoculum) in a small Erlenmeyer flask on a magnetic stirrer in an anaerobic chamber. Red Cedar River sediments, acetone with or without 34-2-CB, and PCB-dechlorinating microorganisms eluted from Hudson River sediments were also added as appropriate to each treatment. Portions (7 mL) of the slurries were then dispensed to Balch tubes, and the tubes were sealed with Teflon-lined rubber stoppers and aluminum crimps. The sampling and analytical procedure was the same as the experiment with slurries made from air-dried sediment.

Results and Discussion

Decreases in the concentrations of certain PCB congeners in the live samples relative to the autoclaved controls were used to compare the effectiveness of the various treatments. These congeners (245-25-CB and 235-24-CB in chromatographic peak 42, and 34-34-CB and 236-34-CB in peak 49) were chosen because each peak represents more than 2 mole percent of the congeners initially present, and because they could not have been formed in significant quantities from the dechlorination of other PCBs present.

In the experiment with slurries made from the air-dried sediment, approximately 50 percent of the congeners present in each indicator peak were dechlorinated in the treatment receiving 34-2-CB (Figure 1). No dechlorination was apparent in any of the other treatments.

In the experiment with slurries made from wet sediments, no inoculation was required for PCB dechlorination, and some dechlorination occurred in all treatments. Thus, merely making a slurry from the River Raisin sediments appears to have stimulated some dechlorination of the PCBs present in them. The most extensive dechlorination, however, occurred in the treatment receiving 34-2-CB, showing that dechlorination could be enhanced by “priming.” Somewhat surprisingly, the addition of Red Cedar River sediments inhibited dechlorination (Figure 2); perhaps the additional organic matter served as a sorptive sink for some of the PCBs.

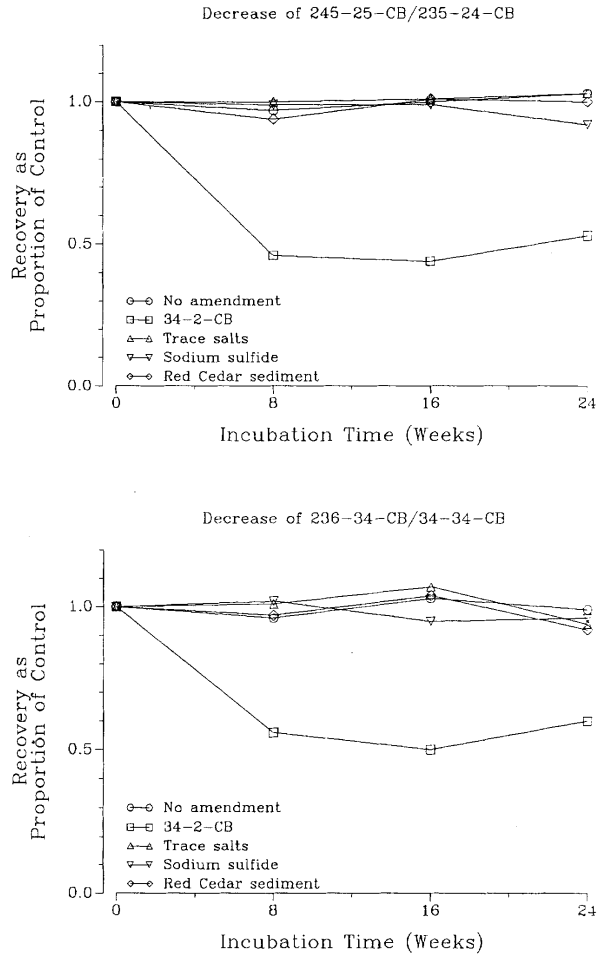

Figure 1. Decrease of indicator PCB congeners due to dechlorination in slurries prepared from air-dried River Raisin sediments.

Silver Lake Sediment Experiments

Although there is evidence that the PCBs present in Silver Lake sediments have undergone in situ dechlorination, these sediments do not support PCB dechlorination in laboratory experiments. These sediments have high concentrations of several metals, especially zinc, copper, lead, and chromium. We suspect that the metals are present mainly in a reduced state in situ and become partially oxidized and therefore more toxic to dechlorinating microorganisms during the sediment handling

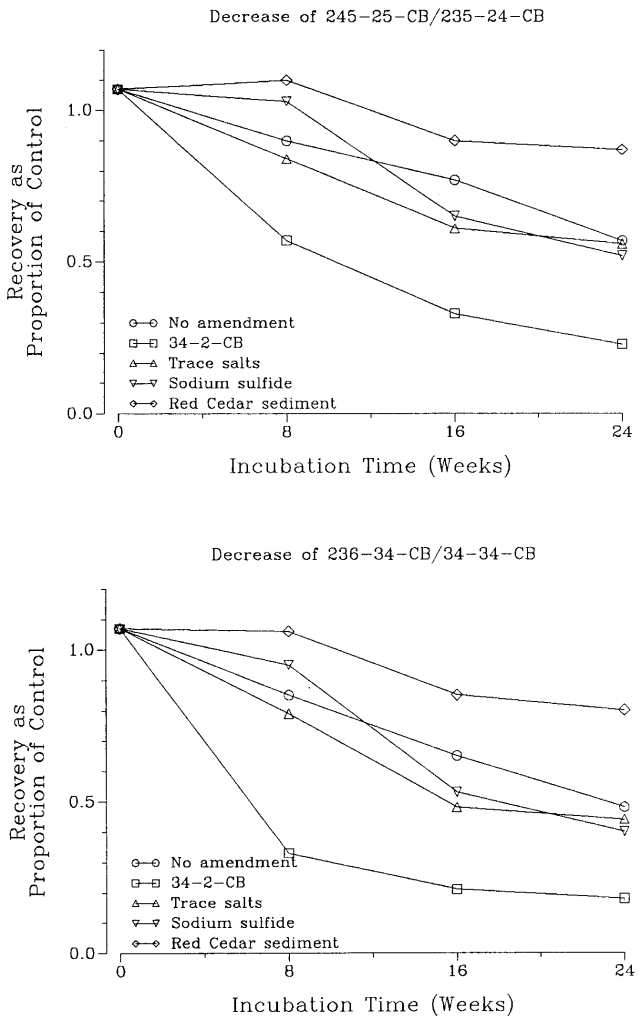

Figure 2. Decrease of indicator PCB congeners due to dechlorination in slurries prepared from wet River Raisin sediments.

required to set up dechlorination experiments. We also have previously shown that high concentrations of zinc can inhibit PCB dechlorination even after highly reduced conditions are restored. The experiments reported here were designed to stimulate dechlorination by reducing the bioavailability of toxic metals through chelation or precipitation in both a model system and in Silver Lake sediments.

Materials and Methods

General Procedure

Anaerobic sediment slurries containing PCBs were inoculated with a PCB-dechlorinating microbial consortium eluted from PCB-contaminated Hudson River sediments. Treatments consisted of the addition of metal salts and/or amendments to precipitate or chelate metals. Autoclaved slurries served as negative or sterile controls, while untreated slurries served as positive controls. Triplicate samples were sacrificed at 4-week intervals, solvent extracted, and analyzed for PCBs using capillary gas chromatography with electron capture detection. The course of PCB dechlorination was followed by calculating the average *meta* plus *para* chlorines for each treatment versus incubation time. No dechlorination from the *ortho* positions was evident. Dechlorination patterns were evaluated by assessing changes in specific congener concentrations over time.

Model System

Anaerobic slurries of non-PCB-contaminated Hudson River sediment were spiked with Aroclor 1242 (500 µg/g sediment) and inoculated with PCB-dechlorinating microorganisms eluted from PCB-contaminated Hudson River sediments. We consistently observed dechlorination of the Aroclor 1242 in such preparations. Zinc (Zn) or Lead (Pb) (as chloride salts) was added at solution concentrations of 500 µg/mL to induce metal toxicity. Amendments of $FeSO_4$, ethylene diamine triacetic acid (EDTA), and citrate were added individually to samples before incubation to test their effectiveness in alleviating the toxicity of Zn and Pb.

Silver Lake Sediment Slurries

Anaerobic slurries of Silver Lake sediments were spiked with 34-2-CB and inoculated with Hudson River microorganisms. The 34-2-CB was added so that we could monitor the dechlorination of a freshly added PCB congener in addition to the PCBs already present in the sediment. Experimental treatments consisted of the addition of $FeSO_4$, EDTA, and citrate, as in the model system described above.

Results and Discussion

In the model system, ZnCl prevented Aroclor 1242 dechlorination while PbCl decreased the extent of dechlorination. EDTA, citrate, and $FeSO_4$ amendments all reversed the inhibitory effect of PbCl while EDTA and $FeSO_4$ eliminated the inhibition by ZnCl. In fact, $FeSO_4$-amended treatments exhibited more extensive dechlorination than the unamended positive controls (i.e., those without PbCl or ZnCl additions). Apparently, the $FeSO_4$ greatly stimulated dechlorination from *para* positions. In all non-$FeSO_4$-amended slurries exhibiting dechlorination, dechlorination occurred primarily from the *meta* positions to yield *ortho* and *para* substituted products (pattern M). But in $FeSO_4$-amended treatments, the major products were 2-CB, 2-2-CB, and 26-CB, indicating that dechlorination occurred from both *meta* and *para* positions (pattern C). We have often noted that the *para* dechlorination activity present in Hudson River sediments is lost during storage of the sediments. It appears that addition of $FeSO_4$ somehow “rescues” this dechlorination activity.

In the Silver Lake sediment slurries, the added 34-2-CB was dechlorinated in citrate- and $FeSO_4$-amended slurries, but not in EDTA-amended slurries. There was no indication of further dechlorination of the PCBs already present in the sediments.

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Bioaugmentation for In Situ Co-metabolic Biodegradation of Trichloroethylene in Ground Water

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Introduction

Trichloroethylene (TCE), a common ground-water contaminant, has been found to be fortuitously degraded (co-metabolized) by organisms grown on a variety of substrates (1, 2). Addition of such substrates can lead to two significant problems in ground-water aquifers. First, organisms stimulated by substrate addition may be unable to degrade TCE. Second, the most promising compounds for inducing TCE degradation, phenol and toluene, are themselves hazardous substances and therefore cause regulatory concern. To address the first issue, aquifers may be bioaugmented with wild-type strains known to be effective at TCE degradation. Perhaps ideally, both problems can be overcome through the use of mutant strains known both to degrade TCE efficiently and to grow on a nontoxic substrate. Laboratory studies were conducted to investigate these two alternatives.

Laboratory Studies

Bacterial Cultures

The wild-type strain evaluated was *Pseudomonas cepacia* G4 (G4), a strain isolated from a holding pond at an industrial waste treatment facility in Pensacola, Florida (2). This organism co-metabolizes TCE using toluene ortho-monooxygenase (TOM), which is induced by phenol or toluene (3). The mutant used was *P. cepacia* G4 $PR1_{301}$ (PR1), a chemically induced mutant of G4 that constitutively expresses TOM while grown on substrates such as lactate. A more complete description of PR1 is presented at this meeting.

Soil Microcosms

Small-column microcosms (17 cm^3) were constructed using aquifer material from a test area at Moffett Federal Air Station. Column fluids were exchanged every 2 to 3 days by pumping 10 mL of solutions held in gas-tight glass barrel syringes through the column influent port with a syringe pump. At the start of each fluid exchange period, 1 mL of bacterial culture was added to the microcosms followed by 9 mL of oxygenated ground water containing about 200 µg/L TCE and/or primary substrates. Microcosm effluent samples were collected during each exchange for analysis.

Detection of Bacteria

A deoxyribonucleic acid (DNA) probe specific for both strains of G4 was constructed using polymerase chain reaction (PCR) to amplify segments of G4 DNA between repetitive extragenic palindromic (REP) sequences. The REP-PCR reaction can be performed directly on environmental samples and therefore does not require extraction of DNA before amplification. The method was tested using ground-water and sediment samples containing indigenous bacterial populations with and without added G4, with parallel plate counts on R2A agar.

Results

Soil Microcosms

The 6.5-mg/L, phenol-fed, nonbioaugmented column followed a pattern similar to that observed in the field (4) and consumed approximately 60 µg/L TCE relative to a nonfed control. Columns augmented with induced G4 without a primary substrate achieved similar levels of TCE degradation. With the addition of 15 mg/L lactate, degradation increased to 100 µg/L TCE in G4-amended columns, but no such increase occurred in a PR1-amended column. In these lactate-fed columns, the G4 was pregrown on phenol while the PR1 was pregrown on lactate. When columns amended with either G4 or PR1 were fed 6.5 mg/L phenol, 130 µg/L TCE was degraded. The results are summarized in Figure 1.

Detection of Bacteria

The REP-PCR probe was able to detect 10 colony forming units (CFUs) of G4 against a background of 10^5 nontarget CFUs contained in 1 µL of template (Figure 2). The probe's sensitivity compares favorably to other PCR-based detection methods (5).

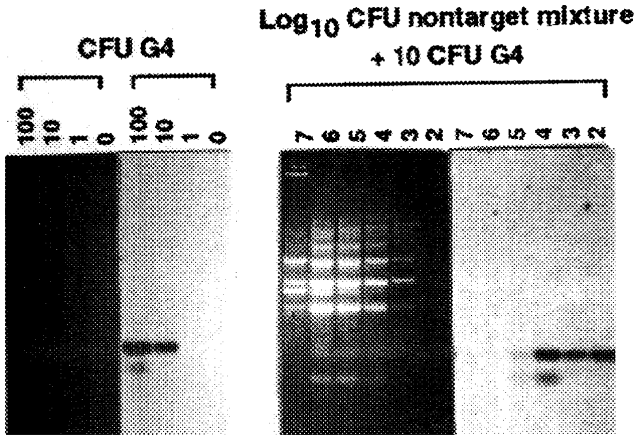

Figure 2. Sensitivity of G4 REP-PCR products using the strain G4 GF13 probe; agarose gel electrophoresis of REP-PCR reactions and hybridization of the Southern blot to GF13.

Application of REP-PCR to aqueous effluent samples from the soil microcosms produced mixed results (Figure 3). G4 was not detected in the control or phenol-only microcosms and gave a strong signal in phenol- and lactate-fed microcosms augmented with G4, as antici-

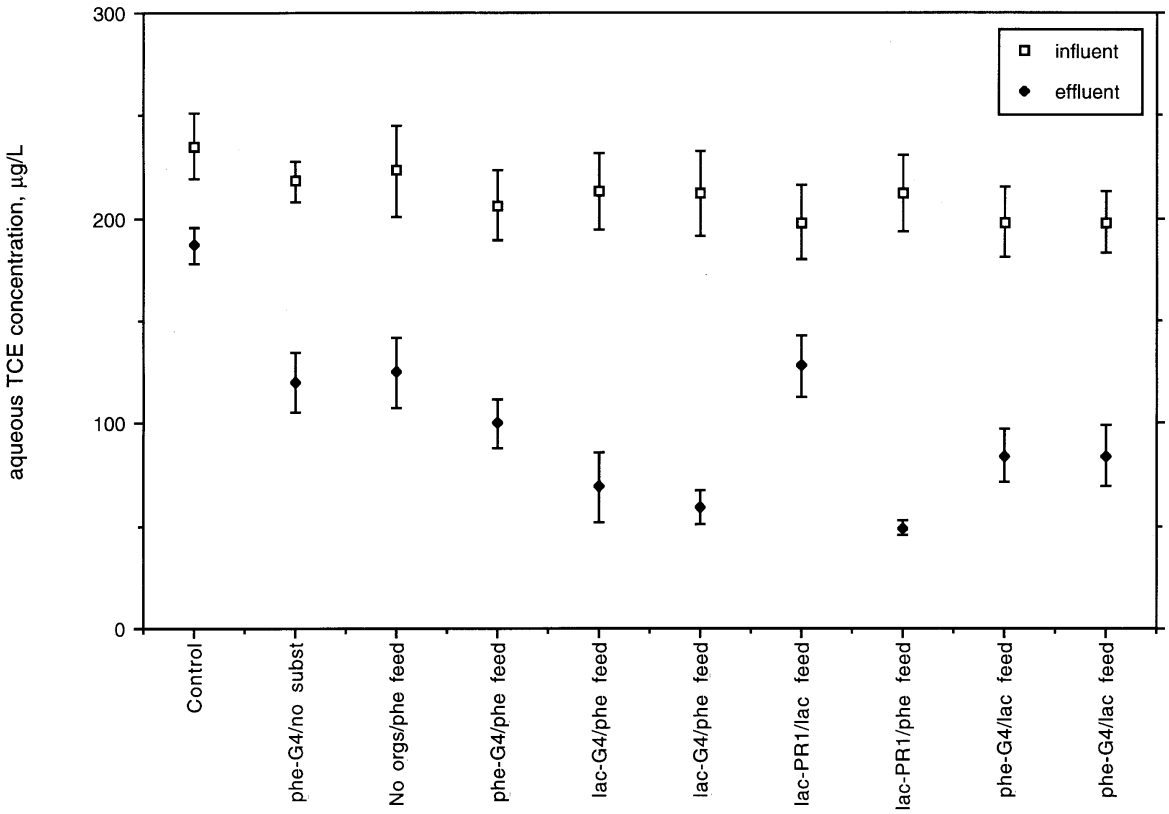

Figure 1. Average TCE levels during column incubation for the period between Days 37 and 56 for various column treatments. (Bars indicate 95-percent confidence intervals.)

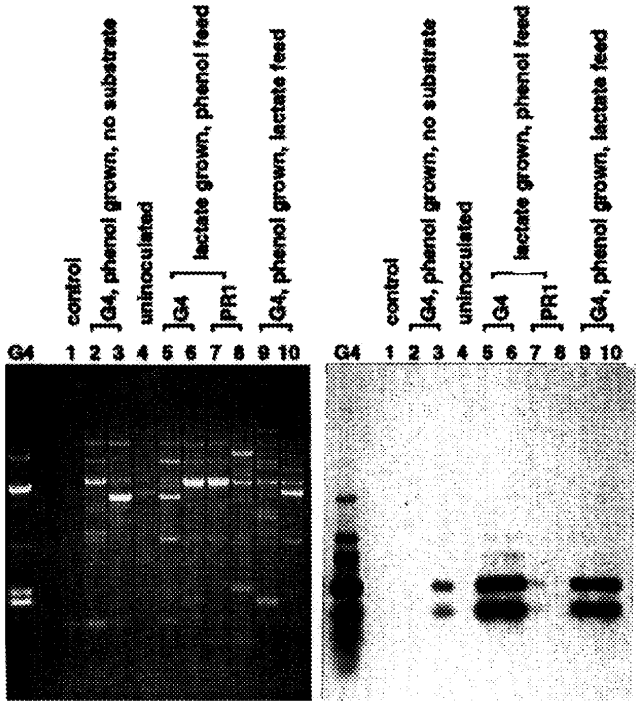

Figure 3. Detection of G4 in column effluents, ninth column exchange (Day 20); agarose gel electrophoresis of REP-PCR reactions of column effluents and probe GF13 hybridization of the Southern blot.

pated. In microcosms containing unfed G4 or phenol-fed PR1, however, responses were weak or absent. The reason for the latter results has not yet been determined.

Conclusion

Bioaugmentation with G4 or PR1 and phenol feed provides a means for enhancing native activity toward TCE. Addition of phenol to aquifers could be avoided by simply adding G4 previously induced for the TCE-degrading enzyme. Lactate enhanced activity of preinduced G4 toward TCE. In PR1-augmented systems, however, lactate did not support the same level of activity toward TCE as did phenol. G4 and PR1 were identified in constructed samples with a sensitivity of 1 in 10^4. Detection in aqueous column samples gave some unexpected results, the causes for which remain to be elucidated.

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Biodegradation of Chlorinated Solvents

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General Scope of Research

Research is being conducted on the bacterial metabolism of chlorinated aliphatic compounds, with a focus on oxidative mechanisms of biodegradation. *Pseudomonas cepacia* G4 oxidizes trichloroethylene (TCE) and related chlorinated alkenes with relatively little loss of activity over time (1), and the molecular basis of this observation is being elucidated. In vivo experiments are delineating the substrate range and concentration limits of *P. cepacia* G4 for chlorinated solvents. In vitro experiments are defining the properties of toluene 2-monooxygenase, the enzyme catalyzing the oxidation of TCE.

Purification and Properties of Toluene 2-Monooxygenase

Toluene 2-monooxygenase activity was monitored in vitro via a sensitive radiometric assay using [^{14}C]-toluene (2). Chromatography of cell-free extracts revealed that this was a three-component oxygenase system. All three components have now been purified to homogeneity. In vitro reconstitution of the three proteins and reduced nicotinamide adenine dinucleotide (NADH) yielded an active enzyme system that oxidizes toluene to *ortho*-cresol and this, subsequently, to 3-methylcatechol. One component is a flavoprotein containing a 2Fe2S cluster that accepts electrons from NADH (Table 1). A second component is a low molecular weight protein that stimulates activity but has no obvious redox-active functional group (Table 1). The largest component has an $\alpha_2\beta_2\gamma_2$ subunit structure (Table 1). This component is implicated as the hydroxylase component as it alone will oxidize toluene in the presence of dithionite + methyl viologen + O_2 or hydrogen peroxide. The hydroxylase component contains four to six iron atoms per holoenzyme. Spectroscopically, this component resembles the soluble methane monooxygenase hydroxylase component from *Methylosinus trichosporium* OB3b (3).

Table 1. Molecular Properties of Purified Components

Property	Hydroxylase	Small Component	Reductase
Subunit structure	$(\alpha\beta\gamma)_2$	Monomer	Monomer
Subunit molecular masses (kDa)	5.4, 37.7, 13.5	10.4	40.0
Molecular mass (kDa)			
Gel filtration	216	19.3	45.8
Native PAGE	190	—	—
SDS-PAGE	211	10.5	41.8
Calculated (aa quantitation)	210	10.4	40.0
Metal content			
Iron content (mol/mol)	5.3	ND	2.3
Inorganic S^- content (mol/mol)	ND	ND	2.9
FAD (mol/mol)	ND	ND	1.2
pI	4.5	4.3	5.8
Absorption maxima	282 nm	277 nm	270, 341, 423, 457 nm
Specific activity (units/mg)	1.7[a]	79.4[a]	512.0[b]
Percent recovery	40	27	30

[a] One unit is defined as 1 nmol [^{14}C]-toluene/min at 23°C.
[b] One unit is defined as 1 µmol cytochrome c reduced/min at 23°C.
ND = not detected.
— = not determined.
PAGE = polyacrylamide gel electrophoresis
SDS = sodium dodecylsulfate
FAD = flavin adenine dinucleotide
pI = isoelectric point

In Vivo Studies With *P. Cepacia* G4

P. cepacia G4 was shown to grow on aromatic ring compounds other than toluene and phenol. *P. cepacia* also oxidized non-growth-supporting aromatic and aliphatic substrates. Examples of the aromatic substrates that were investigated in some detail include naphthalene and indene. The oxidation of TCE by *P. cepacia* G4 has been studied in detail. The major oxidation product is glyoxylic acid. The effects of TCE on *P. cepacia* G4 also were studied to determine how resistant the organism is to variable concentrations of TCE. Unlike *Pseudomonas putida* F1, *P. cepacia* G4 was not detectably toxified by low concentration of TCE or by metabolites generated by oxidative mechanisms. High TCE concentrations, however, exerted a solvent effect that could markedly depress cell division rates and even cause cell death.

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Biological and Nutritional Factors Affecting Reductive Dechlorination of Chlorinated Organic Chemicals

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Introduction

Halogenated organic chemicals are of major public concern because these compounds are usually toxic and persistent in the environment, and they tend to accumulate in soils, sediments, and biota. Polychlorinated biphenyls (PCBs) and organochlorine pesticides are environmental pollutants of great concern due to a history of heavy use, toxicities, persistence in the environment, and wide distribution in environmental media.

PCBs are a mixture of chlorinated biphenyls consisting of 209 possible congeners. These compounds were widely used for almost 50 years, with several hundred million pounds having been released into the environment. An organochlorine pesticide of concern is the insecticide toxaphene, a complex mixture of chlorinated camphenes. Toxaphene consists of more than 177 derivatives and was heavily used in the United States before 1982. Estimates indicate that about 233,688 metric tons of toxaphene was manufactured in the United States from 1964 to 1982. Toxaphene, like PCBs and other organochlorines contaminants, is a global pollutant. Many studies have shown toxaphene to be relatively persistent and bioaccumulated by biota (1).

The objectives of the present research were to study factors affecting anaerobic transformation of PCBs and organochlorine pesticides (e.g., toxaphene) and to develop techniques to enhance their in situ bioremediation. The preliminary goals were 1) to characterize the anaerobic microbial dechlorination of PCBs in Sheboygan River, Wisconsin, sediment, 2) to examine the effects of nutrients, Fe°, and electron carriers on dechlorination of PCBs, and 3) to examine the anaerobic biotransformation of toxaphene using indigenous and PCB-dechlorinating microorganisms.

Toxaphene Biotransformation

Materials and Methods

A Hudson River (HR) pasteurized enrichment culture capable of reductive dechlorination of PCBs was used for initial toxaphene experiments. The enrichment culture was originally pasteurized at 85°C for 15 min and subsequently transferred at monthly intervals (1 percent v/v transfer, repasteurized at 90°C for 10 min at each transfer). The inoculum was serially diluted, and the highest dilution (10^{-6}) retaining PCB-dechlorination activity was inoculated to the medium without PCBs; this culture was used as the inoculum for PCB and toxaphene biotransformation studies. Two milliliters of the PCB-dechlorinating inoculum was anaerobically transferred to 28-mL culture tubes containing 2 mL revised anaerobic mineral medium (RAMM) and 1 g of sterile, uncontaminated (toxaphene-free) pond sediment. Toxaphene was subsequently added at a final concentration of 500 μg/g dry sediment. Control sediment samples were autoclaved three times (121°C for 1 hr each time) on consecutive days, with addition of toxaphene occurring on Day 4. All cultures were incubated at 25°C in the dark.

Results and Discussion

Anaerobic transformation of toxaphene by the pasteurized HR enrichment was evident as indicated by changes in the gas chromatography (GC) isomer-distribution patterns. GC chromatograms of the autoclaved control and a sample inoculated with the HR pasteurized inoculum after 7 months of incubation are presented in Figure 1. Several peaks representative of toxaphene isomers are numbered to facilitate comparison of the control and experimental chromatograms. Only a very minor change was observed for a late

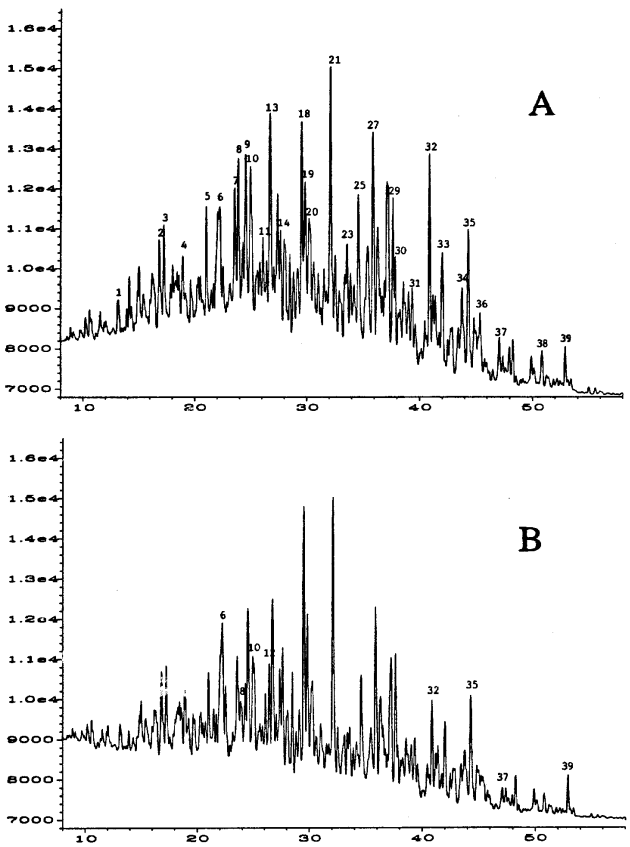

Figure 1. GC profile of toxaphene in A) autoclaved and B) live experimental microcosms (inoculated with enrichment from Hudson River) after 28 weeks of anaerobic incubation. Numbered peaks are for reference only.

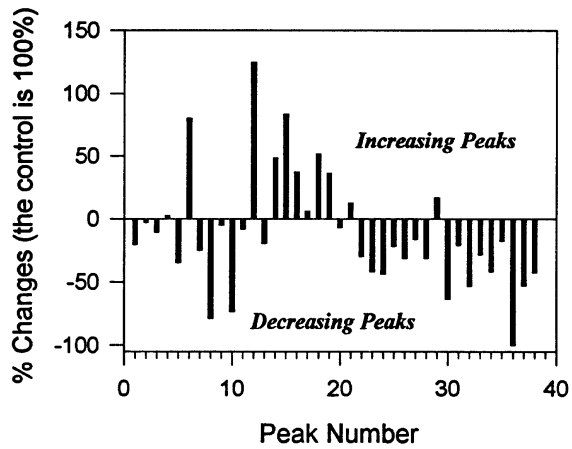

Figure 2. Percentage change (relative to control sample) of select toxaphene peaks in live experimental microcosm after 28 weeks of anaerobic incubation.

eluting peak (#39) in the control and live experimental samples; thus, this peak was chosen as an internal reference peak to which other peaks were normalized (based on peak height). In comparison with the sterile control, appreciable changes in many peaks were noted in live samples after 7 months of incubation. The percentage changes in specific peaks for the live sample compared with the sterile control are presented in Figure 2. Increases in some peaks were observed, indicating accumulation of some dechlorination products. Some early eluting peaks also decreased, however, suggesting that in addition to reductive dechlorination, anaerobic degradation may have occurred. The possible anaerobic degradation of toxaphene will be further investigated by identifying polar transformation products.

Changes in toxaphene isomer-distribution patterns were also observed following GC analyses of autoclaved controls (data not shown). As mentioned previously, sterile controls were prepared by autoclaving sediments at 121°C for 1 hr on 3 consecutive days. Thus, it is highly unlikely that the observed transformations in the sterile controls were biologically mediated. The observed changes are likely due to either abiotic (chemical) transformation or enhanced sorption of toxaphene to the autoclaved soil matrix. It has been previously reported that specific toxaphene isomers were transformed in sterile sediments and in a sand-Fe(II)/Fe(III) system (2). Phototransformation of toxaphene has also been reported (2). On the other hand, the high K_{oc} (soil organic carbon partition coefficient) value reported for toxaphene suggests that the chemical mixture should be strongly sorbed to soil particulates (1); any differences in K_{oc} among the isomers may influence the toxaphene isomer distribution patterns in long-term sediment incubations. Thus, the changes noted in the isomer distribution pattern (after correcting for abiotic transformations observed in sterile controls) are likely isomers of toxaphene that were subject to transformation by the inoculated microorganisms and that were relatively resistant to abiotic transformation. In these inoculated experimental cultures, CH_4 production was not observed, and a *meta*-directed dechlorination of amended PCB congeners (Aroclor 1242) was confirmed in separate experiments. These results suggest that the HR pasteurized enrichment culture was capable of anaerobically transforming toxaphene. The HR pasteurized enrichment is easily maintained and cultivated and, therefore, may be of potential use in the remediation of toxaphene-contaminated soils. Additional studies are under way to evaluate the effectiveness of this enrichment culture for remediation of historically contaminated soils.

PCB Biotransformation

Materials and Methods

PCB biotransformation experiments were performed with PCB-contaminated (approximately 500 ppm) Sheboygan River (SR) sediment. For abiotic transformation experiments, PCB-contaminated sediment was slurried with anoxic site water inside an anaerobic glove box,

homogenized, then amended with Fe°. Of the slurry containing 1 g sediment (dry weight), 1.8 mL was transferred to replicate 28-mL serum tubes. Half of the tubes were spiked with 300 µg Aroclor 1242 as an available PCB source. Fe°, pyrite, and degassed, sterile distilled water were then added. Control samples consisted of autoclaved sediment slurries as described above. All cultures were incubated at 25°C in the dark.

An experiment to assess the effect of pasteurization on microorganisms eluted from SR sediment was conducted as described by Ye et al. (3). Finally, eluted microorganisms from historically contaminated (PCB) SR sediment were subjected to pasteurization (85°C for 20 min) and used as inocula to assess their potential for reductive dechlorination of amended Aroclor 1242, 1248, and 1254. Aroclors were added individually at a final concentration of 500 µg/g dry sediment.

Results and Discussion

Fe°-Amended Experiments

Several studies document the Fe°-mediated reductive dechlorination of trichloroethylene (TCE) and other chlorinated compounds (4). Our preliminary results of Fe°-amended SR sediment slurries, however, indicated that no dechlorination of PCBs occurred after anaerobic incubation for 2 weeks at 20°C in either live or sterile samples. Further, no evidence of reductive dechlorination of PCBs was observed in the SR sediment slurries spiked with Aroclor 1242, indicating that bioavailability of PCB congeners was not a limiting factor for the Fe°-mediated dechlorination.

A prolonged incubation time is usually necessary to achieve biologically mediated reductive dechlorination of PCBs. Thus, it was not surprising to find no evidence of dechlorination in the live experimental samples, especially because the SR sediment had been stored at 2°C to 4°C for approximately 1 yr before use. It is likely that a significant amount of time is necessary for the dechlorinating population to recover to a level to affect significant dechlorination. Additional experimental results with SR sediment (without nutrient amendment) indicated that approximately 4 weeks of incubation was required before detectable PCB dechlorination was observed.

Investigators at the U.S. Environmental Protection Agency Athens Research Laboratory have recently demonstrated Fe°-mediated reductive dechlorination of other halogenated compounds. We have, however, no evidence of PCB dechlorination in Fe°-amended samples under similar experimental conditions. These results suggest that PCBs are more resistant to chemical (abiotic) dechlorination than other chlorinated compounds examined to date.

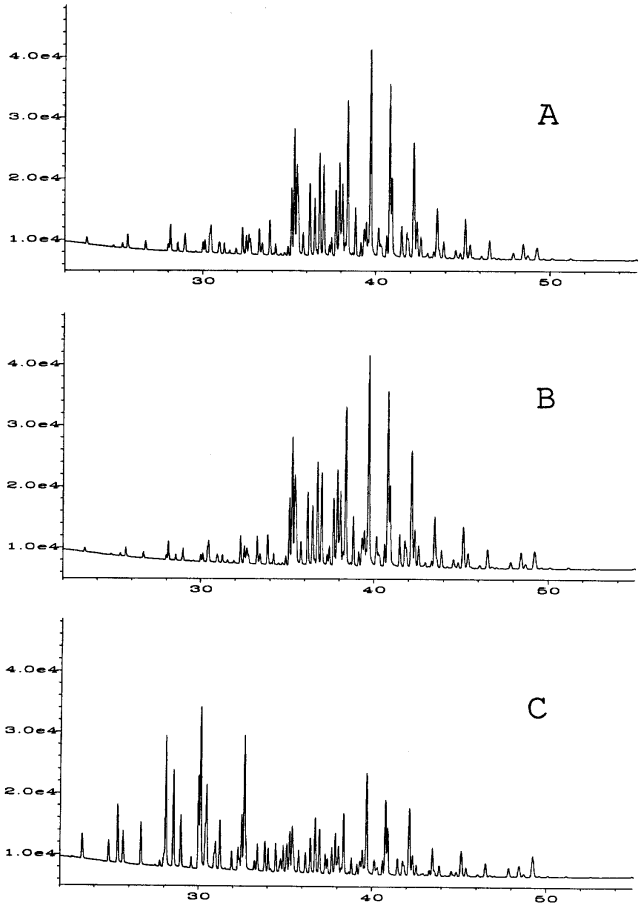

Figure 3. GC profiles of Aroclor 1254 in A) pasteurized, B) autoclaved, and C) live experimental microcosms (inoculated with SR-eluted microorganisms) after 12 weeks of incubation.

Pasteurization of SR Sediment

CH_4 production was not observed in experimental microcosms inoculated with the pasteurized microorganisms from SR sediment; nonpasteurized cultures, however, were actively methanogenic. These observations are consistent with previous pasteurization experiments using HR sediments as inocula (3). The pasteurized cultures preferentially removed *meta* chlorines, while the untreated cultures removed both *meta* and *para* chlorines from selective PCB congeners. In the present study, dechlorination of Aroclor 1254 was observed by the untreated inocula after 6 weeks of incubation; however, no significant dechlorination of Aroclor 1254 was evident in experiments inoculated with the pasteurized inocula after 12 weeks of incubation (Figure 3). These results suggest that anaerobic, spore-forming microorganisms in the SR sediment exhibit similar dechlorinating pathways as the microorganisms in the

HR sediment. Stimulation of in situ PCB dechlorination may be possible through the addition of a suitable spore germinant or growth substrate.

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Predicting Heavy Metal Inhibition of the In Situ Reductive Dechlorination of Organics at the Petro Processor's Superfund Site

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Introduction

Transition metals and synthetic organic compounds are common co-contaminants at waste sites that are candidates for biological treatment. The inhibition of microbial decomposition of natural organic matter by certain transition metals has been widely documented (1); however, the inhibition of anaerobic degradation processes (e.g., reductive dechlorination) is poorly understood.

Inhibition Characteristics

Inhibition characteristics of a model heavy metal (cadmium, Cd) on a model chlorinated aromatic (2,3,4-trichloroaniline, 2,3,4-TCA) was determined in the laboratory. Laboratory microcosm experiments were conducted in three anaerobic flooded soils with varying properties. Dechlorination of 2,3,4-TCA to monochloroanilines occurred when total pore-water Cd concentrations were below a critical threshold level. Inhibition occurred across a continuum of Cd concentrations in several soils, but a completely inhibited threshold concentration was readily identified (Figure 1). Dechlorination kinetics and metabolites differed with soluble metal concentration. Speciation of soluble Cd was necessary to predict whether inhibition would occur, particularly in the presence of high concentrations of organic ligands such as humic acids (Table 1). Estimation of metal pools using selective extractions and measurement of acid-volatile sulfide (AVS) provided additional information but did not adequately predict whether inhibition of dechlorination would occur. These results demonstrated the importance of quantification and speciation of pore-water metals in predicting potential inhibition of anaerobic biodegradation reactions such as reductive dechlorination.

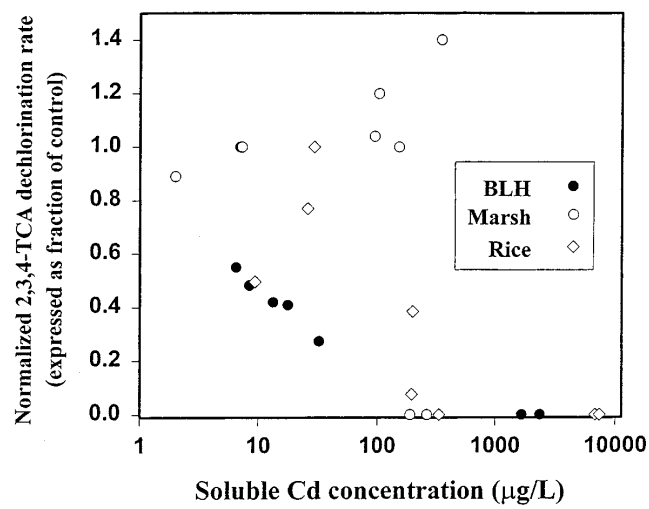

Figure 1. **Normalized 2,3,4-TCA dechlorination rates ($k/k_{control}$) versus soluble Cd concentrations in three flooded soils: bottomland hardwood (BLH), rice paddy, and freshwater marsh.**

Table 1. **MINTEQA2 Results From Pore Water of Representative Rice and Marsh Soil Suspensions (estimated pore-water humic acid concentrations were 1 mg/L in the rice soil and 55 mg/L in the marsh soil)**

Soil	Total Soluble Cd (mg/L)	Cd Species	Equilibrated Mass Distribution, %	2,3,4-TCA Dechlorination Inhibited
RS	0.195	Cd^{+2}	43.7	+
		CdCl+	5.9	
		$CdSO_4$ (aq)	1.6	
		Cd-Humate	48.5	
MS	0.350	Cd^{+2}	1.0	–
		Cd-Humate	98.7	

Site History

The Petro Processor's, Inc., site is a high-priority Superfund site near Baton Rouge, Louisiana. The site served as a chemical waste pit from the early 1960s to the late 1970s. An estimated 60,000 tons of chlorinated organic waste, primarily hexachlorobutadiene and hexachlorobenzene (HCB), was deposited in several unlined, diked pits. A spill event resulted in contamination of stream sediments in an adjacent bottomland hardwood wetland. Heavy metal contamination is contiguous with chlorinated organic contamination (primarily HCB) in these sediments. These sediments are the site of a bioremediation field trial directed at enhancing reductive dechlorination of HCB (2).

Predicting Heavy Metal Inhibition

Sampling is being conducted to determine if metal inhibition of reductive dechlorination can be predicted in the field at the Petro Processor's site. Characteristics of inhibition of model compounds (described above) are being used to develop a strategy for predicting inhibition. Parallel laboratory studies are being used to confirm that these same inhibition characteristics would be observed for HCB in the sediments. Laboratory studies using 2,3,4-TCA indicated that noninhibited soils could be adequately predicted using the AVS/SEM (simultaneously extracted metal) concept. This concept has been used to predict the toxicity of metals to benthic organisms (3). In studies with 2,3,4-TCA, soils in which molar metal concentrations exceeded molar AVS were not always inhibited, requiring further metal speciation and prediction of "free," uncomplexed metal concentrations using MINTEQA2. Spatial and seasonal information of AVS and SEM and observations of lower chlorinated benzene samples are being collected in the field. Sections of the bayou where molar SEM exceeds molar AVS are undergoing further metal speciation studies.

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Effect of Primary Substrate on the Reduction of 2,4-Dinitrotoluene

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Introduction

2,4-Dinitrotoluene (DNT) is one of the priority pollutants (1) commonly found in munitions wastes. It is recalcitrant to biological treatment in aerobic processes (2), such as the activated sludge system, but can be degraded (3) in a sequential anaerobic/aerobic biosystem. 2,4-DNT is completely transformed to 2,4-diaminotoluene (DAT) with ethanol as the primary substrate in an anaerobic reactor. Subsequently, 2,4-DAT is readily mineralized (3) in an aerobic reactor. 2,4-DNT can not be transformed in the anaerobic reactor (4) without a primary substrate. In this study, the anaerobic biotransformation of 2,4-DNT with ethanol, methanol, acetic acid, or hydrogen as primary substrate was investigated. The effect of the primary substrate on the reductive transformation of 2,4-DNT was also studied.

2,4-DNT-transforming anaerobic cultures were acclimated with 2,4-DNT and ethanol, methanol, or acetic acid as the feed organic substrates in three chemostats. The concentrations of 2,4-DNT and the primary substrates in the feed to the three chemostats are listed in Table 1. The chemical oxygen demand (COD) loading for all three chemostats was the same. Minerals and nutrients were added to the chemostat feed to support bacteria growth. $Na_2S \cdot 9H_2O$ (50 mg/L) was added to maintain a reducing environment in the chemostats. The pH and the temperature in the chemostats were maintained constant at 7.2 and 35°C, respectively. The hydraulic retention time in the chemostats was 40 days.

Table 1. Concentrations of Substrates in Feed for the Chemostats

Chemostat	Ethanol Fed	Methanol Fed	Acetic Acid Fed
2,4-DNT, mg/L	91.7	91.7	91.7
Primary substrate, mg/L	500	696	978

2,4-DNT was completely biotransformed to 2,4-DAT in all three chemostats. All the primary substrates (ethanol, methanol, and acetic acid) were converted to methane and carbon dioxide.

After steady-state operation was achieved in the chemostats, the mixed cultures from the chemostats were used as the inocula for the batch tests to determine the kinetics of anaerobic biotransformation of 2,4-DNT with different primary substrates. The cultures were then transferred into the batch reactors in an oxygen-free anaerobic chamber at 35°C. The pH and the temperature in the batch reactors were maintained the same as those in the chemostats. Different initial concentrations of 2,4-DNT were used in the batch tests. To determine the co-metabolic mechanism of the biotransformation of 2,4-DNT and the primary substrate, hydrogen was also used as the primary substrate in the batch tests.

Results and Discussion

All the batch tests were run in duplicate, and the deviations of the results were less than 8 percent. The kinetics of anaerobic biotransformation of 2,4-DNT with ethanol, methanol, and acetic acid as the primary substrates are illustrated in Figure 1 (a), (b), and (c), respectively. 2,4-DNT was completely biotransformed to 2,4-DAT via 4-amino-2-nitrotoluene (4-A-2-NT) or 2-amino-4-nitrotoluene (2-A-4-NT) under anaerobic conditions, regardless of the primary substrate. The rate of the biotransformation of 2,4-DNT and the intermediates (4-A-2-NT and 2-A-4-NT), however, was much higher in the presence of ethanol than that in the presence of either methanol or acetic acid. When ethanol was used as the primary substrate, hydrogen was produced during the acetogenesis of ethanol. The hydrogen then served as the electron donor for the reduction of 2,4-DNT to 2,4-DAT. The bacteria also used ethanol for their growth. When methanol or acetic acid was used as

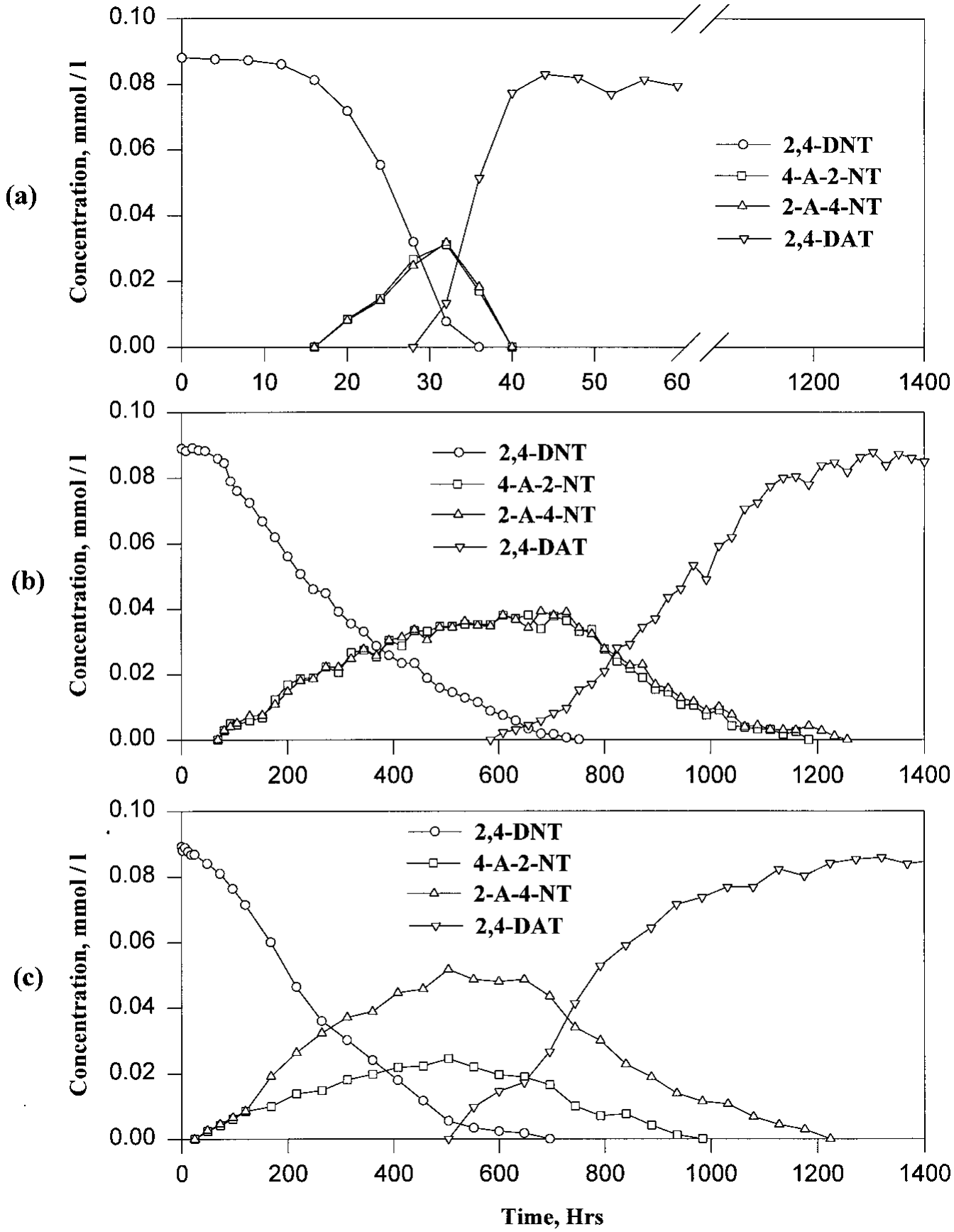

Figure 1. Anaerobic biotransformation of 2,4-DNT with (a) ethanol, (b) methanol, or (c) acetic acid as the primary substrate.

the primary substrate, the substrates were used in the biosystem to support the growth of the bacteria that transformed 2,4-DNT to 2,4-DAT. Neither methanol nor acetic acid was degraded until 2,4-DNT, 4-A-2-NT, and 2-A-4-NT were completely transformed to 2,4-DAT. The hydrogen for the reductive transformation of 2,4-DNT and the intermediates was probably from bacterial endogenous decay. 2,4-DNT was not biotransformed without a primary substrate (4) in both chemostat and batch reactors. 2,4-DNT itself cannot support the growth of the bacteria, and the primary substrate is necessary for maintaining the biological activities to transform 2,4-DNT to 2,4-DAT. The rate of the biotransformation of 2,4-DNT was very low in the initial stage of the process, indicating that 2,4-DNT inhibited its own biotransformation. The presence of 2,4-DNT and its intermediates also exhibited inhibition to the bioconversion of the primary substrate (ethanol, methanol, or acetic acid). The higher the initial concentration of 2,4-DNT, the longer was this period of inhibition to the conversion of the primary substrate. Ethanol, methanol, and acetic acid were rapidly converted by the bacteria after 2,4-DNT and its biotransformation intermediates were completely transformed to 2,4-DAT.

To prove that hydrogen was the electron donor for the reductive biotransformation of 2,4-DNT, the same batch test was conducted with hydrogen as the primary substrate. The results are shown in Figure 2. In the control reactors without 2,4-DNT, hydrogen with CO_2 in the reactors was immediately converted to methane (Figure 2a). When 2,4-DNT was initially present in the reactors, hydrogen was first consumed for the biotransformation of 2,4-DNT to 2,4-DAT, and for supporting the growth of the bacteria. Methane was produced from the excess hydrogen after 2,4-DNT, 4-A-2-NT, and 2-A-4-NT were completely transformed to 2,4-DAT. This phenomenon indicates that 2,4-DNT, 4-A-2-NT, and 2-A-4-NT also inhibited the hydrogen-utilizing methanogenesis. The higher the initial concentration of 2,4-DNT, the more hydrogen was consumed for 2,4-DNT biotransformation and the less hydrogen was left for methane production (Figure 2b-f).

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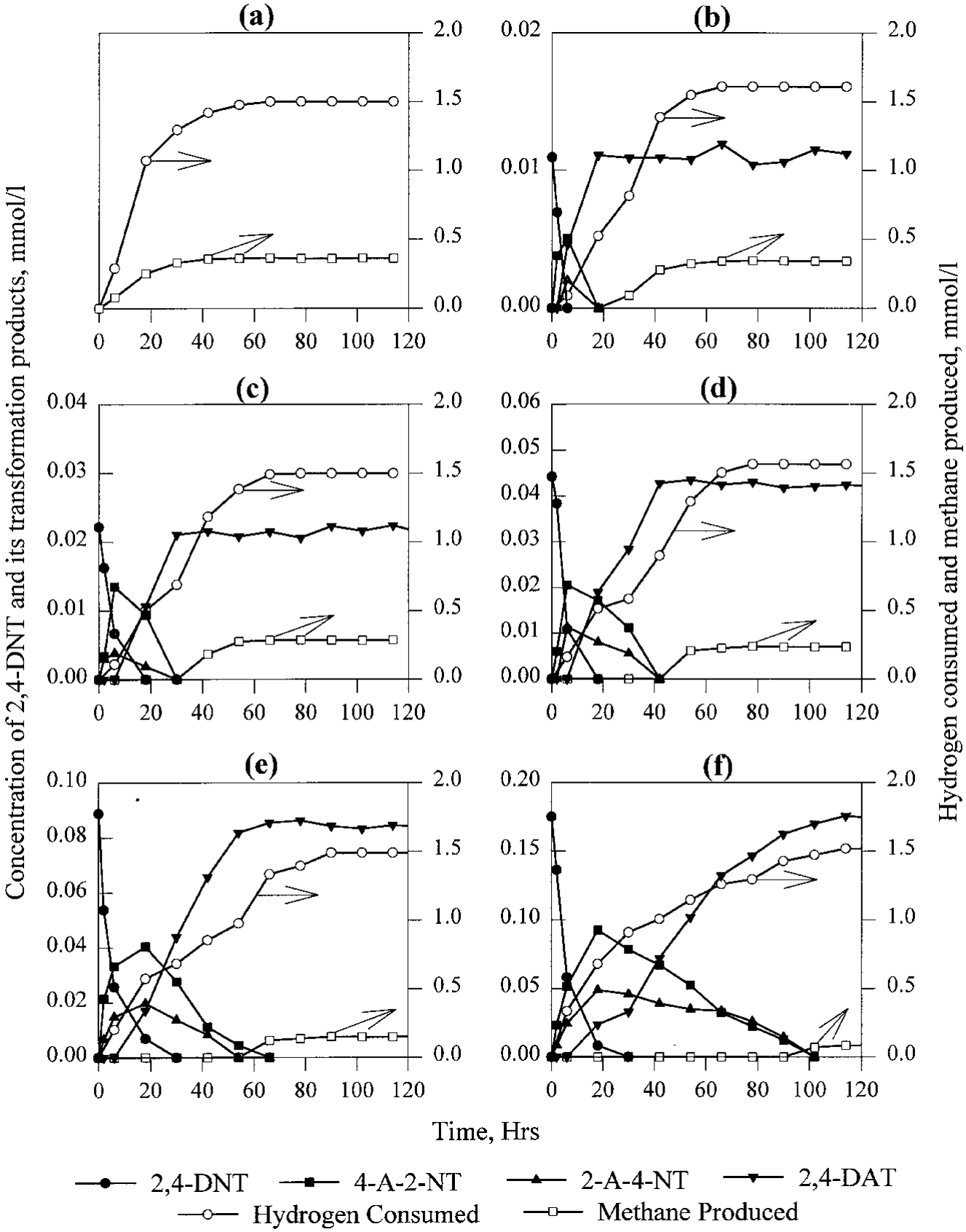

Figure 2. Anaerobic biotransformation of 2,4-DNT with H_2 as the primary substrate.

Surfactants in Sediment Slurries: Partitioning Behavior and Effects on Apparent Polychlorinated Biphenyl Solubilization

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It is generally believed that the biodegradation of poorly water soluble compounds in soil or sediment systems is limited by low bioavailability due to strong sorption of the compounds to natural organic matter. The use of surfactants to increase the apparent water solubility of such contaminants has often been suggested as a way of increasing their bioavailability to degrading microorganisms. A possible limitation of this approach is that solubility enhancement is much greater above the critical micelle concentration (CMC) of the surfactant than below it, and these supra-CMCs are often toxic or inhibitory to bacteria. A few reports, however, indicate that sub-CMC concentrations of surfactants may enhance the anaerobic dechlorination of aromatic compounds. The goals of our present research efforts are to determine if sub-CMCs of surfactants can enhance the microbial dechlorination of polychlorinated biphenyls (PCBs) and, if so, by what mechanism(s). We have determined the partitioning behavior of several surfactants in soil and sediment slurries and their effects on PCB solubilization. These experiments were undertaken to determine if an increase in the apparent aqueous solubility of PCBs by sub-CMCs of these surfactants is a plausible mechanism for any observed enhancement of PCB dechlorination.

The sorption of four commercial nonionic surfactants (Triton X-100, Triton X-405, Triton X-705, and Tween 80) onto the Red Cedar River sediment used in our PCB dechlorination assays was evaluated. Sorption isotherms were plotted, and Freundlich isotherms of the form $C_s=KC_en$ were fitted to the experimental data where C_s is the sorbed concentration of the surfactant (mg/kg), C_e is the aqueous concentration of the surfactant (mg/L), and K and n are constants. K and n values ranged from 1.193×10^{-4} to 1.009×10^{-3} and from 0.232 to 0.696, respectively. The Red Cedar River sediment thus shows orders of magnitude less surfactant sorption than has been reported for soils, as shown by the low K value.

The distribution coefficients of three PCB congeners at sub- and supra-CMC surfactant concentrations (up to four times the CMC) were determined using [^{14}C]labeled PCBs. The aqueous-phase PCB concentrations increased at all surfactant concentrations tested compared with the sediment-water system without surfactants. Notably, this included an increase in the aqueous-phase concentrations of PCBs even at the lowest surfactant concentration tested (0.05 times CMC), especially for the inherently less soluble hexa- and tetra-CBs by Tween 80. In fact, Tween 80 increased the solubility of 2,2',4,4',5,5'-CB by a factor of 3.3 at 25 percent of its CMC, and by a factor of 6.3 at 75 percent of its CMC.

The low sorption of the surfactants by Red Cedar River sediments has important consequences for PCB solubilization. Surfactant monomers sorbed to soils or sediments will increase the total organic matter content of the solids and act as an additional sorptive phase. Consequently, if surfactants strongly sorb to the sediments, they may actually decrease the aqueous phase concentration of nonionic compounds such as PCBs. When the mass of sorbed surfactant is small, however, as in the case of the Red Cedar River sediment, most of the surfactant mass exists in the water, and the PCB solubilization effect of aqueous phase surfactant micelles and monomers dominates the sorptive capabilities of sediment-associated surfactant and native organic matter. Therefore, the aqueous phase concentration of PCB increases even when relatively small amounts of surfactants are added to the system. While these solubility enhancements are small relative to those that occur above the CMCs of these surfactants, the increased solubility may be enough to significantly increase the rate of PCB dechlorination, especially for the more chlorinated and less water soluble congeners.

Partial Characterization of an Anaerobic, Aryl, and Alkyl Dehalogenating Microorganism

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Introduction

To better understand controls and pathways of anaerobic biotransformation of organic pollutants in contaminated environments, pure culture studies are beneficial. To date, only a few strains of anaerobic dehalogenating microorganisms have been isolated and characterized. Among these, *Desulfomonile tiedjei* is probably the most widely studied (1). In this study, we report the partial characterization of an anaerobic bacterium capable of both aryl and alkyl reductive dehalogenation.

Results and Discussion

An anaerobic bacterium, designated as strain XZ-1, was isolated from freshwater pond sediment near Athens, Georgia. Isolate XZ-1 is a sporeforming, motile rod capable of reductive dehalogenation of chlorophenols. Electron acceptors, including sulfite, thiosulfate, and nitrate (but not sulfate), stimulated growth in the presence of yeast extract and pyruvate. None of the following supported growth or dehalogenation of chlorophenol (CP): glucose, fructose, galactose, rhamnose, cellobiose, xylan, ribose, citrate, fumarate, acetate, peptone, tryptone, casein hydrolysate, and casamino acids. The addition of 1 mM carbon dioxide reduced the lag time before growth. No growth was observed in the presence of 4 percent air or higher. Growth was completely inhibited by pentachlorophenol (PCP) ($\geq$ 32 µM), 2,3,4,5-tetraCP ($\geq$ 8 µM), 3,4,5-triCP ($\geq$ 16 µM), 3,5-diCP ($\geq$ 120 µM), 2,4-diCP ($\geq$ 500 µM), and 2-CP ($\geq$ 4,000 µM). The generation time of isolate XZ-1 was 1.8 hr at pH 7.5 (optimal) and 30°C.

Isolate XZ-1 removed *ortho*-chlorines from all *ortho*-chlorine-containing phenols tested (e.g., 2-CP and pentachlorophenol). Hydrogen, formate, ethanol, pyruvate, and yeast extract served as electron donors for dehalogenation of CPs. Only pyruvate and yeast extract, however, stimulated growth either in the absence or presence of electron acceptors, including 3-chloro-4-hydroxyphenylacetate (an analog of *ortho*-CP). The aryl dehalogenation activity was inducible, and induction was inhibited by addition of chloramphenicol to cell suspensions. Experiments with D_2O demonstrated that water was the exclusive proton source for aryl dehalogenation of chlorophenols. Proton nuclear magnetic resonance (NMR) studies indicated that hydrogen was incorporated at the same position where an *ortho*-chlorine was removed. Product solvent isotope effects were 5.4 and 8.5 for dechlorination of 2,3-diCP and 2-CP, respectively. An increase in the assay temperature reduced the product solvent isotope effect in 2,3-diCP dechlorinations.

Cell suspensions of isolate XZ-1 also were capable of reduction of 2,4,6-trinitrotoluene (TNT, 46 ppm) to 2,4,6-triaminotoluene via 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,6-diamino-4-nitrotoluene, 2,4-diamino-6-nitrotoluene, and several unidentified intermediates. The TNT transformation pattern was different in aryl dehalogenation-induced cells and noninduced cells. The identified intermediates of TNT reduction accumulated to lower levels in the induced cells than in the noninduced cells. Addition of pyruvate stimulated TNT transformation. Heat-treated cell suspensions exhibited only traces of TNT transformation activity either with or without addition of pyruvate. Cell suspensions of isolate XZ-1 also metabolized chloramphenicol in the presence of pyruvate. No intermediate(s) of chloramphenicol transformation has been identified to date.

Both noninduced and aryl dehalogenation-induced cell suspensions of isolate XZ-1 dechlorinated tetrachloroethene to trichloroethene (TCE). A comparison of aryl

and alkyl dehalogenation rates in noninduced and induced cells suggests that at least two enzymes are responsible for the two activities. Aryl dehalogenation-induced cells also slowly dechlorinated TCE to cis-1, 2-dichloroethene.

Additional studies are under way to identify the range of transformation activities of dehalogenating isolate XZ-1 and to characterize further the physiology, nutrition, and phylogeny of this anaerobe.

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Microbial Degradation of Petroleum Hydrocarbons in Unsaturated Soils: The Mechanistic Importance of Water Potential and the Exopolymer Matrix

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Background

Total soil water potential, Ψ, is the potential energy per unit volume of unsaturated soil water and is commonly reported in -MPa (1). Matric water potential, Ψ_m, is the largest component of Ψ in most soils and arises from the interaction of soil water with soil surfaces. Matric potential determines water film thickness in soil, and thus controls gas-phase mass transfer through soil pores and solution-phase mass transfer through water films. Bacteria in biofilms are in equilibrium with water in their environment, and adaptation to a given soil water potential or to a changing water potential condition during wetting and drying will affect intrinsic bacterial physiology and biofilm characteristics (2). Because of its role in both mass transfer and bacterial reaction rates, soil water potential is an important environmental factor controlling petroleum biodegradation rates in unsaturated soils.

Project Framework

Our biodegradation model for oil constituents at the biofilm scale contains the following parameters that will vary as a function of Ψ:

q_m = intrinsic molar removal rate per area of biofilm (moles/#-t)
K_s = intrinsic half saturation constant (moles/L^3)
L_b = total biofilm thickness (L)
D_e = effective diffusivity of contaminant through biofilm (L^2/t)
ρ_m = number density of bacteria per mass of biofilm (#/m)
ρ_b = density of biofilm or mass of biofilm per biofilm volume (m/L^3)
C_{sat} = aqueous solubility of petroleum hydrocarbon (moles/L^3)

Experimental protocols include determining each parameter as a function of Ψ and determining the overall removal rate in unsaturated soil as a function of Ψ. We are also examining how physicochemical properties of the bacterial matrix are altered with Ψ to effect hydrocarbon solubility and spreadability.

Biofilm Reactors and Preliminary Results

Phenanthrene, hexadecane, and methyl-decalin are the selected test substrates representing the three major classes of petroleum constituents. Pristane is the conservative tracer. Polyethylene glycol, a nonpermeating solute with a molecular weight of 8,000 (PEG 8,000), is used to set matric water potential in well-mixed and biofilm culture systems.

Custom-designed biofilm reactors for developing biofilms under unsaturated conditions have been constructed and are being tested using various growth substrates. Transmission electron micrographs taken through biofilms grown under Ψ-controlled conditions reveal architectural changes, specifically cell packing and morphological, with Ψ. Preliminary diffusion studies suggest that diffusional mass transfer through biofilms is related to the Ψ-condition during growth.

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Metabolic Indicators of Anaerobic In Situ Bioremediation of Gasoline-Contaminated Aquifers

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Bioremediation is one of a limited number of options for restoring aquifers contaminated with the hazardous aromatic hydrocarbons that occur in unleaded gasoline, such as benzene, toluene, ethylbenzene, and the xylenes (BTEX). Considering the cost and technical difficulty associated with introducing oxygen into some aquifers, in situ bioremediation using indigenous, anaerobic bacteria merits serious consideration for some contaminated sites. A major impediment to the acceptance of in situ bioremediation is the difficulty of demonstrating that decreases in the concentrations of BTEX in ground water truly represent bacterial metabolism of these compounds rather than abiotic processes such as sorption, dilution, or volatilization.

Work in our laboratory has included the characterization of byproducts of alkylbenzene metabolism by pure and mixed anaerobic cultures (1, 2). This research, which has focused on sulfate-reducing cultures, has involved the extensive use of gas chromatography/mass spectrometry for metabolite characterization. We have recently integrated such laboratory findings with field data from a controlled-release experiment conducted at the Seal Beach Naval Weapons Station in California.

Based on the concordance of laboratory studies of anaerobic bacteria and field observations from the aquifer in Seal Beach, we propose a group of compounds including benzylsuccinic acid, benzylfumaric acid (or a closely related isomer), and the *o*-, *m*-, and *p*-methyl homologs of these compounds as biogeochemical indicators of in situ anaerobic alkylbenzene metabolism in gasoline-contaminated aquifers. Under the controlled conditions of the field study, a strong correspondence was observed between the disappearance of alkylbenzenes from ground water over time and the appearance of associated metabolic byproducts. This correspondence was both qualitative (i.e., only products specific to the metabolism of toluene, *o*-xylene, and *m*-xylene were observed, and only these three hydrocarbons were depleted) and quantitative (i.e., metabolic byproduct concentrations tended to increase as the associated alkylbenzene concentrations decreased).

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Contaminant Dissolution and Biodegradation in Soils Containing Nonaqueous-Phase Organics

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Several models have been developed to describe the dissolution, adsorption to soil, and biodegradation of nonaqueous-phase contaminants (i.e., hydrocarbons) in the subsurface, and remediation times have been estimated for various conditions (1-6). The significant rate-limiting factors determining the required bioremediation time appear to be the rates of transport of the electron acceptor or oxygen and organic contaminants in pores and soil aggregates in the vicinity of the hydrocarbon phase. The contaminants' solubilities in the aqueous phase affect their dissolution and transport. Contaminant dissolution and transport are more rapid than oxygen transport for more water-soluble compounds such as toluene, and less rapid for less soluble compounds such as pyrene. As long as the nonaqueous phase is present, the higher the solubility of a compound, the greater the extent of removal by pump-and-treat operations rather than by oxygen-limited biodegradation. The sizes of aggregates and hydrocarbon blobs significantly affect remediation time, which has been found to be proportional to the square of the characteristic length of the blob.

The available experimental data for pyrene and anthracene fit well with the results of simulation obtained with one of the models. Besides dissolution, adsorption, desorption, and biodegradation, this model takes into account the hydrocarbon-phase size distribution; moreover, it expresses the rate of biodegradation by Monod kinetics.

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Protein Expression in Mycobacteria That Metabolize Polycyclic Aromatic Hydrocarbons

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Three species of mycobacteria have been isolated from petroleum contaminated soil (*Mycobacterium* sp. PYR-1) (1) or coal gassification sites (*Mycobacterium* sp. PAH135 and *M. gilvum*) (2, 3). These organisms have potential application in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) because each can mineralize various PAHs, including naphthalene, phenanthrene, pyrene, and fluoranthene. The present study was initiated to investigate the molecular basis for the degradation of PAHs by these species of mycobacteria. To determine part of the physiological response of the organisms to the presence of a metabolizable PAH in the environment, we have analyzed the expression of proteins by each organism in response to pyrene using two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For each organism, the pattern of separated proteins was distinct, and proteins increased in expression following addition of the PAH. Major proteins increased in induced cells of *Mycobacterium* sp. PYR-1 had approximate masses of 105, 79, 53, 42, and 15 kDa. In comparison, three proteins were induced in *Mycobacterium* sp. PAH135 (95, 70, and 53 kDa) and in *M. gilvum* (72, 27, and 15 kDa). To determine whether increased expression of these proteins is associated with metabolism of pyrene in *Mycobacterium* sp. PYR-1, uninduced cells were incubated with the PAH for varying periods up to 8 hr, and the amounts of pyrene metabolism and protein expression were quantified by high-performance liquid chromatography (HPLC) analysis and densitometry of proteins detected in two-dimensional SDS-PAGE gels, respectively. After a delay of about 1 hr, uninduced cells metabolized all of the pyrene within 8 hr. The 79 kDa protein, undetectable in uninduced cells, was expressed at 1.2 percent of proteins within 2 hr and was fully expressed at about 2 percent of total protein within 4 hr. Partial characterization of this protein by N-terminal sequencing and hybridization of a synthetic oligonucleotide probe corresponding to the amino acid sequence to *Bam*III-digested *Mycobacterium* sp. PYR-1 deoxyribonucleic acid (DNA) show that this protein is similar to the *kat*G gene product (catalese-peroxidase) expressed in many other mycobacteria. Kinetics of increased expression of the 15 kDa protein followed those for the 79 kDa protein. In contrast, the 42 kDa protein was not increased until 6 hr and was not fully expressed even at 8 hr after addition of pyrene. A variant of the organism was isolated that failed to metabolize pyrene and fluoranthene added to soft agar overlays or in liquid cultures. The variant retained the ability to metabolize naphthalene and phenanthrene. None of the proteins studied was induced in this organism after exposure to 3 μg/mL pyrene for 24 hr. These results indicate that additional components are required for metabolism of pyrene and fluoranthene compared with those for metabolism of naphthalene and phenanthrene in *Mycobacterium* sp. PYR-1. Our results suggest that the proteins studied are associated with metabolism of pyrene in induced cells of this organism. These results provide fundamental information about the proteins expressed by these mycobacteria during PAH degradation. Clearly, this information will be important for future application of these mycobacterial strains as inoculants in the bioremediation of PAH-contaminated sites.

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Section 6
Hazardous Substance Research Centers

The Hazardous Substance Research Centers (HSRCs) conduct research on bioremediation under the direction of ORD's National Center for Extramural Research and Quality Assurance. Research is sponsored by the following centers: the Northeast Hazardous Substance Research Center (Regions 1 and 2), the Great Lakes and Mid-Atlantic Hazardous Substance Research Center (Regions 3 and 5), the South/Southwest Hazardous Substance Research Center (Regions 4 and 6), the Great Plains and Rocky Mountain Hazardous Research Center (Regions 7 and 8), and the Western Region Hazardous Substance Research Center (Regions 9 and 10).

The symposium's poster session included presentations on the co-metabolic biodegradation kinetics of trichloroethylene in unsaturated soils; the effect of water potential on biodegradation kinetics and population dynamics; developments in anaerobic and aerobic bioventing; developments in the treatability protocol for co-metabolic bioventing; the environmental safety of commercial oil spill bioremediation agents; the effectiveness of gas-phase bioremediation stimulating agents for unsaturated zone in situ bioremediation; protein expression of mycobacteria that metabolize polycyclic aromatic hydrocarbons; a field evaluation of pneumatic fracturing enhanced bioremediation; and the solids suspension characteristics related to slurry biotreatment performance.

Co-metabolic Biodegradation Kinetics of Trichloroethylene in Unsaturated Soils

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The ability of methanotrophic and heterotrophic bacteria to aerobically transform chlorinated solvents is well established. Methane monooxygenase (MMO) and aryl monooxygenase enzymes, produced by these microorganisms respectively during growth on primary substrates, catalyze the cooxidation and dehalogenation of chlorinated ethenes including trichloroethylene (TCE) and vinyl chloride. Bioventing may prove useful for stimulating co-metabolism and achieving in situ remediation of vadose zone soils contaminated with chlorinated alkenes. This possibility motivated an investigation of co-metabolic dechlorination by indigenous microbial populations in soils collected from Wurtsmith Air Force Base (AFB) in Oscoda, Michigan.

Contaminated aquifer and vadose regions at Wurtsmith AFB contain perchloroethylene, TCE (up to 1,000 μg/L), trans-dichloroethylene, vinyl chloride, dichlorobenzenes, and benzene, toluene, ethylbenzene, and xylene (BTEX) compounds. High methane concentrations have also been detected in soil gas at the site, indicating potentially favorable conditions for methanotrophic bacteria. Sandy soils from several depths are being characterized and studied in aerobic batch microcosm systems at room temperature to discern the relative importance of methanotrophic and heterotrophic organisms, and to optimize methods for their stimulation. Methanotrophs are supplied with oxygen and methane, while heterotrophs are supported on toluene as the primary inducing substrate. A range of environmentally relevant concentrations is studied, and following an acclimation period TCE is added at approximately one-tenth the level of primary substrate. The effect of soil moisture on biodegradation kinetics is examined by comparing microcosms containing soil maintained at the local water content of 4 percent to microcosms containing saturated soil. In addition, substrate degradation by soil-derived cultures is monitored in liquid medium without soil.

Bacterial growth on methane and toluene has been stimulated, and ongoing work will evaluate optimum primary to co-metabolic substrate ratios and elucidate the effect of moisture content on TCE co-metabolism in soil systems. Through development of a simple methodology for screening soils and microbial populations indigenous to a particular site, this study may clarify the potential of bioventing to enhance chlorinated solvent transformation in unsaturated zones containing mixed wastes.

The Effect of Water Potential on Biodegradation Kinetics and Population Dynamics

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Although bioventing is currently being applied in the field, much remains to be learned about the underlying parameters controlling biological degradation kinetics in these systems. These parameters need to be systematically studied to improve modeling and design of bioventing applications. In this investigation, the impact of subsurface moisture content on biokinetic parameters is studied, and the applicability of biological kinetics obtained in saturated batch systems to the unsaturated zone is evaluated. Specific emphasis is placed on studying the effects of water potential on oxygen availability, microbial metabolism, and growth.

Mixed culture studies with indigenous microorganisms derived from the unsaturated zone at the Wurtsmith field site, Oscoda, Michigan, have been performed in batch systems. No degradation of toluene was detected at a field moisture of about 3 percent (by weight) even after a month of incubation. Moisture contents between 12 and 16 percent moisture exhibited the fastest degradation of toluene. Differences in biodegradation kinetics observed as a function of moisture content and independent of population shifts are being verified using pure cultures of a toluene-degrading microorganism, isolated from the same unsaturated soil samples. Water potential, the thermodynamic variable expressing water activity and therefore water availability for the microorganisms, is used as the experimental variable rather than the gravimetric moisture content. Varying water contents of the soil as a result of drying due to airflow in bioventing operations influence the different components of the water potential in the soil matrix. The osmotic and matric water potential components are studied separately in their effect on bacterial growth, energy production, and degradation kinetics. Bacteria isolated from an unsaturated zone below Wurtsmith Air Force Base are grown in liquid culture on toluate at different concentrations of membrane diffusable solutes (NaCl) and nondiffusable solutes (polyethyleneglycol, PEG). PEG is used to simulate the effect of matric potential independent of the effect of mass transfer limitations resulting from moisture changes in porous media. Salt additions to the liquid medium resulted in higher growth rates of toluate degraders up to 0.2 M NaCl and increased CO_2 evolution. The amount of adenosine triphosphate produced appeared to be independent of the salt addition. Studies will be extended to assess growth in homogenous solids of defined pore structure, and mixed population studies will be performed to assess the separate effect of population shifts. The results from these studies will serve as a model for water potential induced microbial stress in unsaturated soil horizons during bioventing.

Anaerobic-Aerobic Bioventing Development

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Motivation

Surface spills and leaking pipelines and underground storage tanks can result in vadose zone soils contaminated with hazardous chemicals. The vadose zone must be cleaned to minimize contamination of ground water and emissions of volatile organic chemicals to the atmosphere.

Aerobic bioventing is now a common approach to treating aerobically biodegradable contaminants (e.g., fuels) in the vadose zone. Many highly chlorinated aromatics and aliphatics can be destroyed microbiologically, most rapidly by sequential anaerobic-aerobic treatment. Usually, the biochemical pathway providing the highest rate for the initial steps of microbial destruction of the highly chlorinated organic is anaerobic reductive dechlorination. Once partially dechlorinated, the resulting compounds typically degrade faster under aerobic, oxidizing conditions. For example, perchloroethylene, wood treating wastes containing pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), and many chlorinated pesticides can be at least partially dechlorinated by microbial consortia under anaerobic conditions. The resulting dechlorinated product often can be destroyed microbiologically under aerobic conditions.

For treatment of large volumes of soil at depth, the most cost-effective approach is likely to be an in situ process that takes advantage of the above well-known anaerobic and aerobic biological activities. Our approach to this challenge is to develop “anaerobic/aerobic bioventing.”

Approach

Anaerobic-aerobic bioventing requires development of a new process: anaerobic bioventing. Conditions must be established in the contaminated vadose zone to induce anaerobic microbial biodegradation. Anaerobic conditions may be established by injecting nitrogen into the vadose zone to displace all oxygen. A volatile cosubstrate in the nitrogen stream may be needed to induce the soil microorganisms to consume all the oxygen and to establish a low oxidation-reduction potential (ORP). The low ORP should induce dechlorination of the contaminant. The cosubstrates must be volatile and consumable by anaerobic microorganisms (e.g., ethanol, acetone, hydrogen).

Once the contaminant is fully or mostly dechlorinated, aerobic bioventing is initiated by injecting air into the vadose zone. The aerobic microorganisms should then complete the mineralization of the contaminant.

To move the technology to the field, the following questions are currently being addressed with pilot-scale tests:

- Can venting with nitrogen at a low rate establish adequate anaerobic conditions for dechlorination in unsaturated soil? What is the cost to supply nitrogen at the required flow rate?
- Can a cosubstrate introduced with the nitrogen stream promote the dechlorination of target compounds such as PCP in unsaturated soil?
- What are the most effective volatile cosubstrates, biologically and economically, to promote anaerobic dechlorination? (Several will be evaluated.)
- Will switching to an aerobic environment (by replacing nitrogen and the primary substrate with air in the injection stream) promote mineralization of the dechlorination byproducts?
- What classes of compounds are amenable to anaerobic/aerobic bioventing? (Several will be evaluated.)

- Will the addition of hydrogen gas (H_2) to the nitrogen stream aid in the development of reducing environments and promote dechlorination?

Initial experiments were conducted beginning in June 1995. The tests were conducted in pilot-scale soil columns. Two 4-ft long, 4-in. diameter glass columns were built to simulate an in situ horizontal column of unsaturated soil. Gas sampling ports were placed along the length of the column. The column was filled with sand inoculated with secondary effluent to add biomass and moisture. Essential inorganic nutrients were also added. Nitrogen gas passed through an oxygen scrubber was used to flush the columns of oxygen. At field scale, the nitrogen could be supplied by tank or, perhaps more economically, by separating it from atmospheric air using onsite molecular sieves. The first cosubstrates evaluated were ethanol and acetone.

Development of Co-metabolic Bioventing: Laboratory Tests

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Background

The objective of this project is to accumulate the information necessary to write a protocol for determining site-specific treatability of co-metabolic bioventing. "Co-metabolic bioventing" is a bioventing-like process that delivers air and a volatile co-metabolite to a vadose zone contaminated with chlorinated solvent (e.g., trichloroethylene) to induce in situ biodegradation of the contaminant.

The process attempts to induce the following biologically catalyzed (unbalanced) reactions:

$$\text{Cosubtrate} + O_2 \Rightarrow CO_2 + H_2O$$

$$TCE + O_2 + H_2O \Rightarrow CO_2 + HCl$$

where the cosubstrate is chosen because it stimulates the productions of enzymes in the microbial culture that oxidize trichloroethylene (TCE). Known volatile cosubstrates include methane, propane, butane, toluene, jet fuel, gasoline, and isoprene. The co-metabolic bioventing system delivers the cosubstrate and oxygen from gas injection wells to induce the in situ biodegradation of the contaminant TCE.

The Remediation Technology Development Forum Workgroup on In-Situ Bioremediation of Chlorinated Solvents, a research team with members from industry and government, plans to conduct field demonstrations of three in situ bioremediation technologies for the cleanup of chlorinated solvents at two sites. Laboratory studies using site-contaminated soils are supporting the field research. The three technologies are 1) co-metabolic bioventing of TCE and dichloroethylene contaminated vadose zone soils, 2) accelerated anaerobic biotreatment of chlorinated ethylene contaminated ground water, and 3) intrinsic bioremediation of chlorinated ethylene contaminated aquifers. Dover Air Force Base (AFB), in Delaware was selected as the first site.

One task under the co-metabolic bioventing project was established to develop a protocol to test site-specific treatability of co-metabolic bioventing. The U.S. Environmental Protection Agency's National Risk Management Research Laboratory has assumed primary responsibility for this task. Information from laboratory testing using soil from Dover AFB and two to four other sites will be used to generate the recommended protocol. The efficacy of the protocol will be evaluated by comparing treatability test results with results of at least two field demonstrations.

Approach

To simulate co-metabolic bioventing in a closed reactor, a biodegradation test using soil from Dover AFB was conducted in the following manner:

- Reactors were closed bottles containing 100 g of soil.
- Soil was unsaturated and not mixed.
- Cosubstrate (toluene or propane) was added to the bottle.
- No moisture, nutrients, or microorganisms were added.
- Killed controls were established.
- Two sets of bottles were established:
  - Reactors that were sacrificed in triplicate at various times during the test.
  - Reactors that were monitored by automated respirometry.

- Contaminant and cosubstrate loss with time were monitored with the sacrificial reactors.
- Oxygen use and carbon dioxide production with time were monitored by the respirometer.

The biodegradation test was conducted during June and July 1995. Earlier tests conducted with clean soils spiked with TCE and cosubstrates indicated that care must be taken in the reactor design to minimize abiotic losses of TCE. Bottles that expose the soil and its atmosphere only to glass and Teflon showed significant (greater than 40 percent) loss of TCE in killed controls within a week. This loss was attributed to sorption into the Teflon gaskets. We have redesigned the bottles to maximize glass surfaces and virtually eliminate Teflon surface area. Preliminary tests showed that these reactors minimized abiotic TCE loss very well.

Evaluating the Environmental Safety of Using Commercial Oil Spill Bioremediation Agents

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The use of commercial bioremediation agents (CBAs) for reducing the ecological impact of oil spills raises several risk assessment questions. The presence of petroleum hydrocarbons may contribute some toxicity; CBAs, with their associated chemical constituents (e.g., nutrients, dispersants, enzymes), microbes, and inert ingredients, may add to this toxicity either directly or indirectly through decreases in dissolved oxygen or increases in particulates. In addition, interaction of CBAs with oil may have other environmental effects, either through increasing the amount of petroleum hydrocarbons available to aquatic organisms (i.e., through biosurfactant activity) or by generation of toxic metabolites. A related issue is whether use of a CBA could reduce the toxicity of the oil (an efficacy issue).

A tiered approach, with increasing complexity, cost, and effort, has been proposed to address the environmental safety of CBA usage. Originally developed for assessing effluents, 7-day chronic estimator tests using a fish (*Menidia beryllina*) and a crustacean (*Mysidopsis bahia*) were adapted to evaluate CBAs; the tests utilize endpoints of survival, growth, and, in the case of the mysids, a measurement of egg production. Tier II evaluates the toxicity of the CBA, alone and in the presence of a water-soluble fraction of oil, to provide baseline information on CBA toxicity and potential synergism with petroleum hydrocarbons. Tier III examines effluents from flow-through test systems that model a variety of aquatic habitats (open water, beach, marsh) to assess toxicity under more realistic conditions, where a CBA and oil are allowed to interact.

Data are presented on the toxicity of a variety of CBAs classified by vendors as microbial, nutrient, enzyme, dispersant, and “other.” In the flow-through test systems, the CBAs exhibited relatively low toxicity, either by themselves or in the presence of an artificially weathered oil. During a particular period, an apparent interaction between one CBA and oil appeared to increase toxicity in the marsh system. Toxicity reduction in the sand component of the beach test system could not be developed into an efficacy endpoint because very small quantities of oil produced measurable effects on a benthic amphipod.

UNIFAC Phase Equilibrium Modeling To Assess the Bioavailability of Multicomponent Nonaqueous-Phase Liquids Containing Polycyclic Aromatic Hydrocarbons

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This work is part of a project to evaluate bioremediation of contaminants that are nonaqueous-phase liquid (NAPL) mixtures of polycyclic aromatic hydrocarbons (PAHs). This poster presents the first phase of this work, aimed at gaining a thorough understanding of multicomponent NAPL/water-phase equilibria for very complex mixtures. This provides basic information about maximum bioavailable concentrations of PAHs.

The thermodynamics of multicomponent NAPL/water-phase equilibria can be described with knowledge of the mixture composition, NAPL-phase activity coefficients, and pure solute aqueous solubilities. This analysis involves application of the UNIFAC model to predict NAPL-phase activity coefficients for constituent compounds in four different tar materials for which detailed composition data are available. This group contribution method has proven to be useful for complex mixtures such as coal tars because a mixture is represented by a relatively small number of functional groups, making thermodynamic analysis using excess Gibbs energy models tractable. For this work, the molecular structures of the uncharacterized portions of the tars are approximated through nonparametric regressions of functional group characteristics with molecular weight. The UNIFAC model was found to predict nearly ideal behavior for most tar constituents. The activity coefficients range from 0.14 (quinoline) to 1.27 (ethylbenzene), but the vast majority of the constituents are predicted to have activity coefficients between 0.9 and 1.1.

These results provide a firm theoretical basis for making an assumption of solution ideality for many tar constituents (i.e., Raoult's law). The robustness of this conclusion is indicated through comparable results across different tar materials, and through a sensitivity analysis to the estimated characteristics of the uncharacterized fractions. These results, in conjunction with laboratory measurements of PAH biodegradation rates (for individual compounds and for multiple substrate NAPL systems), will eventually be integrated into a mathematical model describing the rate of biotransformation of PAH-containing NAPL contaminants and the dynamics of the composition of the NAPL residual.

Field Evaluation of Pneumatic Fracturing Enhanced Bioremediation

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In situ bioremediation is often limited by the rate of transport of nutrients and electron acceptors (e.g., oxygen, nitrate) to the microorganisms mediating the process, particularly in soil formations with moderate to low permeability. To overcome these rate limitations, an investigation was conducted to integrate the process of pneumatic fracturing with bioremediation. Pneumatic fracturing is an innovative technology that uses high-pressure air to create artificial fractures in contaminated geologic formations, resulting in enhanced subsurface air flow and transport rates. Following fracturing, the pneumatic fracturing system can also be used to inject electron acceptors and other biological amendments directly into a formation to stimulate biodegradation. The specific bioremediation process evaluated in this project used amendment injections and low-rate in situ vapor extraction to provide oxygen and other supplements, which resulted in the formation of aerobic, denitrifying, and methanogenic biodegradation zones, spatially distributed with increasing distance from the fracture interfaces. A “countercurrent” bioremediation process was thus established with respect to the diffusion of contaminants towards the fracture interface.

A field pilot demonstration of the integrated technologies was carried out at a gasoline refinery site over a 20-month period. Initial site characterization indicated the presence of BTX at concentrations of up to 1,500 mg/kg soil, as well as other hydrocarbons. The soil at the site was overconsolidated clayey silt with very low permeability. The site was pneumatically fractured followed by periodic injections of subsurface amendments over a period of 50 weeks. Results demonstrated that fracturing increased subsurface permeability by an average of 36 times. Information gained from periodic vapor sampling indicated that following subsurface injections, the production of carbon dioxide was enhanced due to increased biological activity. Following a lag phase, the methanogens became active, and an increase in methane production was observed. There was no carbon dioxide or methane detected in the predemonstration vapor samples. The mass of carbon converted to carbon dioxide and methane was used as an independent measure for the depletion of total carbon. Based on this balance, the $C_{generated}$ to $C_{biodegraded}$ ratio was computed to be 3.3:1, indicating that other carbon sources in gasoline also served as substrate and participated in the biodegradation process. After 1 year of process operation and monitoring, soil samples obtained from the site indicated a 79-percent reduction in soil-phase BTX concentrations, and over 85 percent of the BTX reduction was attributed to biodegradation.

Solids Suspension Characteristics Related to Slurry Biotreatment Performances

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Introduction

Slurry biotreatment has been demonstrated to be an effective process for bioremediation of contaminated soils, sediments, and sludges (1). Solid-phase biotreatment such as composting or formal land treatment units cannot compete with slurry treatment for the extent of treatment in short timeframes. Slurry biotreatment has been commonly conducted in reactor systems such as agitated tanks or lined lagoons. Slurry systems provide conditions of improved contact between the pollutant and the microorganisms responsible for the desired biotransformation. The extent of particle suspension by agitation is a crucial factor controlling treatment efficiency. Power input from an impeller system dictates not only the homogeneity of the slurry medium but also the degree of particle suspension. Equally importantly, the power input requirement is an important economic factor in assessing the feasibility of bioslurry treatment for particular solids to be treated (2). Very little attention is given, however, to this important component of the treatment system. This work presents our current technical efforts on identifying the conditions for optimal slurry agitation for bioremediation of contaminated solids.

Results

Four flow regimes in terms of particle suspension characteristics have been identified; in increasing order of impeller power input, they are: nonsuspension, semisuspension, off-bottom suspension, and complete-suspension regimes. Experimental results indicate that unique relationships exist between the flow regimes and power input. In addition, kinetic energy rather than impeller rotational speed dictates particle suspension dynamics in a slurry medium. A flow regime map (Figure 1) is constructed using power input as the primary parameter. At extremely low power inputs, particles remain stationary and settle on the tank bottom. As the power input exceeds a certain value, the upper layer of the settled particles starts to become mobile. With a further increase in power, the layer of settled particles reduces in thickness, and eventually all particles are mobilized with a portion of particles moving along the tank bottom. Such a state corresponds to the minimum off-bottom suspension as conventionally reported in the literature. The suspended particles tend to fall back to the tank bottom, however, due to insufficient momentum transferred from the liquid medium to particles. Dynamics of particles in this regime can be described by an up-and-down motion, and the particle distribution is nonuniform along the height of the slurry tank. An increase in the impeller power input reduces the degree of nonuniformity, and the particle distribution becomes rather uniform as the power input exceeds the minimum complete-circulation value.

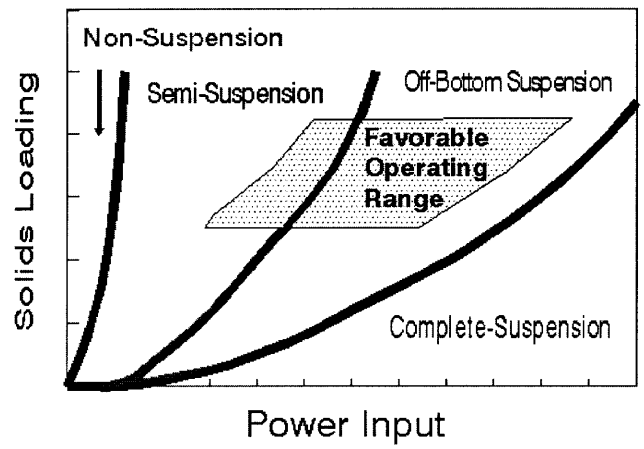

Figure 1. Flow regime map of particle suspension in slurry agitation systems.

Location of impellers (e.g., bottom clearance) greatly affects the particle suspension. A substantial reduction in power input required for both on-bottom and off-bottom particle suspension is obtained as an impeller is placed near the tank bottom. The conventional design of agitated tanks, with bottom clearance equal to the

impeller diameter or tank radius, is inadequate for particle suspension applications.

Conclusion

Feasibility of slurry bioreactors for bioremediation of contaminated solids depends on the energy consumption required to achieve adequate fluid mixing and particulate suspension in the slurry medium. Four particle suspension regimes are identified with respect to the power input to the slurry medium in an agitated tank. Solids properties affecting the suspension behavior include 1) size and shape distribution, 2) density difference, and 3) solids loading. Operation under the complete suspension regime for achieving maximum uniformity of particle suspension may not be necessary because the treatment efficiency may only be marginally improved. This is because mass transfer resistance between particles and the bulk liquid phase is not the only rate-limiting step in the soil-slurry treatment process.

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