BIOREMEDIATION OF HAZARDOUS WASTES RESEARCH, DEVELOPMENT, AND FIELD EVALUATIONS, 1994

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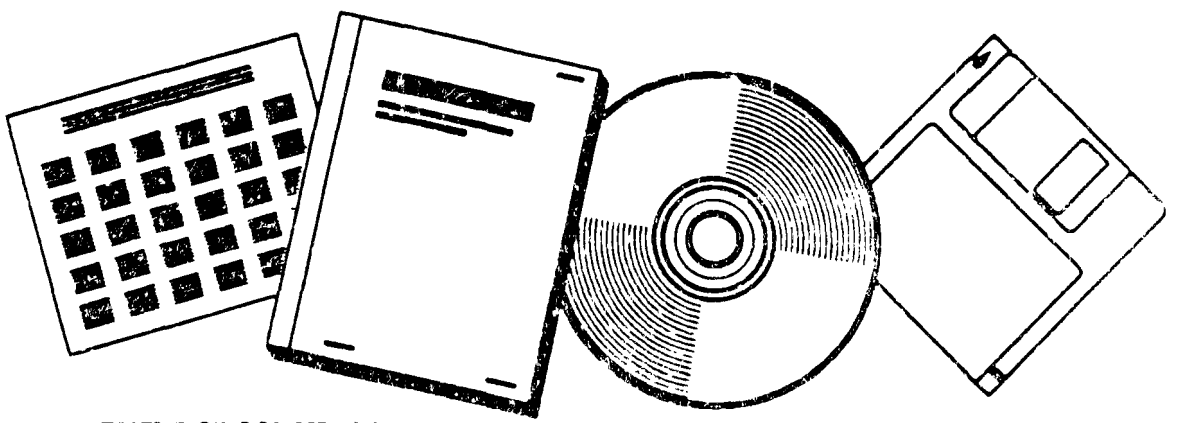

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BIOREMEDIATION OF HAZARDOUS WASTES
Research, Development, and Field Evaluations
1994

Biosystems Technology Development Program
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The proceedings of the 1994 Symposium on Bioremediation of Hazardous Wastes, hosted by the Office of Research and Development (ORD) of the EPA in San Francisco, California. The symposium was the seventh annual meeting for the presentation of research conducted by EPA's Biosystems Technology Development Program (BTDP) and by affiliated Hazardous Substance Research Centers (HSRCs). The document contains abstracts of recent research projects, ranging in scope from laboratory application to cleanup evaluations in the field. 41 papers and numerous posters presented at the symposium are organized into six program areas: Bioremediation Field Initiative, Performance Evaluation, Field Research, Pilot-Scale Research, Process Research, and Hazardous Substance Research Centers. The proceedings also contain a brief synopsis of introductory remarks made by opening speakers.

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RE: **Bioremediation in the Field Bulletin**
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Introduction

Some of the most promising new technologies for solving hazardous waste problems involve the use of bioremediation, an engineered process that relies on microorganisms such as bacteria or fungi to transform hazardous chemicals into less-toxic or nontoxic compounds. Until recently, 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 cleaning up hazardous waste, 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. The Bioremediation Research Program is made up of three major research components: the Biosystems Technology Development Program, the In Situ Application Program, and the Bioremediation Field Initiative.

Related bioremediation studies are being carried out at five EPA Hazardous Substance Research Centers (HSRCs) under the direction of ORD's Office of Exploratory Research (OER). 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. This symposium was held to present and discuss recent developments in bioremediation research undertaken during 1993 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
- Performance Evaluation
- Field Research
- Pilot-Scale Research
- Process Research

In the last section of this document are abstracts of poster presentations on bioremediation research performed as part of the HSRC program.

Executive Summary

The U.S. Environmental Protection Agency's (EPA's) Office of Research and Development (ORD) hosted the seventh annual *Symposium on Bioremediation of Hazardous Wastes: Research, Development, and Field Evaluations*, in San Francisco, California, June 27-29, 1994. The symposium was held in cooperation with EPA's Region 9 offices. More than 500 people attended, including leading bioremediation researchers and field personnel from federal, state, and local agencies as well as representatives from industry and academia. Three speakers opened the symposium with background information on bioremediation research.

Fran Kremer, Coordinator of the Bioremediation Field Initiative, provided an introduction and overview 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. These scientists work out of the following laboratories and organizations, all of which are institutional participants in the program:

- Environmental Research Laboratory–Ada, Oklahoma
- Environmental Research Laboratory–Athens, Georgia
- Center for Environmental Research Information–Cincinnati, Ohio
- Risk Reduction Engineering Laboratory–Cincinnati, Ohio
- Environmental Research Laboratory–Gulf Breeze, Florida
- Health Effects Research Laboratory–Research Triangle Park, North Carolina

A regional perspective on bioremediation was presented by Jeffrey Zelikson, Director of EPA's Region 9 Hazardous Waste Management Division in San Francisco, California. According to Mr. Zelikson, accelerated development and use of innovative technologies are critical to protecting the environment and ensuring the competitiveness of U.S. industry both at home and abroad. Although research is the key, from a regional perspective, two other factors are necessary for su cess. These factors are diffusion of information or "ge ting the word out" and community acceptance. T recent legislative efforts were designed to address the issues: the Environmental Technology Initiative, wh promotes the use of bioremediation, and the Superfu Reform Act, which expands the role of communities decisionmaking at hazardous waste sites across country.

Robert Menzer, Director of the EPA's Environmen Research Laboratory in Gulf Breeze, Florida, discuss ORD's Bioremediation Program. ORD has teamed with the Department of Defense (DOD) and the Depa ment of Energy (DOE) to form the Strategic Resea and Development Program, which provides funds bioremediation projects in the field. Part of this ef involves setting up the former Wurtsmith Air Force Ba (AFB) near Lake Huron in Michigan as a national ce for testing bioremediation research and developme An industry group, the Bioremediation Technologies D velopment Forum, has already committed itself to c ducting field test work at the Wurtsmith site.

The 41 papers delivered at the conference highlight recent program achievements and research proje aimed at bringing bioremediation into more widespre use. Taken as a whole, these topic areas represen comprehensive approach to bioremediation of haza ous waste sites. The presentations were organized i five key research and program areas:

1. *Bioremediation Field Initiative.* This initiative w instituted in 1990 to collect and disseminate perfor ance data on bioremediation techniques from fi application experiences. The Agency assists the gions and states in conducting field tests and carrying out independent evaluations of site clea ups using bioremediation. Through this initiati tests are under way at Superfund sites, Resou Conservation and Recovery Act corrective action cilities, and Underground Storage Tank sites. Ei paper presentations were devoted to this k program area, covering field evaluations at si using bioventing, biochemical techniques, a

bioremediation under a variety of aerobic and anaerobic conditions.

2. *Performance Evaluation* Performance evaluation of various bioremediation technologies involves assessing the extent and rate of cleanup for particular bioremediation methods as well as monitoring the environmental fate and effects of compounds and their by-products. Because attempts to remediate a contaminated site can result in the production of additional compounds, an important aspect of performance evaluation involves assessing the potential health effects of processes. Two papers were presented concerning EPA's Health Effects Research Laboratory (HERL) and its integrated program developed to address the risk of potential health effects and to identify bioremediation approaches that best protect public health.

3. *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 (e.g., cost effectiveness) cannot be thoroughly evaluated until the approach is scaled up and field tested. Four paper and several poster presentations provided information on recent or ongoing field research.

4. *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. As such, it is a necessary step in anticipation of full-scale application of a technology. Given the expanding base of experience with various bioremediation methods, the need for pilot-scale research is increasing. Six papers and numerous posters were presented concerning research based on microcosms of field sites.

5. *Process Research.* Process research involves isolating and identifying microorganisms that carry out biodegradation processes and the environmental factors affecting these processes. Such research is fundamental to the development of new biosystems for treatment of environmental pollutants in surface waters, sediments, soils, and subsurface materials. Twenty-one papers and numerous poster presentations addressed this critical area.

In addition to presentations on research being carried out under the BTDP, the symposium's poster session included presentations from the five EPA Hazardous Substance Research Centers (HSRC). The scientists and engineers involved in the latter program conduct EPA research sponsored by the following centers:

- Northeast Hazardous Substance Research Center (Regions 1 and 2)
- Great Lakes and Mid-Atlantic Hazardous Substance Research Center (Regions 3 and 5)
- South/Southwest Hazardous Substance Research Center (Regions 4 and 6)
- Great Plains and Rocky Mountain Hazardous Substance Research Center (Regions 7 and 8)
- Western Region Hazardous Substance Research Center (Regions 9 and 10)

Section One
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 150 Superfund sites, RCRA corrective action facilities, and Underground Storage Tank sites nationwide, and will soon expand its database to include sites under private sector jurisdiction and international sites. Performance evaluations currently are being conducted at nine sites, six of which were reported on at this symposium.

Investigations at the St. Joseph, Michigan, National Priority List (NPL) site revealed that natural anaerobic degradation of trichloroethylene (TCE) contamination was occurring in ground water at the site. Later sampling was performed to estimate the contaminant mass flux, and to estimate apparent degradation constants. Other studies also were performed to determine whether enhancement of the anaerobic process might be beneficial, what microorganisms are responsible for the natural transformation, and what is an effective primary substrate to add to the ground water for enhancing the remediation in situ.

Other ongoing evaluations include a 3-year field investigation, which began in 1991, of the use of bioventing to remediate jet fuel spills at two Air Force Base (AFB) sites. At the Eielsen AFB near Fairbanks, Alaska, studies were performed to demonstrate bioventing in a cold climate and to evaluate several low-intensity soil warming methods. At the Hill AFB site near Salt Lake City, Utah, studies were performed to investigate bioventing in deep vadose zone soils and to determine the influence of air flow rate on the biodegradation and volatilization rates of organic contaminants.

Research continued on the Reilly Tar and Chemical Corporation site in St. Louis Park, Minnesota, as part of a 3-year evaluation program that began in November 1992. The research is designed to evaluate the potential of bioventing to remediate soils contaminated with wood preservatives. In situ bioremediation of a pipeline spill in Park City, Kansas, using nitrate as an electron acceptor also is being investigated.

At the Libby Ground-Water Site in Libby, Montana, performance evaluation has been completed of full-scale bioremediation of creosote wastes in ground water and soils. This evaluation addressed three separate biological treatment processes: 1) surface soil bioremediation in a prepared-bed, lined treatment unit; 2) treatment of extracted ground water from the upper aquifer in an aboveground fixed-film bioreactor; and 3) in situ bioremediation of the upper aquifer. These three processes represent a treatment train approach to site decontamination, where each process

was chosen for remediation of a specific phase (i.e., soil, oil, and water). Published results of the study will be available from EPA later this year.

Finally, a demonstration study of bioaugmentation of soil contaminated with pentachlorophenol (PCP) using selected strains of lignin-degrading fungi was performed at an abandoned wood treating site in Brookhaven, Mississippi.

The symposium's poster session included information on the Bioremediation in the Field Search System (BFSS), a PC-based software application developed by EPA's Bioremediation Field Initiative. BFSS provides access to a database of information compiled by the Initiative on hazardous waste sites where bioremediation is being tested or implemented, or has been completed.

Intrinsic Bioremediation of TCE in Ground Water at an NPL Site in St. Joseph, Michigan

John T. Wilson, James W. Weaver, and Don H. Kampbell
Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK

The ground water at the St. Joseph, Michigan, National Priority List (NPL) site is contaminated with chlorinated aliphatic compounds (CACs) at concentrations in the range of 10 mg/L to 100 mg/L. The chemicals are thought to have entered the shallow sandy aquifer either through waste lagoons, which were used from 1968 to 1976, or through disposal of trichloroethylene (TCE) into dry wells at the site (1). The contamination was determined to be divided into eastern and western plumes, as the suspected sources were situated over a groundwater divide. Both plumes were found to contain TCE, cis- and trans-1,2-dichloroethylene (c-1,2-DCE and t-1,2-DCE), 1,1-dichloroethylene (1,1-DCE), and vinyl chloride (VC).

Previous investigation of the site indicated that natural anaerobic degradation of the TCE was occurring because of the presence of transformation products and significant levels of ethene and methane (2,3). The purpose of this presentation is to provide the results of later sampling of the western plume near Lake Michigan, to estimate the contaminant mass flux, and to estimate apparent degradation constants. The estimates are based on visualization of the data representing each measured concentration by a zone of influence based on the sample spacing. The presentation of the data is free from artifacts of interpolation, and extrapolation of the data beyond the measurement locations is controlled.

Data Summary

In 1991 three transects (1, 2, and 3 on Figure 1) were completed near the source of the western plume (2).

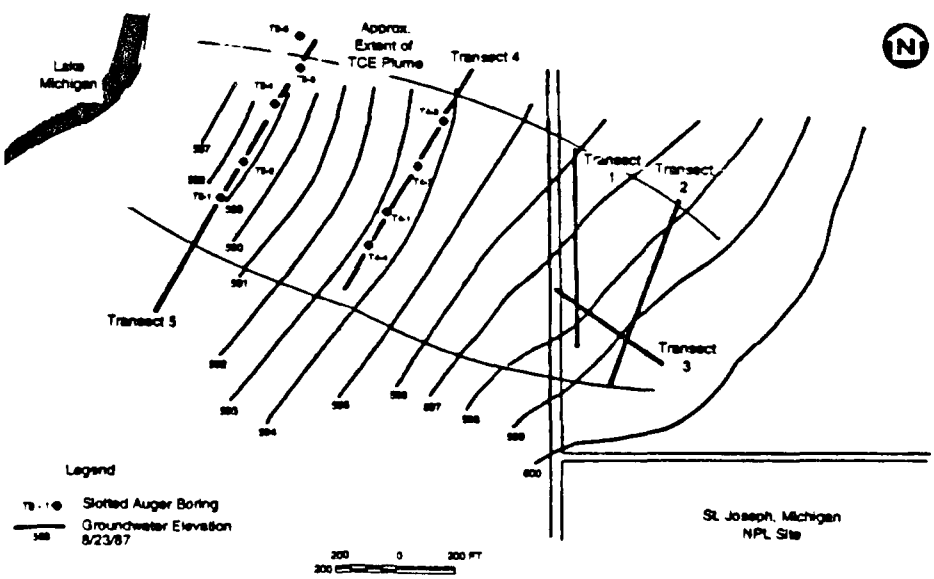

Figure 1. St. Joseph, Michigan, NPL site plan.

The three transects consisted of 17 borings with a slotted auger. In 1992, two additional transects (4 and 5 on Figure 1) were completed consisting of 9 additional slotted auger borings. In each boring, water samples were taken on roughly 1.5 m (5 ft) depth intervals. Onsite gas chromatography was performed to determine the width of the plume and to find the point of highest concentration. Three of the transects (2, 4, and 5) were roughly perpendicular to the contaminant plume. Of the remaining transects, transect 1 crosses the plume at an angle and transect 3 lies along the length of the plume. The perpendicular transects form logical units for study of TCE biotransformation.

The site data from the transects are visualized as sets of blocks centered around the measurement point. The blocks are defined so that the influence of a particular measured concentration extends halfway to the next measurement location both horizontally and vertically. Thus, the presentation of the data is simple and direct. The visualization of the data is performed on a Silicon Graphics Indigo workstation using a two-dimensional version of the fully three-dimensional field-data analysis program called SITE-3D, which is under development at the Robert S. Kerr Environmental Research Laboratory.

The mass of each chemical per unit thickness and the advective mass flux of each chemical are calculated by summing over the blocks. By following this procedure, the measured chemical concentrations are not extrapolated into the clay layer under the site. Neither are they extrapolated beyond a short distance from the measurement locations (5 ft vertically and 50 to 100 ft horizontally). Other interpolation schemes, such as inverse distance weighting or kriging, also could be used to estimate the concentration field and perform the mass estimates. Figures 2 and 3 show the distributions of VC and TCE at transect 5 using a logarithmic, black-and-white "color" scale. Notably, the maximum VC concentration at transect 4 was 1,660 µg/L and at transect 5 was 205 µg/L. The maximum TCE concentration at transect 4 was 8,720 µg/L and at transect 5 was 163 µg/L . As noted previously for other portions of the site (2,4), the contamination is found near the bottom of the aquifer. The highest concentrations of VC and TCE do not appear to be co-located. In Table 1, mass estimates are presented for the perpendicular transects ordered from furthest upgradient (transect 2) to furthest downgradient (transect 5). The data in Table 1 represent the mass in a volume of aquifer that has an area equal to the cross-sectional area of the transect and is 1.0 m thick in the direction of ground-water flow.

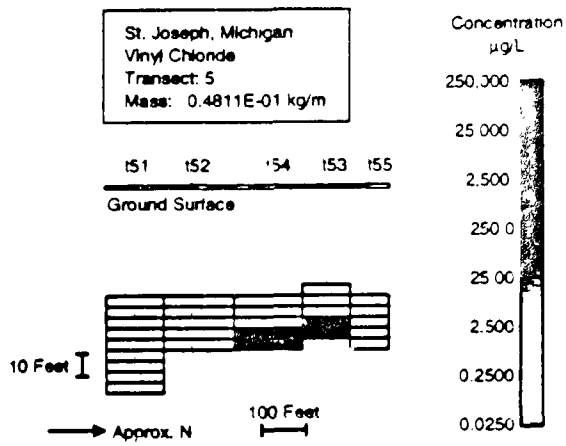

Figure 2. VC distribution at transect 5.

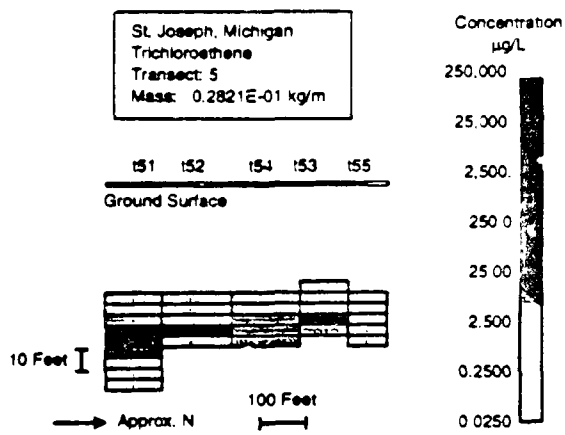

Figure 3. TCE distribution at transect 5.

Advective Mass Flux Estimates

Results from the calibrated MODFLOW model of Tiedeman and Gorelick (4) were used to estimate the ground-water flow velocity at each transect (Table 2). The estimate is an upper bound because the modeled vertical component of flow was neglected in the present analysis. The head drop from one location to the next was assumed to generate horizontal flow only. Tiedeman and Gorelick (4) also represented the aquifer by single values of hydraulic conductivity and porosity. They gave, however, 95 percent confidence limits for the hydraulic conductivity. Well yields estimated for each sample location indicate declining hydraulic conductivity toward the west, i.e., toward Lake Michigan and transects 4 and 5. Thus, using the single parameter values from the MODFLOW simulations may overestimate the flux of water into the lake.

As would be expected, the advective mass fluxes decline toward the downgradient edge of the plume. There, the concentrations are lower due to either transient flow or degradation of the TCE. Notably the mass fluxes

Table 1. Mass per Unit Thickness (kg/m) at St. Joseph, Michigan

Chemical	Transect 2	1	4	5
VC	1.523	1.8969	0.4868	0.04811
1,1-DCE	0.2377	0.0816	0.01451	0.001047
t-1,2-DCE	0.566	0.5059	0.03628	0.007041
c-1,2-DCE	12.32	5.1127	1.890	0.2832
TCE	10.67	5.5804	1.397	0.02821
Methane	5.855	5.4826	4.620	1.373
Ethene	0.6847	0.8925	0.1747	0.004901
Ethane	no data	no data	0.2085	0.001689
TOC	no data	no data	12.63	8.314
Chloride	129.9	148.8	213.1	156.2
Sulfate	37.05	34.376	95.78	66.19
NO_3- Nitrogen	2.904	2.471	4.421	8.247
NH_4- Nitrogen	1.835	2.5609	0.4562	0.2256
TKN- Nitrogen	2.987	3.8357	0.6353	0.3646

using the average hydraulic conductivity result in a total flux of 13 kg/y of TCE, c-1,2-DCE, t-1,2-DCE, 1,1-DCE, and VC at transect 5. This value contrasts with the total flux of these CACs of 310 kg/y at transect 2, near the source of contamination, a 24.4-fold decrease in mass flux of CACs across the site. Given the 95 percent confidence limits on the hydraulic conductivity determined by Tiedeman and Gorelick (4), the total range of mass flux of these five chemicals is from 205 kg/y to 420 kg/y at transect 2 and from 8.4 kg/y to 17 kg/y at transect 5. The range of fluxes at transect 5 is an upper bound on, and the best estimate of, the flux into Lake Michigan.

Apparent Degradation Constants

The mass per unit thickness of TCE at transects 2, 4, and 5 was used to estimate apparent first-order degradation constants. The constants are estimated by applying the first order rate equation

$$\ln\left(\frac{c_{j+1}}{c_j}\right) = \lambda\,\Delta t \qquad (1)$$

to the site data, where c_j is the average concentration in the transect j, c_{j+1} is the average concentration in the downgradient transect j+1, t is the advective travel time for TCE to move between the transects, and λ is the apparent degradation constant. The mass per unit thickness data for TCE and the cross-sectional area were used to determine the average concentrations c_j and c_{j+1} in the up- and downgradient transects. The porosity, bulk density, fraction organic carbon, organic carbon partition coefficient (5), ground-water gradient, and distance between the transects were used to determine the advective travel times. The values used in Equation 1 are given in Table 3. From these quantities, the apparent degradation constant for TCE was determined to be -0.0076/week from transect 2 to 4 and -0.024/week from transect 4 to 5.

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Table 2. Mass Flux (kg/y) at St. Joseph, Michigan

Chemical	Transect 2	Transect 1	Transect 4	Transect 5
VC	18.81	36.03	10.69	1.676
1,1-DCE	2.934	1.551	0.3185	0.03648
t-1,2-DCE	6.995	9.609	0.7963	0.2453
c-1,2-DCE	152.1	97.11	41.48	9.868
TCE	131.7	106.0	30.67	0.9829
Methane	72.29	104.1	101.4	47.86
Ethene	8.453	16.95	3.836	0.1708
Ethane	no data	no data	4.577	0.05885
TOC	no data	no data	277.2	289.7
Chloride	1,604	2,826	4,678	5,444
Sulfate	457.4	652.9	2,102	2,306
NO_3- Nitrogen	35.85	46.93	97.05	287.4
NH_4- Nitrogen	22.66	29.64	10.01	7.861
TKN- Nitrogen	36.88	72.65	13.95	12.70

Table 3. Chemical and Hydraulic Values Used in Estimating Apparent Degradation Rates

Transect	Area with Non-zero TCE Concentration (m^2)	Mass Per Unit Thickness from SITE-3D (kg/m)	Average TCE Concentration in the Transect (kg/m^3) c_i and c_{i-1} in Equation 1	Distance Between Transects (m)	Gradient Estimated from Tiedeman and Gorelick (1993)	[a]Retarded Seepage Velocity for TCE (m/d)	Estimated Travel Time Between Transects (weeks) Δt in Equation 1
2	1,592	0.67	6.70e-3	—	—	—	—
				260	7.3e-3	0.11	340
4	2,774	1.397	5.04e-4	—	—	—	—
				160	1.1e-2	0.156	145
5	1,943	0.0282	1.44e-5	—	—	—	—

[a]Constants used in seepage velocity calculation:
Hydraulic conductivity: 7.5 m/d
Retardation factor for TCE: $1.78 = 1 + K_{oc} f_{oc} \rho_b / \theta$
Porosity θ, : 0.30
Bulk density ρ_b: 1.86 g/cm^3
K_{oc}: 126 mL/g
f_{oc}: 0.001

Enhanced Reductive Dechlorination of Chlorinated Ethenes

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Reductive dehalogenation of trichloroethylene (TCE) to cis-1,2-dichloroethylene (c-1,2-DCE), trans-1,2-dichloroethylene (t-1,2-DCE), vinyl chloride (VC), and ethene was found to be occurring at a site in St. Joseph, Michigan, by indigenous microbial populations under anaerobic conditions (1). This has raised two possibilities for further study: 1) that the natural anaerobic processes at the site may be sufficient to bring about site remediation alone; or 2) that the natural process will be incomplete without some enhancement. Further site characterization is now under way by the EPA Robert S. Kerr Environmental Research Laboratory to determine the extent of natural onsite transformation. This study aims to determine whether enhancement of the anaerobic process might be beneficial, what microorganisms are responsible for the natural transformation, and what is an effective primary substrate to add to the ground water for enhancing the remediation *in situ*. For comparison, aquifer material from a site in Victoria, Texas, also is being evaluated. This site is contaminated by tetrachloroethylene (perchloroethylene, or PCE) and is being actively bioremediated by the addition of benzoate and sulfate (2).

Methods

Aquifer material for this study was obtained aseptically in the absence of oxygen from both St. Joseph and Victoria sites. The potential of the St. Joseph aquifer material for TCE transformation and the effect of adding different primary substrates were studied using 25 mL test tubes as small laboratory columns (3). The fluid within the test tubes was exchanged after incubation periods ranging from 1 to 4 months with filter-sterilized site ground water that was amended with a primary substrate and TCE. Control columns received TCE-amended, filter-sterilized ground water without an added primary substrate. Between fluid exchanges, the openings were sealed, and the columns were incubated without fluid exchange in a room temperature anaerobic glovebox containing 1 to 10 percent hydrogen. Each primary substrate was fed to yield 100 mg/L chemical oxygen demand (COD) to provide similar reducing equivalents for each column. Each column was fed only one substrate from the time the column was prepared.

In addition, microcosms consisting of 125 mL bottles containing aquifer material and site ground water were used to simulate *in situ* conditions with the Victoria aquifer material. Only 110 mL of saturated aquifer material was used in the bottles to allow for sampling of the liquid from the remaining 15 mL, and to provide for bed fluidization during mixing. These microcosms were incubated without headspace.

Enrichments were developed by the addition of Victoria aquifer-material to a basal medium (4). This enrichment was subsequently transferred to aquifer-material-free media. The effect of different metabolic inhibitors was studied using an inoculum from a benzoate enrichment culture into 160 mL bottles filled with 120 mL of defined media amended with PCE, benzoate, yeast extract, and the respective inhibitor.

Results

The possibility of enhancing biodegradation by the addition of various primary substrates was studied using columns of St. Joseph material. Table 1 shows the resulting concentrations of TCE dechlorination products after a typical 6-week incubation period. Following this exchange, the ethanol-fed column was switched to benzoate and immediately performed similar to the column that had been fed benzoate from the start.

Of the primary substrates tested, benzoate addition consistently stimulated the most complete dechlorination. Similar results were obtained with the microcosms containing Victoria aquifer material (data not shown). No significant lag time before the onset of dechlorination was observed with either material.

In the St. Joseph unfed column control, partial dechlorination of TCE to c-1,2-DCE was observed over several exchanges spanning several months. This dechlorination may have been associated with oxidation of natural

Table 1. Concentration of TCE Dechlorination Products after 6 Weeks of Incubation in St. Joseph Aquifer Material Columns*

Added Substrate	Compounds Remaining after 6 Weeks of Incubation (μM)					
	TCE	cDCE	1,1-DCE	VC	Ethene	Sum
None	20.5	4.8	0	0	0	25.3
Benzoate	0	0	0	11.6	14.4	26.0
Lactate	0.5	4.1	0	13.5	5.8	23.9
Sucrose	2.4	5.4	0.7	16.1	5.1	29.7
Ethanol	3.6	3.7	0.6	14.1	2.4	24.4
Methanol	9.3	6.3	0.7	7.9	1.6	25.8
Acetate	10.4	3.4	0.9	7.1	1.9	23.7

*x-1,2-DCE was also present in some columns in trace amounts.

organics within the aquifer material or of hydrogen that diffused into the column from the glovebox gases. Victoria microcosms also showed some dechlorination of PCE to TCE in the unfed controls.

For column studies with St. Joseph material, site ground water was used that included 0.49 mM nitrate and 0.50 mM sulfate. During incubation in the substrate-amended columns, nitrate and sulfate were consumed completely, and varying amounts of methane were produced. Nitrate also disappeared in the unfed control, but no sulfate was consumed or methane produced. Dechlorination accounted for less than 2 percent of the substrate utilized; nitrate reduction, sulfate reduction, and methanogenesis accounted for the rest.

After several exchanges, the primary substrate-fed columns became clogged. Small entrapped bubbles were visible in the columns as well as a noticeable amount of black precipitate. Considering the amount of primary substrate added to the columns, up to about a fifth of the pore volume could have been filled by methane formation. The extent of the clogging caused by iron sulfide precipitate or biomass is unknown, but after a few months, during which the columns sat unfed, the entrapped bubbles visibly decreased and the columns became unclogged. Bubbles also formed in the Victoria microcosms, but they were allowed to come to the surface during daily shaking and were removed during analysis.

PCE was not dechlorinated within 2 months in microcosms containing a defined mineral media amended with only benzoate, while the addition of benzoate and 0.05 percent yeast extract stimulated dechlorination of all the PCE completely to ethene (data not shown). The addition of benzoate and sulfate stimulated partial dechlorination, as did the addition of yeast extract alone.

Studies of the effects of various metabolic inhibitors were conducted to better understand the role of sulfate-reducing and methanogenic bacteria. Table 2 lists duplicate live bottles from a 3-month incubation with 0.416 mM benzoate, 0.01 percent yeast extract, and various amendments, including 2 mM sulfate, 0.5 mM bromoethanesulfonic acid (BESA), and 0.5 mM molybdate, where applicable. No dechlorination was observed in uninoculated or sterile controls. t-1,2-DCE and 1,1-dichloroethylene were not observed in the enrichment cultures.

Summary and Conclusions

Studies with aquifer material from both contaminated sites have shown that all primary substrates tested were capable of stimulating dechlorination of some PCE or TCE to ethene, with benzoate consistently stimulating the most complete degradation. High sulfate concentrations appear to inhibit dechlorination, although no dechlorination was observed in microcosms incubated without some sulfate or yeast extract. The addition of molybdate reversed sulfate inhibition, but here dechlorination stopped at c-1,2-DCE. These data show that the anaerobic dechlorination of PCE or TCE to ethene can be enhanced by the appropriate addition of a primary substrate and yeast extract or sulfate.

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Table 2. Effects of Inhibitors on Dechlorination*

Amendments	Moles Remaining in Duplicate Bottles after Incubation					
	PCE	TCE	cDCE	VC	Ethene	Sum
Benzoate and Yeast Extract	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	1.70 1.76	1.70 1.76
Benzoate, Yeast Extract, and BESA	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	1.63 1.62	1.63 1.62
Benzoate, Yeast Extract, and Molybdate	0.05 0.01	0.06 0.01	1.52 1.65	0.00 0.00	0.00 0.00	1.63 1.67
Benzoate, Yeast Extract, and Sulfate	1.06 1.07	0.30 0.31	0.15 0.13	0.00 0.00	0.00 0.00	1.51 1.50
Benzoate, Yeast Extract, Sulfate, and Molybdate	0.00 0.00	0.00 0.00	1.78 1.65	0.00 0.00	0.00 0.00	1.78 1.65
Benzoate, Yeast Extract, Sulfate, and BESA	0.37 0.96	0.33 0.35	0.24 0.24	0.00 0.00	0.00 0.00	1.54 1.55

*Values for PCE and its dechlorination from duplicate cultures incubated for 3 months at room temperature.

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Bioventing of Jet Fuel Spills I: Bioventing in a Cold Climate with Soil Warming at Eielson AFB, Alaska

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Bioventing is a process that supplies oxygen *in situ* to oxygen deprived soil microbes by forcing air through unsaturated contaminated soil at low flow rates (1). Unlike soil venting or soil vacuum extraction technologies, bioventing attempts to stimulate biodegradative activity while minimizing stripping of volatile organics, thereby destroying the toxic compounds in the ground. Previous work (2) has demonstrated that biodegradation rates associated with bioventing are temperature dependent. Briefly, the goal of the current study is to demonstrate bioventing in a cold climate and to evaluate several low-intensity soil warming methods for the ability to maintain greater than average soil temperatures and rates of biodegradation.

The EPA Risk Reduction Engineering Laboratory, with resources from EPA's Bioremediation Field Initiative, began a 3-year field study of *in situ* bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Eielson Air Force Base (AFB) near Fairbanks, Alaska. The site has JP-4 jet fuel contaminated unsaturated soil where a spill has occurred in association with a fuel distribution network. The contractor operating the project is Battelle Memorial Institute, Columbus, Ohio. This report summarizes the first 2½ years of operation.

Methodology

Site history, characterization, installation, and monitoring were summarized previously (3,4,5). Figure 1 shows a plan view of the project.

Briefly, four 50 ft *x* 50 ft test plots have been established, all receiving relatively uniform injection of air. The four test plots are being used to evaluate three soil warming methods:

- *Passive warming:* Enhanced solar warming in late spring, summer, and early fall using a clear plastic covering over the plot; and passive heat retention the remainder of the year by applying insulation to the surface of the plot.
- *Active warming:* Warming by applying heated water from soaker hoses 2 ft below the surface. Water is applied at roughly 35°C and at an overall rate to the plot of roughly 1 gal/min. Five parallel hoses 10 ft apart deliver the warm water. The surface is covered with insulation year-round.
- *Buried heat tape warming:* Warming by heat tape buried at a depth of 3 ft and distributed throughout the plot 5 ft apart. The tape heats at a rate of 6 W/ft, giving a total heat load to the plot of roughly 1 W/ft^2.

The contaminated control consists of contaminated soil vented with injected air with no artificial method of heating.

The passively heated, actively heated, and control test plots were installed in the summer of 1991, and the heat tape plot was installed in September 1992. Air injection/withdrawal wells and soil gas and temperature monitoring points are distributed throughout the site. (See Figure 1.) Heating of the actively heated plot was

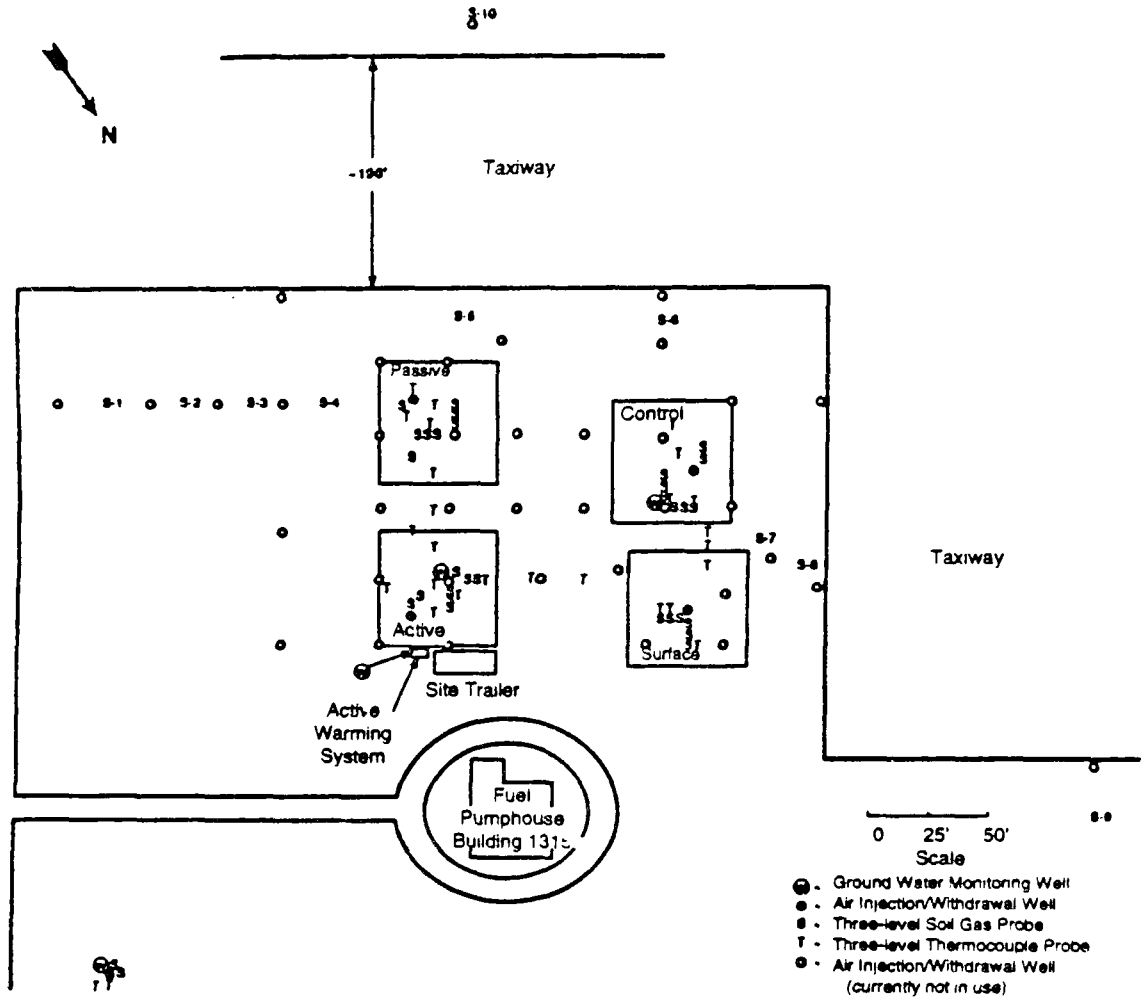

Figure 1. Plan view of the EPA/U.S. Air Force bioventing system at Eielson AFB near Fairbanks, Alaska. "S" represents three-level soil gas monitoring points, "T" represents three-level temperature probes, and "o" and "•" represent inactive and active air injection wells, respectively. Instrumentation in the lower left is the uncontaminated background location.

discontinued in July 1993 to compare heated and unheated biodegradation rates at the same location.

Periodically *in situ* respirometry tests (6) are conducted to measure *in situ* oxygen uptake rates by the microorganisms. These tests allow estimation of the biodegradation rate as a function of time and, therefore, as a function of ambient temperature and soil warming technique. The rate of oxygen use can be converted into the rate of petroleum use by assuming a stoichiometry of biodegradation (4). Quarterly comprehensive and monthly abbreviated *in situ* respiration tests were conducted.

Final soil hydrocarbon analyses will be conducted in the summer of 1994 and compared with initial soil analyses to document actual hydrocarbon loss due to bioventing.

Results

Evaluation of Soil Warming Methods

Figure 2 displays the average temperature of each plot and at an uncontaminated background location as a function of time during the study. By applying warm water to the plot, the temperature of the actively heated plot was maintained in the range of 10°C to 25°C, compared with the contaminated (unheated) control plot where the minimum winter temperature is roughly 0°C. When heating of the actively heated plot was terminated in July 1993, its temperature followed the temperature of the unheated control plot closely, as expected.

The ability to control temperature in the passively heated plot was not as successful. The temperature of the

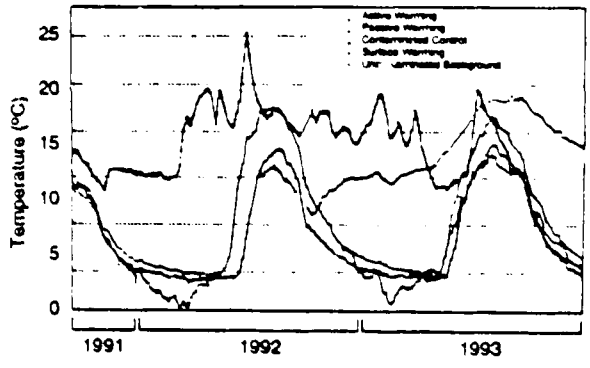

Figure 2. Average temperature of each plot and at an uncontaminated background location at the Eielson AFB bioventing site as a function of time during the study.

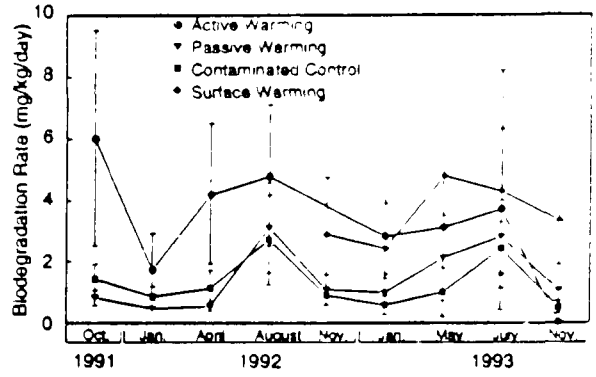

Figure 3. Average rate of jet fuel biodegradation of each plot at the Eielson AFB bioventing site, as measured by in situ respirometry, as a function of time during the study.

passively heated plot roughly mimicked the contaminated control plot temperature except during the summer of 1992, when the passively heated plot was roughly 5°C warmer than the control plot. The insulation applied during the winter has been marginally successful at best, providing 1°C to 2°C temperature elevation in the passively heated plot relative to the control plot.

Heating by buried heat tape in the surface heated plot has been successful at maintaining temperatures between 10°C and 22°C year-round. Temperatures achieved in this plot in the summer were much higher than those maintained in the winter because, although the heat input was constant, the ambient temperature was much higher in the summer.

Rate of Biodegradation

The rate of jet fuel biodegradation, estimated by *in situ* respirometry tests, as a function of time for each plot is shown in Figure 3. The influence of temperature on the rate is clear: the actively warmed and surface warmed plots maintained rates two to three times greater than the unheated control plot year-round. The small difference in temperature between the passively warmed and the control plots (see Figure 2) is reflected in the small difference in respective rates measured in these plots.

Researchers commonly believed that bioremediation systems should be shut down for the winter in any cold climate because microbial activity is thought to approach zero at these low temperatures. The rate was nonzero (roughly 0.5 mg/kg/day), however, in the unheated control plot in the middle of winter in Alaska, when the average temperature of the plot was roughly 0°C (see Figure 2).

After July 1993, when heating of the actively warmed plot was discontinued, the rate observed in this plot was not significantly different than the rate measured from the unheated control plot, consistent with the similar temperatures of these two plots.

Conclusions

Application of warm water and heat generated by electrical resistance has been successful at maintaining summer-like temperatures in the soil year-round. The enhanced temperatures in the plots provided elevated rates of biodegradation. The passively warmed plot has performed only marginally better than no heating (the contaminated control) with respect to temperature and rate.

At the conclusion of this study, a cost-benefit analysis will be conducted to compare the performance of the heating methods in terms of rate enhancement versus cost of heating.

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Bioventing of Jet Fuel Spills II: Bioventing in a Deep Vadose Zone at Hill AFB, Utah

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Bioventing is a process that supplies oxygen *in situ* to oxygen deprived soil microbes by forcing air through unsaturated contaminated soil at low flow rates (1). Unlike soil venting or soil vacuum extraction technologies, bioventing attempts to stimulate biodegradative activity while minimizing stripping of volatile organics, thus destroying the toxic compounds in the ground. Bioventing technology is especially valuable for treating contaminated soils in areas where structures and utilities cannot be disturbed because bioventing equipment (air injection/withdrawal wells, air blowers, and soil gas monitoring wells) is relatively noninvasive.

The EPA Risk Reduction Engineering Laboratory, with resources from EPA's Bioremediation Field Initiative, began a 3-year field study of *in situ* bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Hill AFB near Salt Lake City, Utah. The site has JP-4 jet fuel contaminated unsaturated soil, where a spill occurred in association with overfilling of an underground storage tank. The contractor operating the project is Battelle Laboratories, Columbus, Ohio. This report summarizes the first 2 ½ years of the study.

The objectives of this project are to increase our understanding of bioventing large volumes of soil and to determine the influence of air flow rate on biodegradation and volatilization rates of the organic contaminant.

Methodology and Results

See previous reports (2,3) for additional details.

Site Description/Installation

The site is contaminated with JP-4 from depths of approximately 35 ft to perched water at roughly 95 ft. Here, bioventing, if successful, will stimulate biodegradation of the fuel plume under roads, underground utilities, and buildings without disturbing these structures. A plan view of the installation is shown in Figure 1. The single air injection well installed in December 1990, continuously screened from 30 ft to 95 ft below grade, is indicated.

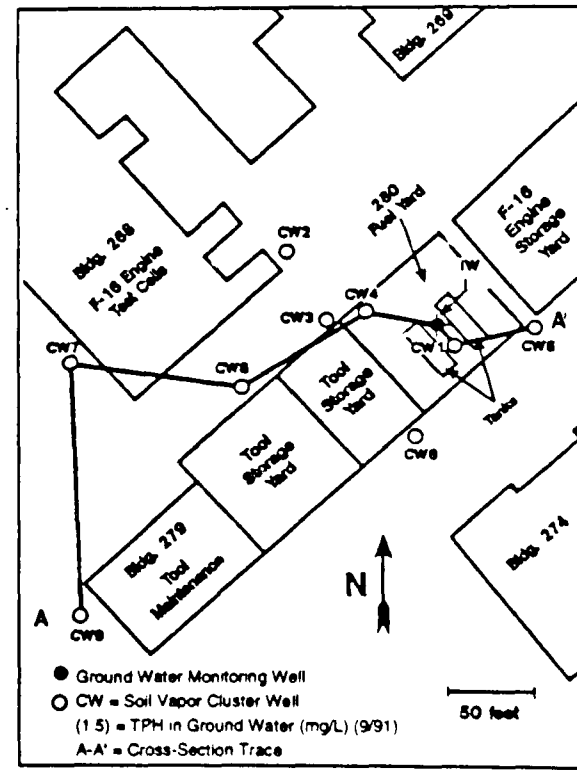

Figure 1. Plan view of the joint EPA and U.S. Air Force bioventing activities at Hill AFB, near Salt Lake City, Utah. IW is the air injection well, and CW are cluster soil gas monitoring wells.

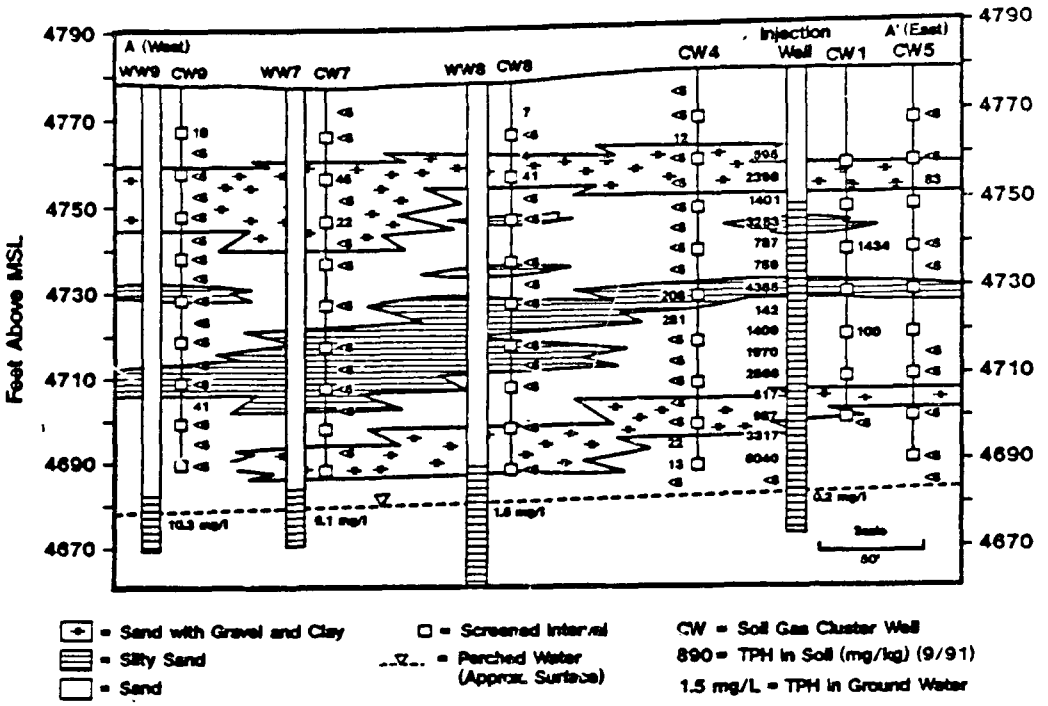

Figure 2. Cross-section view of the bioventing installation at Hill AFB. Cross section follows the path AA' in Figure 1. Initial soil TPH concentrations measured at various depths at the wells are indicated.

"CW" wells are soil gas "cluster wells," where independent soil gas samples can be taken at 10-ft intervals from 10 ft to 90 ft deep; CW1 through CW3 were installed in April 1991, CW4 through CW9 were installed in September 1991. A cross section of the site along path AA' in Figure 1 is shown in Figure 2. The injection well and the soil gas monitoring wells are indicated. Initial soil total petroleum hydrocarbon (TPH) concentrations measured from the locations indicated are given also.

Air Injection

To determine the influence of air injection rate on biodegradation and volatilization rates, various air injection rates have been used during this study:

- August 1991 to October 1992 and December 1992 to April 1993, 67 ft^3/min
- October to December 1992 and April to June 1993, 40 ft^3/min
- July to November 1993, 117 ft^3/min
- November 1993 to present, 20 ft^3/min

Soil Gas Composition

Monthly soil gas measurements during venting are conducted. Soil gas O_2, CO_2, and total hydrocarbons are measured at each depth in all wells, providing a three-dimensional map of soil gas composition in the vadose zone.

In Situ Respiration Tests

For each flow rate used, an *in situ* respirometry test (4) is conducted to evaluate the *in situ* biodegradation rate. Rates are measured at each soil gas monitoring location. Table 1 shows rates at three original well locations averaged over depth versus time over a 2-year period. These wells are close enough to the injection well that changes in the air injection flow rate did not significantly change oxygen levels at these locations (data not shown). Lower rates with time suggest that bioventing is removing petroleum hydrocarbons from the site at a significant rate.

Operational Paradigm for Bioventing in Deep Vadose Zones

Bioventing of this system appears to degrade jet fuel by two mechanisms: 1) providing oxygen for bioremediation

Table 1. Rates of Biodegradation, Averaged over the Depth and Measured by In Situ Respirometry, at the Three Original Soil Gas Monitoring Wells

Well	Rate (mg/kg/day) September 1991	September 1992	October 1993
CW1	1.1	0.59	0.31
CW2	0.26	0.13	0.16
CW3	0.54	0.26	0.12

of jet fuel contaminated soils near the injection well (Figure 2); and 2) transporting oxygen and volatilized jet fuel components into the surrounding, relatively uncontaminated soils (Figure 2), where the organic vapors are biodegraded. Other studies have demonstrated *in situ* hydrocarbon vapor biodegradation (5-8). Evidence also exists here to support this operational paradigm. Based on soil gas measurements averaged from August and September 1993 from all depths in all monitoring wells, Figure 3 shows CO_2 produced versus O_2 consumed as the air stream passes from the injection well to the monitoring point. The approximately linear relationship indicates that oxygen is being converted stoichiometrically to carbon dioxide at all locations, contaminated or not. Thus, hydrocarbon vapors are degraded as they are transported through the uncontaminated soils.

Based on data taken in April and September 1991, a preliminary best-fit linear model for the rate of oxygen uptake versus soil gas TPH and soil TPH was developed:

$$\text{Rate}(\%O_2/\text{hr}) = 2.5 \times 10^{-5}\, C_{\text{soil gas TPH}}(\text{ppmv}) + 5.7 \times 10^{-6}\, C_{\text{soil TPH}}(\text{mg/kg}) \quad (1)$$

where $C_{\text{soil gas TPH}}$ and $C_{\text{soil TPH}}$ are soil gas TPH and soil TPH concentrations, respectively. Clearly, the soil gas hydrocarbon vapors contribute significantly to the total oxygen demand. Thus, jet fuel vapor degradation is a significant mechanism for total jet fuel removal at Hill AFB. The rate function Rate($C_{\text{soil gas TPH}}$,$C_{\text{soil TPH}}$) is plotted in Figure 4. This model will be reassessed as additional soil gas data are reviewed.

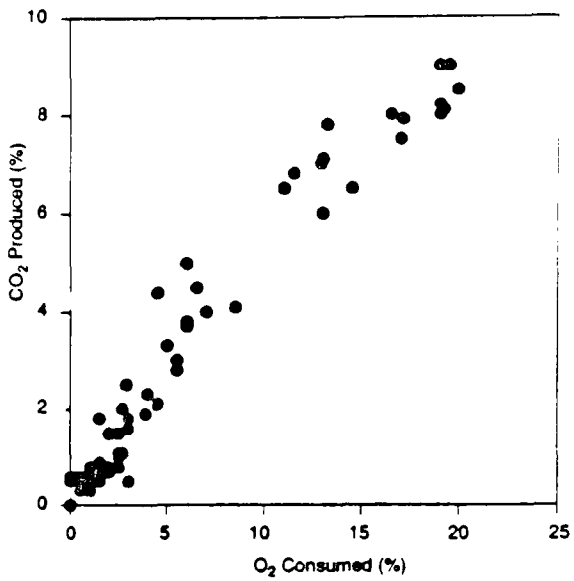

Figure 3. CO_2 produced versus O_2 consumed as the air stream passes from the injection well to each soil gas monitoring point. Data indicate biological activity at all soil gas monitoring well locations.

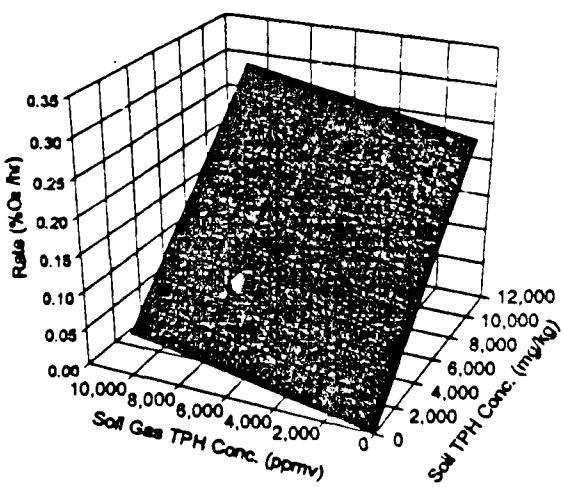

Figure 4. Plot of the model (Equation 1), the rate of oxygen use as a function of soil gas TPH and soil TPH levels.

Soil Sampling

Final soil hydrocarbon analyses will be conducted in the summer of 1993 and compared with initial soil analyses to document actual hydrocarbon loss due to bioventing.

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In Situ Bioremediation of a Pipeline Spill Using Nitrate as the Electron Acceptor

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In the late 1970s, leakage of refined petroleum products from an underground pipeline contaminated approximately 24,000 square meters of a shallow water-table aquifer in Park City, Kansas. Aerobic *in situ* bioremediation was initiated but was unsuccessful due to plugging of the injection wells or sediments adjacent to the well screen by gas and iron precipitates.

Nitrate was selected as an alternative electron acceptor that might avoid some of the problems with plugging.

Approach

Ground water from the aquifer was amended with sodium nitrate and ammonium chloride and returned to the area of the hydrocarbon spill through a series of infiltration wells that were installed in a grid. The wells were spaced 6.1 m apart. The study area contained 157 infiltration wells, spaced over 5,800 m^2, which received 3,000 m^3 of water in a tracer test, followed by 39,400 m^3 of water containing 4,136 kg of sodium nitrate (an average of 17 mg/L nitrate nitrogen). The circulated water also contained 50 to 60 mg/L sulfate.

Figure 1 plots the cumulative flow of ground water to the infiltration wells against time. Flow was unhindered for the first 150 days of operation, then the system plugged over the next 100 days.

A total of 7.3 m of recharge was applied to the spill, of which 6.8 m contained nitrate.

Procedure to Distinguish Flushing from Biodegradation of BTEX

The site was cored, and vertically stacked continuous cores from the same borehole were analyzed to determine the total mass of benzene, toluene, ethylbenzene, and xylene (BTEX) compounds in the aquifer. To estimate the mass of BTEX compounds in ground water in contact with the hydrocarbon spill, monitoring wells were installed in the boreholes used to acquire the cores. The screened interval on the monitoring well was equivalent to the depth interval containing NAPL hydrocarbons.

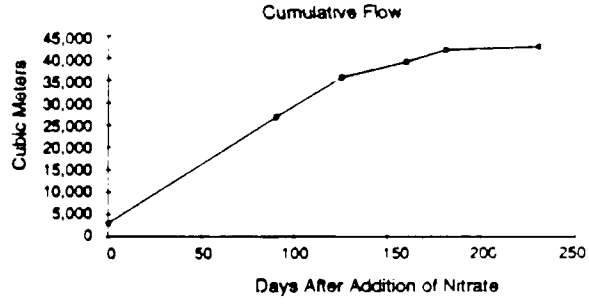

Figure 1. Cumulative flow of ground water amended with nitrate to the study area (m^3).

The following procedure was used to determine the total mass of BTEX compounds in the aquifer under a unit surface area. The concentrations of BTEX compounds in individual core samples (g/kg) were multiplied by the vertical interval that each core represented (M), then multiplied by the bulk density of sandy aquifer material (1,800 kg/m^3). The masses in the depth intervals represented by the cores then were summed to determine the total mass of each BTEX compound under each square meter (Table 1).

The concentration of BTEX compounds in water under each square meter was determined by multiplying a square meter by the length of the well screen to determine the volume sampled, then by 0.3 to estimate the volume of ground water, then by the concentration of BTEX compounds in ground water sampled from the well (Table 1). The volume of aquifer sampled by the well to estimate mass in ground water and the volume summed to estimate total mass were equivalent.

The ratio of mass in water to total mass determines the fraction of total mass that can be flushed away each time water in the sampled volume is exchanged by the infiltrating ground water (Table 1).

The volume of water in the sample volume was considered equivalent to a pore volume in a column experiment; the infiltration of ground water was expressed in

Table 1. Concentration of BTEX Compounds in Ground Water and in the Aquifer at Site 60A, the Most Contaminated Site in the Study Area*

Compound	Mass in Water (g/m^2)	Total Mass (g/m^2)	Water/ Total
Benzene	2.01	17.6	0.114
Toluene	2.57	102	0.0252
Ethylbenzene	1.02	72	0.0142
p-Xylene	0.958	68	0.0141
m-Xylene	1.26	161	0.00783
o-Xylene	0.776	78.3	0.00991

* Subsurface concentrations are expressed as the total mass in the vertical interval under a square meter of land surface area.

pore volumes. The mass of each BTEX compound remaining after one pore volume of flushing should equal the initial mass, multiplied by 1.0 minus the ratio of mass in water to total mass. The mass of each BTEX compound remaining after any number of pore volumes of flushing should equal the initial total mass, multiplied by 1.0 minus the ratio of water/total, raised to an exponent equal to the number of pore volumes flushed through the spill.

$$\text{Final Mass} = \text{Initial Mass}\,(1.0 - \text{Water/Total})^{\text{pore volume}}$$

This approach was used to predict the reduction in contaminant concentration because of flushing and to separate the effects of flushing from biodegradation. More than 90 percent of benzene was removed from ground water during the demonstration. However, flushing accounted for most, if not all, of this removal (Figure 2). More than 95 percent of toluene and ethylbenzene was removed, and biodegradation accounted for most of the removal (see Figure 3 for toluene removal). Removal of xylenes varied from 68 to 76 percent; most of the removal was accounted for by biodegradation (Figure 4).

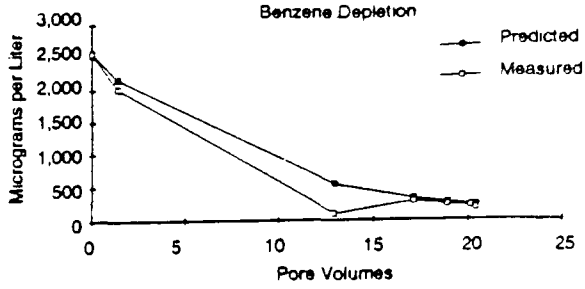

Figure 2. Comparison of benzene depletion to that expected from flushing alone. Concentrations in µg/L.

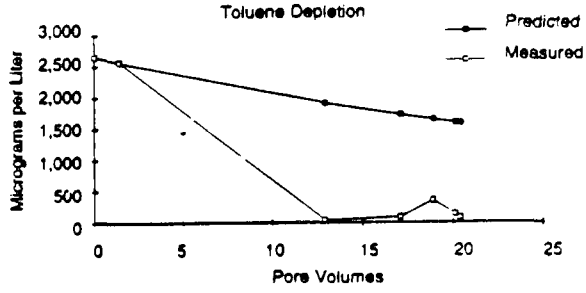

Figure 3. Comparison of toluene depletion to that predicted from flushing alone. Concentrations in µg/L.

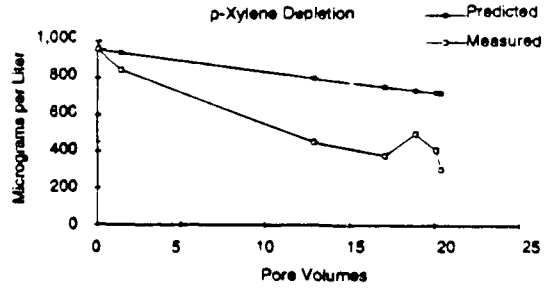

Figure 4. Comparison of p-xylene depletion to that expected from flushing alone. Concentrations in µg/L.

Estimate of Treatment Effectiveness

If the concentration of BTEX compounds in ground water and in the NAPL are in equilibrium, Raoult's Law can be used to put an upper boundary on the total mass of contaminant removed by *in situ* bioremediation. Concentrations of individual BTEX compounds were compared before and after remediation to determine fractional removal in ground water. The fractional removals in ground water were multiplied by the initial total mass of each BTEX compound to estimate total mass removals.

The amount of BTEX degraded during denitrification is equivalent to the amount of nitrate-nitrogen applied. Apparently, considerably more BTEX was removed than could be explained by the quantity of nitrate supplied (Table 2). In fact, there was more removal than could be accounted for by either denitrification or flushing. Sulfate in well 60A was less than 1.0 mg/L prior to the start of infiltration; during infiltration concentrations ranged from 57 mg/L to 93 mg/L. During the course of the demonstration, concentrations of sulfate in monitoring well 60G in the study area were near 10 mg/L, when concentrations of sulfate were in the range of 50 mg/L to 60 mg/L in the infiltrated water. Removal of 40 mg sulfate per liter by sulfate reduction could have accounted for as much as 230 gm/m^2 of total BTEX removal. If this is the case, naturally occurring sulfate in the infiltrated ground water was more important as an electron acceptor than the nitrate that was intentionally added. Concentrations of methane in the infiltrated water ranged from 4.8 mg/L to 6.3 mg/L while concentrations in well 60A ranged from

Table 2. Use of Raoult's Law to Estimate the Total Mass of Contaminants Removed by Nitrate-Based Bioremediation at 60A, the Most Contaminated Site in the Study Area

Compound	Concentration in Well 60A (µg/L)		Fraction Removed from Water	Initial Concentration in Core Material	Mass Removed
	Initial	Final	(percent)	(gm/m^2)	(gm/m^2)
Benzene	2,010	174	0.913	17.6	16.1
Toluene	2,570	77.9	0.970	102	98.9
o-Xylene	776	209	0.732	78.3	57.2
m-Xylene	1,260	297	0.764	161	123
p-Xylene	958	304	0.683	68	46.4
Ethylbenzene	1,020	26.5	0.974	72	70.2
Total BTEX removed					411.2
Maximum attributed to nitrate as electron acceptor					118
Maximum attributed to flushing					131
Balance, attributed to sulfate as electron acceptor					163

2.8 mg/L to 3.7 mg/L. Methanogenesis cannot explain the missing mass of BTEX compounds.

The assumption of chemical equilibrium also may be in error, and much of the BTEX may not have been in contact with the ground water. In this case, the total BTEX removed would be overestimated, and the nitrate demand that was exerted would represent that portion of the hydrocarbons that exchanged readily with the ground water.

Performance Evaluation of Full-Scale In Situ and Ex Situ Bioremediation of Creosote Wastes in Ground Water and Soils

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The Champion International Superfund Site in Libby, Montana, was nominated by the Robert S. Kerr Environmental Research Laboratory as a candidate site for performance evaluation as part of the EPA-sponsored Bioremediation Field Initiative. Two forms of wood preservatives were used at the site: creosote, containing polycyclic aromatic hydrocarbons (PAHs), and loose pentachlorophenol (PCP). PAHs are currently the primary components of concern at the site. The performance evaluation project is directed by Dr. Ronald Sims of Utah State University.

The bioremediation performance evaluation consisted of three phases: 1) summarize previous and current remediation activities; 2) identify site characterization and treatment parameters critical to the evaluation of bioremediation performance for each of the bioremediation treatment units; and 3) evaluate bioremediation performance based on this information.

Three biological treatment processes are addressed in the bioremediation performance evaluation: 1) surface soil bioremediation in a prepared-bed, lined land treatment unit (LTU); 2) treatment of extracted ground water from the upper aquifer in an aboveground fixed-film bioreactor; and 3) *in situ* bioremediation of the upper aquifer at the site. A description of the site with accompanying figures appears in the abstract book from the 1993 EPA-sponsored Symposium on Bioremediation of Hazardous Wastes (1).

Biological Treatment Processes

The LTU has been used for bioremediation of contaminated soil taken from three primary sources, including tank farm, butt dip, and waste pit areas. Contaminated soil was excavated and moved to one central location, the waste pit. Soil pretreated in the waste pit area is further treated in one of two prepared-bed, lined land treatment cells (LTCs). Total estimated contaminated soil volume for treatment is 45,000 yd^3 (uncompacted). Contaminated soil cleanup goals (dry-weight basis) are 1) 88 mg/kg total (sum of 10) carcinogenic PAHs; 2) 8 mg/kg naphthalene; 3) 8 mg/kg phenanthrene; 4) 7.3 mg/kg pyrene; 5) 37 mg/kg PCP, and 6) 0.001 mg/kg 2,3,7,8-dioxin equivalent.

The LTU comprises two adjacent 1-acre cells. Components of the soil bioremediation system for each LTC include the treatment zone, liner system, and leachate collection system. Each cell is lined with low-permeability materials to minimize leachate infiltration from the unit. Contaminated soil is applied and treated in lifts (approximately 9-in. thick) in the designated LTC. When reduction of contaminant concentrations in all lifts placed in the LTU has reached the cleanup goals specified in the Record of Decision (ROD), a protective cover will be installed over the total 2-acre unit and maintained in such a way as to minimize surface infiltration, erosion, and direct contact.

Degradation rates, volume of soil to be treated, initial contaminant concentration, degradation period, and LTC size determine the time required for remediation of a given lift. Based on an estimated 45-day ti...e frame for remediation of each applied lift as determined by Champion International, an estimated 45,000 yd^3 of contaminated soil, and a 2-acre total LTU surface area, the projected time to complete soil remediation is 8 to 10 years.

The upper aquifer aboveground treatment unit provides biological treatment of extracted ground water for removal of PAHs and PCP prior to reinjection via an infiltration trench. The biological treatment consists of two fixed-film reactors operated in series. The first reactor is heated and has been used for roughing purposes, while the second has been used for polishing and reoxygenation of the effluent prior to reinjection. The system was commissioned in February 1990.

Extracted ground-water treatment system components include equalization and biotreatment. Equalization system components include four ground-water extraction wells and an equalization tank, which consists of a cylindrical horizontal flow tank with a nominal hydraulic residence time of 6 hours at a flow rate of 10 gpm. The bioreactor treatment system components include nutrient amendment, influent pumping, bioreactor vessels, aeration, heating, and effluent pumping. The components of the aboveground treatment system for extracted ground water are shown in the 1993 Symposium abstract book (1).

The pilot upper aquifer area *in situ* bioremediation system involves the addition of oxygen and inorganic nutrients to stimulate the growth of microbes. The initial source of oxygen was a hydrogen peroxide injection system that was designed to maintain a concentration of approximately 100 mg/L of hydrogen peroxide. Injection flow rate was approximately 100 gpm into three injection clusters. Inorganic nutrients in the form of potassium tripolyphosphate and ammonium chloride are continuously added to achieve concentrations in the injection water of 2.4 mg/L nitrogen and 1 mg/L phosphorus.

The ROD calls for cleanup levels in the upper aquifer of 40 parts per trillion (ppt) total carcinogenic PAHs, 400 ppt for total noncarcinogenic PAHs, 1.05 mg/L for PCP, 5 µg/L for benzene, 50 µg/L for arsenic, and a human health threat no greater than 10^{-5} for ground-water concentrations of other organic and inorganic compounds.

Performance Evaluation Activities

Performance of the soil bioremediation system in the LTCs involved evaluating the reduction in concentration of PAHs and PCP with time and with depth within the LTU. The primary purpose of the LTU soil sampling program in this project was to determine the statistical significance and extent of contaminated soil treatment at this site. A quantitative expression of data variability is necessary to determine an accurate estimate of biodegradation of these contaminants at field scale. Such an expression will allow data generated to be used by others to help estimate the biodegradation potential of similar type wastes under similar conditions at other sites.

In most soils and disturbed soil materials, physical and chemical properties are not distributed homogeneously throughout the volume of the soil material. The variability of these properties may range from 1 percent to greater than 100 percent of the mean value within relatively small areas. Chemical properties, including contaminants, often have the highest variability. A first approximation of the total variance in monitoring data can be defined by the following equation:

$$V_t = V_s/k + V_a/k*n$$

where k is the number of samples, n the number of analyses per sample, k*n the total number of analyses, V_t the total variance, V_a the analytical variance, and V_s the sample variance. In general, sampling efforts to minimize V_t result in the most precision. Analytical procedures frequently achieve precision levels ($V_a/k*n$) of 1 to 10 percent, while soil sampling variation (V_s) may be greater than 35 percent. Sampling designs that reduce the magnitude of V_s should be employed where possible. Therefore, the sampling procedures used in this evaluation were designed to minimize V_s and to provide representative information about the transformation of PAHs and PCP within the LTCs.

The LTU was sampled in May, June, July, and September 1991, and in September 1992. Field-scale investigations concerning PAH and PCP concentrations were supported by laboratory mass-balance investigations of radiolabeled compounds for determination of mineralization as well as humification potential for target contaminants.

Performance evaluation of the upper aquifer aboveground fixed-film treatment system involved evaluating the bioreactor system. Treatment evaluation focused on characterizing performance regarding system capability to remove PAHs and PCP from the ground water, and on optimizing operation within the bioreactors. The aboveground treatment system was sampled during 1991 and 1992 for chemical, physical, and biological parameters. In addition, a pilot-scale reactor was constructed and operated to evaluate abiotic reactions of chemicals present in the water phase within the bioreactors. The information generated from the sampling and monitoring of the full-scale reactor and from the operation of the pilot-scale reactor was combined with data provided by Champion International to provide an in-depth evaluation of performance.

Performance evaluation of the *in situ* bioremediation system focused on characterization of the water phase, the solid phase (aquifer materials), and oil associated with the aquifer solid material. The aquifer was sampled during 1991 and 1992. An evaluation of the water phase included measurements of dissolved oxygen (DO) concentrations, the inorganic chemicals iron and

manganese to evaluate potential abiotic demand for injected hydrogen peroxide, and the concentrations of PAHs and PCP. An evaluation of the aquifer solid phase has included PAHs and PCP concentrations in treated and background areas at the site. Laboratory mass balance experiments using radiolabeled target compounds were used in conjunction with field-scale measurements to provide additional information concerning biotic reactions (mineralization) and potential abiotic reactions (poisoned controls).

Summary of Results

Analyses of more than 300 soil samples were performed from which greater than 5,000 individual chemical concentrations were determined for the 16 priority pollutant PAH compounds using gas chromatography/mass spectrometry (GC/MS) and for pentachlorophenol (PCP) using a gas chromatography/electron capture detector (GC/ECD). Results of chemical analyses indicated that target remediation levels for target chemicals were achieved using mean values at each depth evaluated in each LTC, with only two exceptions where mean concentrations were only slightly higher than the target remediation levels. As a result of obtaining vertical samples at each sampling event, downward migration of target chemicals through the LTU was not observed. Soil within the LTU was detoxified to control uncontaminated soil levels. Toxicity information was based upon results of using both the Microtox assay to measure water extract toxicity and the Ames *Salmonella typhimurium* mammalian microsome mutagenicity assay (Ames assay) to measure mutagenicity of soil solvent extracts. Detoxification to nontoxic levels was evident in all samples evaluated for both Microtox and Ames assays.

Results of the laboratory evaluation of soil microbial metabolic potential demonstrated that PCP and phenanthrene, the two chemicals evaluated using radiolabeled carbon, could be metabolized to carbon dioxide by indigenous microorganisms present in the contaminated soil matrix present at the site at temperature and moisture conditions representative of the site. In addition, significant volatilization of PCP or phenanthrene is unlikely based upon the laboratory evaluation. The information obtained in the laboratory evaluation corroborated the interpretation of apparent decrease in target chemical concentrations in field samples within the LTU and in the *in situ* aquifer samples at the Libby site as due to biological processes rather than physical/chemical processes.

Results of the aboveground fixed-film bioreactor indicated that removal of PCP and PAHs from extracted ground water was strongly influenced by hydraulic retention time (HRT). The system removed greater than 80 percent of PCP and 90 percent of PAHs at a flow rate of 10 gpm, with an HRT of 30 hours. At a flow rate of 10 gpm, the effluent concentrations of PCP and total PAHs were 0.3 mg/L to 0.9 mg/L and less than detection (30 µg/L), respectively. When the flow rate was increased to 15 gpm, with an HRT of 20 hours, removal of both PCP and PAHs decreased significantly. At the 15-gpm flow rate, effluent concentrations of PCP and total PAHs were 6 mg/L to 12 mg/L and 0.6 mg/L to 6 mg/L, respectively. Additional limitations of DO and nutrients are addressed in the final report.

Results of the *in situ* treatment evaluation indicated that, with respect to the ground-water phase, total PAHs and PCP were present at lower concentrations in wells considered to be under the influence of the treatment injection system consisting of nutrients and hydrogen peroxide, while total PAHs and PCP were present at higher concentrations in wells considered to be outside of the influence of the injection system. An evaluation of the water phase in monitoring wells demonstrated the presence of reduced inorganic compounds, including iron and manganese, with concentrations inversely related to DO concentrations. These chemicals may exert a demand on the oxygen supplied by the hydrogen peroxide and reduce the oxygen available for microbial utilization.

With respect to the nonaqueous phase liquid (NAPL) phase, both total PAHs and PCP were found in the highest concentrations in the NAPL, greater than 10,000 mg/L and 1,000 mg/L, respectively, than in any other phase sampled at the Champion International Site. These results indicate that there is potential contamination of the upper aquifer remaining in the form of a nonaqueous phase that represents significant potential contamination of the ground water by transfer of contaminants from the NAPL phase to the ground-water phase.

Total PAH and total petroleum hydrocarbons (TPH) were present within the aquifer sediment/NAPL samples at concentrations of 5 mg/kg to 687 mg/kg and 70 mg/kg to 2,525 mg/kg, respectively. The heterogeneous distribution of total PAH, PCP, and TPH contaminants was consistent among three boreholes evaluated from the water table to the deepest sampling point. Target chemicals associated with sediment/NAPL interfaces may be more difficult to bioremediate *in situ* than chemicals in the aqueous phase due to limitations of mass transport of oxygen and nutrients from the water phase to the NAPL phase that contain target chemicals.

Chemical mass balance evaluations conducted using radiolabeled target chemicals in the laboratory demonstrated that aquifer materials from the site contained indigenous microorganisms that had the ability to mineralize phenanthrene. Up to 70 percent of the radiolabeled carbon became incorporated into the aquifer matrix and was nonsolvent extractable. No significant phenanthrene mineralization or incorporation of radiolabeled

carbon was observed in poisoned controls. PCP mineralization, however, was insignificant (less than 2 percent), with results similar for nonpoisoned and poisoned samples.

The three biological treatment processes evaluated at the Libby, Montana, site represent a treatment train approach to site decontamination, where each of the treatment processes are biological. The soil phase is treated in the LTU system, and any leachate produced can be treated in the aboveground bioreactor before it is returned to the LTU as part of soil moisture content control and treatment of low levels of PAHs and PCP in the effluent. The *in situ* treatment system addresses the oil and solid phases in the subsurface. At the Libby site, therefore, a different biological process was chosen for remediation of each contaminated phase (soil, oil, and water).

Performance Evaluation Reports

While the extended abstract presented in this report has been abridged concerning site characterization and treatment results, separate reports have been prepared for EPA that address each of the three biological treatment systems at the site in detail: 1) soil bioremediation in the prepared-bed LTU; 2) aboveground fixed-film system for extracted ground water; and 3) *in situ* treatment. Information generated from full-scale characterization and monitoring, pilot-scale studies, and laboratory treatability studies was combined with information provided by Champion International to provide an integrated evaluation of bioremediation performance at the Libby, Montana, site. The information obtained can be used to evaluate and select rational approaches for characterization, implementation, limitations, and monitoring of bioremediation at other sites.

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Bioventing Soils Contaminated with Wood Preservatives

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The Reilly Tar and Chemical Corporation operated a coal tar distillation and wood preserving plant, known as the Republic Creosote Company, in St. Louis Park, Minnesota, from 1917 to 1972. During this period, wastewater discharges as well as drips, spills, and dumping from the wood preserving processes resulted in creosote and coal tar contamination of about 80 acres of this site and the underlying ground water. In 1972, the City of St. Louis Park purchased the site from the Reilly Tar and Chemical Corporation for land use. All onsite buildings were dismantled and removed, and the soil was graded and covered with 3 ft of topsoil for beautification and odor control.

In 1978, the Minnesota Department of Health began analysis of ground water from municipal wells in St. Louis Park and neighboring communities for carcinogenic and noncarcinogenic polycyclic aromatic hydrocarbons (PAHs). The discovery of significant concentrations of regulated PAHs in six St. Louis Park wells resulted in their shutdown during the period of 1978 to 1981. St. Louis Park is currently maintaining gradient control of the contaminated ground-water plume by pumping and treating. With the exception of a tar plug in one well, little PAH source contamination has been removed. Without source control of the PAHs, pumping and treating of contaminated ground water may be required for several hundred years.

Background

Bioventing is a proven technology for *in situ* remediation of various types of hydrocarbon contaminants. The technology has been used successfully to remediate sites contaminated with gasoline (1), aviation fuels (JP-4 and JP-5) (2,3), and diesel fuel (4). A biological treatment process, bioventing uses low-rate atmospheric air (or oxygen enriched air up to 100-percent oxygen) injection to treat contaminated unsaturated soil *in situ*. The air flow provides a continuous oxygen source that enhances the growth of aerobic microorganisms naturally present in soil, with minimal volatilization to the atmosphere of any volatile organic compounds that may be present in the soil. The size of the treatment area is defined by the number of wells installed, the size of the air blower used, and site characteristics such as soil porosity. The current research evaluates the potential of bioventing to remediate soils contaminated with PAHs.

Methods

Site Description

A 50 ft *x* 50 ft control and a 50 ft *x* 50 ft bioventing treatment plot were established on the site during the original soil gas survey (Figure 1). The first 3 ft of soil at the test plots is uncontaminated topsoil applied after the cessation of industrial use (Figure 2). A dense, 3-in. to 6-in., hard-packed layer separates the topsoil from the porous sandy layer, which extends to below the water table (8 ft to 10 ft below the ground surface). Most of the PAH contamination was found in the sandy layer.

PAH Sampling

Composite soil samples (120 soil borings per plot) were taken for PAH analysis and prepared by homogenizing the soil obtained from the 4 ft to 8 ft depth of each boring. The resultant boreholes were filled immediately with bentonite. The PAH soil analyses were recorded as zero-time PAH concentrations.

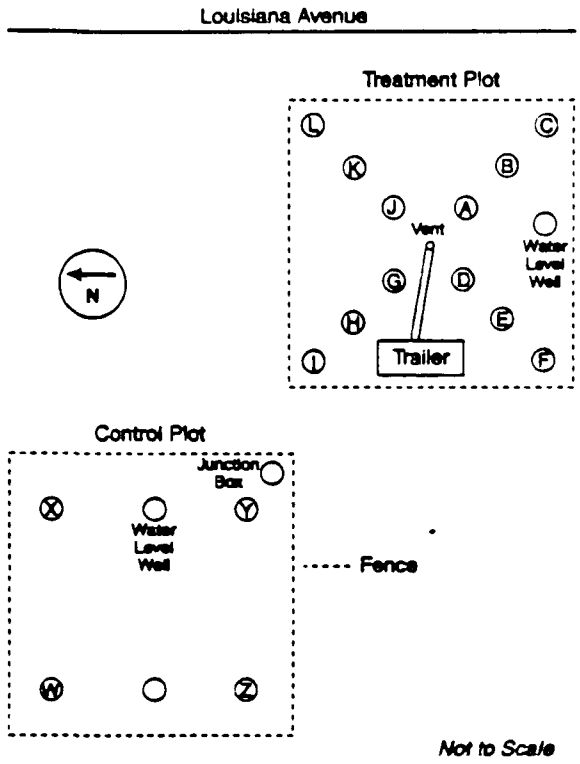

Figure 1. Placement of injection and soil gas sampling wells in the control and treatment plots.

Venting Well

A single-vent bioventing system was installed at the center of the treatment area (Figure 2). The vent (injection) well was screened from 7 ft to 11 ft below the surface and packed with sand. The vent well then was sealed with bentonite from the 5 ft depth to the surface.

Soil Gas Sampling Well

Twelve soil gas probes were installed along diagonals drawn from the corner of the square treatment area (Figure 2), and four were installed in the corners of the no-treatment control area. The soil gas probes were constructed so that the soil gas withdrawal points and thermocouples were located at 4, 6, and 8 ft below the ground surface.

Respirometry

Initial O_2 and CO_2 measurements were obtained using stainless steel gas probes withdrawing air from measured intervals below the ground surface to Gas Teck O_2 and CO_2 meters. The gas measurements were expressed as percentages of total soil gas. Gas samples for the zero-time sampling in November were extracted using the newly installed soil gas sampling wells. Initial sampling indicated that, due at least in part to the highly pervious soil at the Reilly site, injected air was migrating from the test plot 125 ft to 180 ft into the unaerated

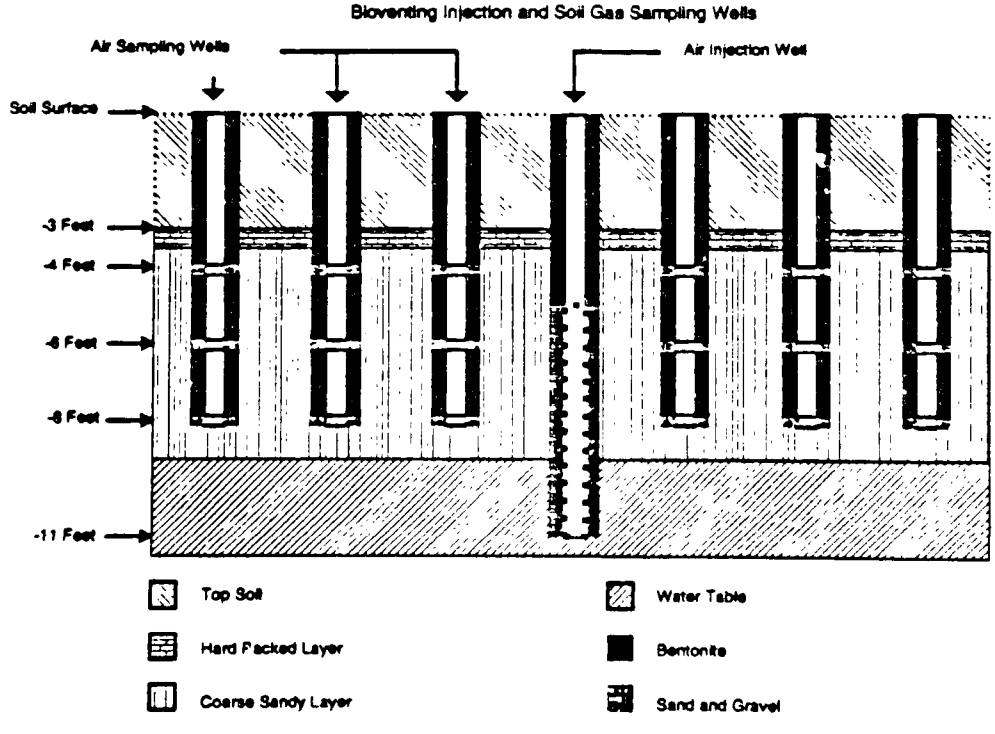

Figure 2. Air injection and soil gas sampling wells installed in the treatment plot.

control plot. A 10-ft deep bentonite slurry wall was constructed across the near wall of the control plot. The slurry wall and reduced air injection pressures and flow rates effectively prevented further unwanted aeration of the control plot.

Shutdown Respiration Tests

Shutdown respiration tests are being conducted for 2 weeks at quarterly intervals. Soil gases are brought to atmospheric O_2 and CO_2 levels in the test plots by pumping ambient air into the ground. When ambient O_2 and CO_2 levels are achieved and documented, the air flow into the ground is stopped. Soil gases levels are taken over measured intervals until an O_2 utilization rate is defined. The air flow was set at 10 ft^3/min, which translated at this site to a pressure of 3.5 in. of H_2O.

Results

In the summer of 1992, a field team from the Risk Reduction Engineering Laboratory (RREL), Biosystems Branch, conducted a soil gas survey at the Reilly site and determined that soil gases were below the estimated 5-percent oxygen threshold required for aerobic metabolism (5). Under a cooperative project involving the Bioremediation Field Initiative, the Superfund Innovative Technology Evaluation (SITE) Demonstration Program, and RREL's Biosystems Program, a pilot-scale bioventing field demonstration for PAH bioremediation was initiated at the Reilly site in November 1992.

Soil PAH analysis demonstrated significant contamination in both plots. The treatment plot demonstrates an order-of-magnitude decrease in PAH concentration on the eastern side of the plot. The control plot is contaminated to about the same degree as the western half of the treatment plot.

Quarterly shutdown respiration tests have shown respiration rates ranging from below detection (Figure 3) to 0.484 percent O_2 per hour (Figure 4). The highest respiration rates were found in the western half of the treatment area, where PAH contamination also was shown to be the heaviest. Current average measured respiration rates are consistent with a 14-percent reduction in PAH contamination per year.

Summary and Conclusion

A 3-year evaluation program was initiated in November 1992 with the zero-time sampling. *In situ* respiration tests are being performed four times each year to determine oxygen utilization and CO_2 evolution rates. These data can be converted to estimated biodegradation rates to estimate the disappearance of PAHs (6). Because of the strong partitioning of PAHs to soil, long-term bioventing is expected to be necessary to fully remediate the site. The target PAH removal rate for this 3-year project is 30 percent. Successful achievement of this rate would project total cleanup in 10 to 15 years.

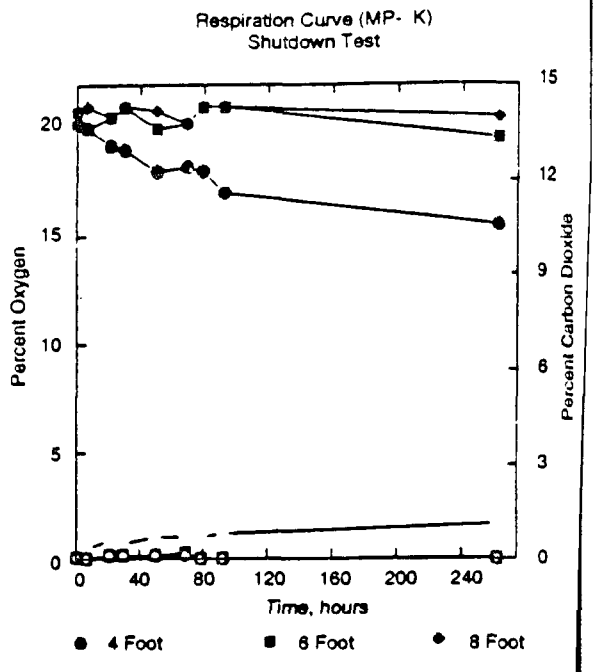

Figure 3. Solid symbols represent O_2. Hollow symbols represent CO_2.

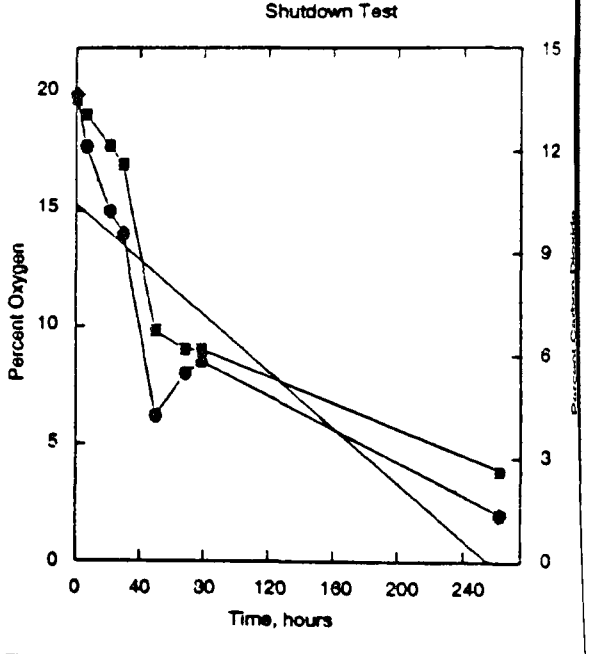

Figure 4. Solid symbols represent O_2. Hollow symbols represent CO_2.

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Field Evaluation of Fungal Treatment Technology

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Bioaugmentation of soil contaminated with pentachlorophenol (PCP) using selected strains of lignin-degrading fungi has been shown to result in extensive and rapid decrease in the PCP concentrations for two soils under field treatment conditions (1,2). In different soils studied under laboratory conditions, the same behavior was observed and extensively evaluated by means of determining the pollutant mass balance in the soils (3,4). Initial products of fungal biotransformation were identified. PCP concentrations in excess of 1,000 mg/kg were 80 to 90 percent biotransformed in soil by selected fungi in 56 days (Figure 1).

A two-phase project, consisting of a treatability study in 1991 and a demonstration study in 1992, was conducted at an abandoned wood treating site in Brookhaven, Mississippi, to evaluate fungal treatment effectiveness under field conditions. The study site, located 60 miles south of Jackson, was identified as a removal action site for EPA Region 4. While the wood treating facility was in operation, two process liquid lagoons were drained and excavated. The sludge was mounded above the ground surface in a Resource Conservation and Recovery Act (RCRA) hazardous waste treatment unit. The excavated material provided the contaminated soil for both phases of the project. The demonstration phase was undertaken as a Superfund Innovative Technology Evaluation (SITE) Program Demonstration Project.

The fungal treatment processes reported herein were conducted at Brookhaven because the site characteristics were suitable for conducting field investigations, not because the investigators desired to promote fungal treatment as one of the treatment options for the site.

Methodology

The demonstration study was designed to evaluate the ability of a single fungal strain *(Phanaerochaete sordida)*

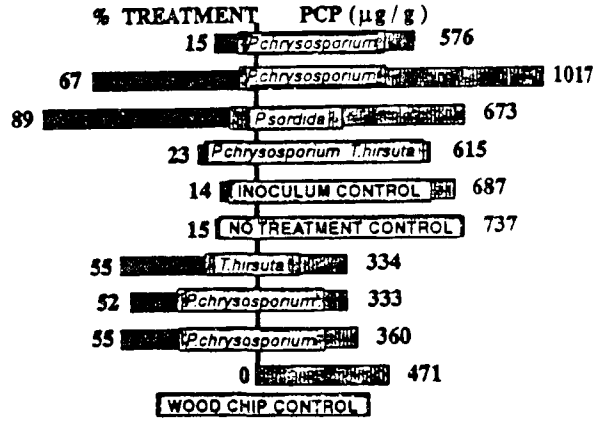

Figure 1. Treatability study performance.

to degrade PCP in soil. The soil pile was sampled and analyzed for PCP and creosote components (i.e., polycyclic aromatic hydrocarbons [PAHs]) prior to developing the test site. Analysis of the laboratory results identified sections of the pile with PCP concentrations of less than 700 mg/kg. These sections were used to supply the contaminated soil for both phases of the study.

A test location was constructed on an uncontaminated portion of the wood treating site. The base for the test plots was formed by using uncontaminated soil to provide a 1-percent to 2-percent slope to promote better drainage. Soil beds (Figure 2) were constructed of galvanized sheet metal. For the demonstration study, the *P. sordida* treatment plot measured 30.5 m *x* 30.5 m and the treatment and inoculum control plots measured 7.6 m *x* 15.25 m. Plot dimensions were determined in conjunction with SITE program personnel. A concrete

pad was constructed to assist tiller entry into the different plots and to decontaminate the tiller as it was moved from plot to plot.

Within each plot, the base soil was graded for a V-shaped indentation in the central portion of the plot to permit leachate collection. A leachate collection system was installed to direct the liquid discharge from all test plots to a central location for testing and treatment. After installation of the leachate system, 25 cm (10 in.) of clean sand was layered into each test plot followed by a 25-cm (10-in.) lift of contaminated soil.

The treatment plot received 10 percent by weight of an infested inoculum containing *P. sordida*. The no-treatment control received no amendments. The inoculum control plot consisted of contaminated soil amended with noninfested inoculum carrier. All plots were tilled on the same schedule, weather permitting. The fungal inoculum was developed jointly with the L.F. Lambert Spawn Co. of Coatesville, PA. The prepared inoculum and inoculum carrier were shipped to the site by refrigerated transport.

The contaminated soil was sized through a 2.5-cm (1-in.) mesh screen using a Read Screen All shaker screen having a capacity 8.4 m^3/hr (10 yd^3/hr). The soil was deposited in separate piles on a polyethylene tarp. Further homogenization was accomplished by mixing different portions of screened soil. The soil then was mixed with the 10 percent by weight fungal inoculum in a Reel Auggie Model 2375 Mixer and applied to the treatment plots using a front end loader.

After inoculation with fungi, each plot was irrigated and tilled with a garden rototiller. Soil moisture was monitored on a daily basis throughout the study and maintained at a minimum of 20 percent. Ambient and soil plot temperatures were recorded daily throughout the study. Plot tilling was scheduled on a weekly basis for the duration of the study. A time series analysis of treatment performance was accomplished by sampling the plots before application of the treatments, immediately after treatment application, and after 1, 2, 4, 8, 12, and 20 weeks of operation (Figure 3).

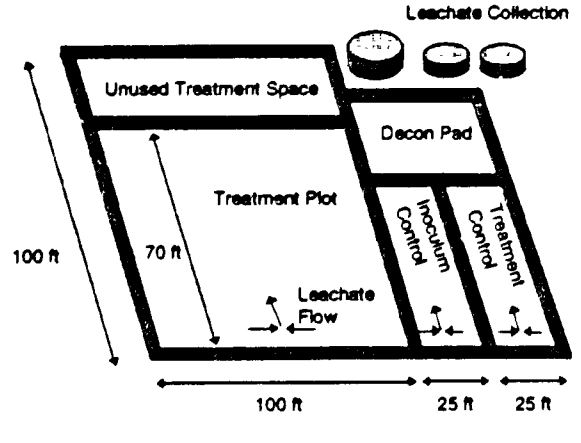

Figure 2. Brookhaven demonstration treatment plot perspective.

Results

The demonstration study was conducted over a 5-month period between June and November 1992. The greatest removal of PCP (Table 1) was achieved in the plot inoculated with *P. sordida*. Over the course of the study, this treatment regime produced 69-percent transformation of PCP from the contaminated soil initially having a pH of 3.8. Significant precipitation occurred throughout the study, leading to unexpected excursions from the prescribed treatment protocol specified by the Risk Reduction Engineering Laboratory (RREL) Forest Products Laboratory (FPL) developers. Lack of tilling clearly compromised the ability to evaluate the fungal treatment technology.

Information collected by both the SITE program and the RREL/FPL effort demonstrated that fungal activity in the treatment plot was significantly lower than expected at the beginning of the study. Fungal activity in the inoculum control increased significantly during the study, which is most likely attributable to infestation with a wild-type fungal species.

Summary demonstration removal data for the soil contaminants is presented in Table 1 for the treatment using *P. sordida*. Concentration decreases of the three- and four-ring PAHs were consistently greater following fungal treatment. Larger ring PAHs persisted in both the treatment and control plots.

Summary and Conclusions

Treatment of PCP by fungal application had a significantly greater effect when compared with controls. Loss of fungal activity was detected in both the fluorescein

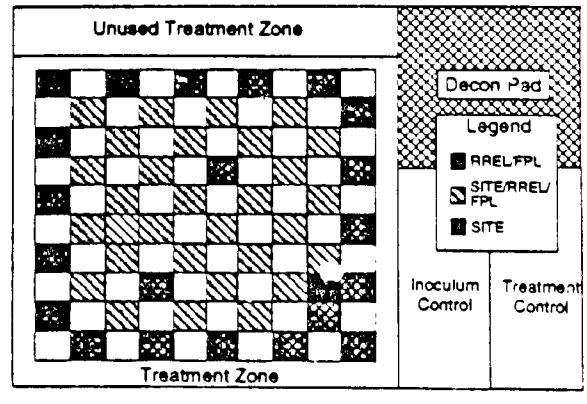

Figure 3. Sampling plan layout.

Table 1. Summary Results for Demonstration Study (5,6)

Analyte	Percentage Removal		
	No Treatment Control	Inoculum Control	Treatment (*P. sordida*)
PCP.	13	71	69
(RREL1/FPL data)	19	30	69
2-Ring PAHs	70	48	46
3-Ring PAHs	83	72	64
4-Ring PAHs	46	67	58
5-Ring PAHs	14	25	27
Total PAHs	65	66	59

diacetate and ergosterol analyses (Figures 4 through 8). The specified RREL/FPL treatment protocol could not be followed in the required time frame because of excessive precipitation during the testing period. The missing component of the protocol was the specified tilling of the treatment beds. The treatment data clearly show that the inoculum control was infested with a wild-type fungal species, which contributed to the biotransformation of the targeted pollutants in that plot.

Treatment by the selected fungal species was observed for PCP concentrations in excess of 1,000 mg/kg, which is greater than any reported concentrations treated using bacterial inocula (Figure 9). Despite the remarkable differences in soil composition and characteristics for the Wisconsin and Mississippi sites, consistent biotransformations of 80 to 90 percent were observed for PCP. One notable soil feature that apparently does not affect fungal treatment is soil pH, which, for the Wisconsin and Mississippi sites, was 3.5 and 9.2, respectively.

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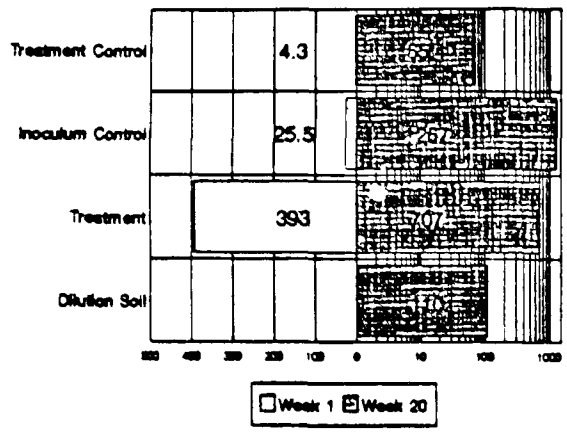

Figure 4. Total fungal biomass (mg/kg) by fluorescein diacetate staining.

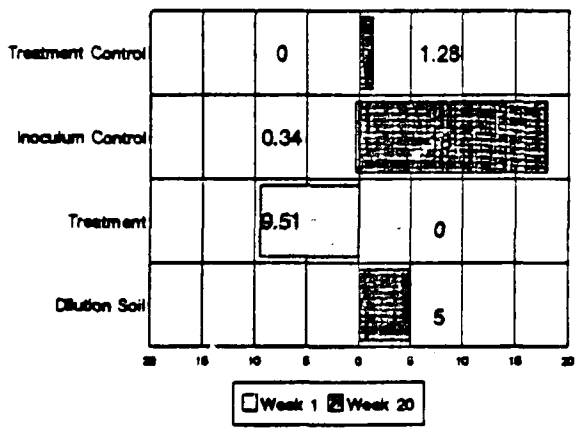

Figure 5. Active fungal biomass (mg/kg) by fluorescein diacetate staining.

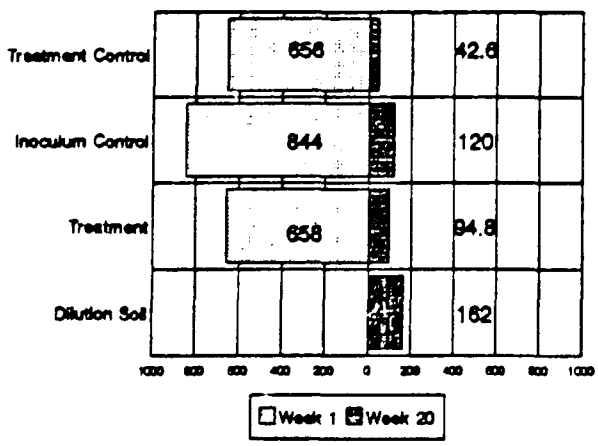

Figure 6. Total bacterial biomass (mg/kg) by fluorescein diacetate staining.

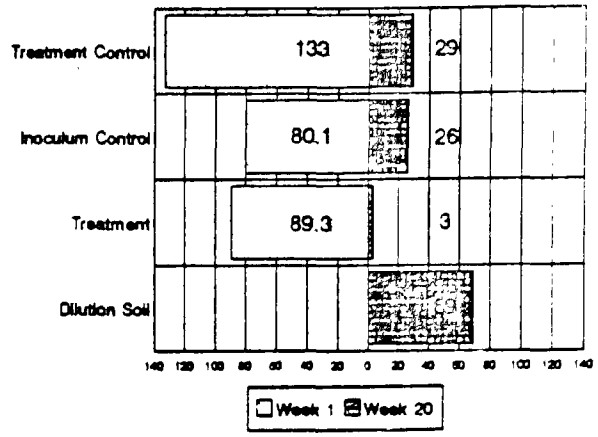

Figure 7. Active bacterial biomass (mg/kg) by fluorescein diacetate staining.

	Conc (mg/kg)	
	Found	Expected
Inoculum	241	
Raw soil	0.2	
Inoculated soil	4	24

Figure 8. Ergosterol evaluation.

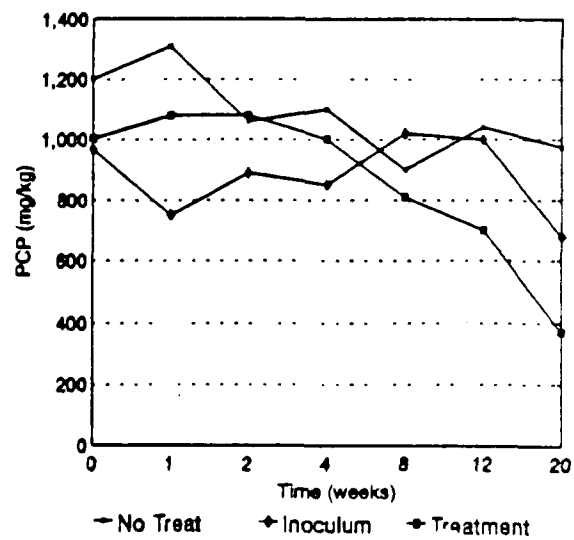

Figure 9. PCP concentration depletion.

The Bioremediation in the Field Search System (BFSS)

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The Bioremediation in the Field Search System (BFSS) is a PC-based software application developed by EPA's Bioremediation Field Initiative. BFSS provides access to a database of information compiled by the Initiative on hazardous waste sites where bioremediation is being tested or implemented, or has been completed. Sites include Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) sites, Resource Conservation and Recovery Act (RCRA) sites, Toxic Substances Control Act (TSCA) sites, and Underground Storage Tank (UST) sites. The database currently contains information on approximately 160 sites, primarily those under federal authority. This summer the Initiative plans to expand the database by soliciting information from industry, contractors, and vendors—an effort that is expected to double or triple the number of sites in the database.

BFSS contains both general site information and data on the operation of specific biological technologies. General site information includes the location of the site, site contacts, the predominant site contaminants, and the legislative authority under which the site is being remediated. Technology-specific information includes the stage of operation, the type of treatment being used, the wastes and media being treated, the cleanup level goals, and the performance and cost of the treatment. Both *ex situ* and *in situ* technologies are represented, including activated sludge, extended aeration, contact stabilization, fixed-film, fluidized bed, sequencing batch, and slurry reactor treatments; aerated lagoon, pile, and land treatments; and bioventing, air sparging, *in situ* ground-water treatment, and confined treatment facilities.

BFSS allows the user to search the system based on location, regulatory authority for cleanup, media, contaminants, status of the project, and treatment utilized. Based on the search criteria specified by the user, BFSS generates a list of qualifying sites. BFSS allows the user to view on-line information about these sites and to print site reports based on information contained in the database.

The Initiative established the BFSS database to provide federal and state project managers, consulting engineers, industry personnel, and researchers with timely information regarding new developments in field applications of bioremediation. BFSS data and the operation of the search system have been reviewed by representatives of the target user community, including personnel from EPA regional offices and other professionals in the field of bioremediation. Information in the database is updated semiannually and is reported in EPA's quarterly *Bioremediation in the Field* bulletin, which is published by the Office of Research and Development (ORD) and the Office of Solid Waste and Emergency Response (OSWER). The bulletin provides a valuable information-sharing resource for site managers using or considering the use of bioremediation.

Version 1.0 of BFSS will be available by August 1994 on several EPA electronic bulletin boards—Cleanup Information (CLU-IN), Alternative Treatment Technology Information Clearinghouse (ATTIC), and ORD bulletin board systems—and on diskette from the EPA Center for Environmental Research Information.

Section Two
Performance Evaluation

In an effort to evaluate the performance of various bicremediation 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 by-products, since 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 Health Effects Research Laboratory (HERL) has 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 and chemical/microorganism interactions occur; and 4) the development of methods to better extrapolate toxicological bioassay results to the understanding of potential human toxicity.

Specific research ongoing within the HERL program includes a study of the construction of noncolonizing *E. coli* and *P. aeruginosa*. Researchers obtained strains of *E. coli* that are unable to colonize the lung tissue or the intestines of humans and animals, thus minimizing the possibility of opportunistic infections that can result in debilitating disease. These strains could be useful as detoxifiers of chemicals, agricultural biopesticides, and in the prevention of ice nucleation on plants.

The symposium's poster session included presentations on toxicant generation and removal during crude oil degradation, the effects of *Lactobacillus reuteri* on intestinal colonization of bioremediation agents, and potentiation of 2,6-dinitrotoluene bioactivation by atrazine in Fischer 344 rats.

Integrating Health Risk Assessment Data for Bioremediation

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Scientific literature clearly indicates that our environment contains individual substances, combinations of substances, and complex mixtures that are hazardous to human health. Additionally, some environmental microorganisms historically considered nonpathogens have been shown to cause disease when humans are exposed under "nontypical" conditions. To protect public health, those involved in remediation efforts must understand the potential for adverse health effects from environmental contaminants and microorganisms before, during, and after any type of remediation. When bioassay information coupled with chemical characterization indicates a measurable loss of toxicity and testing of applied microorganisms (if any) shows no adverse effects, one can have increased confidence that the remediation effort will have its intended effect.

Because any human exposure to toxicants in bioremediation sites is most likely to be of the chronic, low-concentration type, the toxicological endpoint of greatest concern typically is carcinogenesis. Some investigators report an increased frequency of cancers in counties surrounding hazardous waste sites. One study reported that age-adjusted gastrointestinal (GI) cancer mortality rates were higher than national rates in 20 of 21 of New Jersey's counties. The environmental variables most frequently associated with GI cancer mortality rates were population density, degree of urbanization, and presence of chemical toxic waste disposal sites (1). In a study of 339 U.S. counties (containing 593 waste sites) where contaminated ground drinking water is the sole source water supply, the association between excess deaths due to cancers of the lungs, bladder, stomach, large intestine, and rectum and the presence of a hazardous waste site (HWS) was significant when compared with all non-HWS counties (2). Although studies such as these do not prove causality between cancer incidence and release of hazardous substances from waste sites, they do raise serious questions that should be examined through more precise research.

There are numerous reasons why large gaps exist in our ability to assess the health significance of environmental exposures to chemicals in our environment. Exposure cannot be readily quantified by measuring body burdens of contaminants, because rapid metabolism of toxic agents prevents measurable accumulation. Because of the complexities of toxin uptake, toxicologists do not fully understand the relationships between environmental exposure and body burden (i.e., the amount of a toxin reaching and interacting with biological targets). Even more problematic are the possible antagonistic and synergistic interactions that can possibly nullify predictions based on the toxicity of individual compounds.

Bioremediation involves increasing the numbers of pollutant-degrading microorganisms to a level at which they can have a significant effect in a timely fashion. This increase in the microbial population also increases the likelihood of human exposure to these microorganisms. Because environmental organisms do have some potential to cause adverse health effects, researchers must develop methods to screen bioremediation microorganisms for the ability to induce adverse effects.

The Health Effects Research Program

To address the adverse health effects questions associated with bioremediation, the EPA's Health Effects Research Laboratory (HERL) has developed an integrated program that addresses key issues. In collaboration with other EPA laboratories, HERL examines 1) the toxicity of known HWS 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 and chemical/microorganism interactions occur; and 4) the development of methods to better extrapolate toxicological bioassay results to the understanding of potential human toxicity. The program is carried out using known HWS pollutants, samples from microcosm studies that model the biode-

gradation within waste sites, and actual waste site samples. The HERL program attempts to coordinate its own efforts with those of the other cooperating EPA laboratories and academic researchers funded through cooperative agreements.

HERL projects can be grouped into four categories: 1) the infectivity and pathogenicity of environmentally released microorganisms; 2) the toxicity of metabolites of environmental toxicants; 3) the toxicity of products of bioremediation; and 4) development of microbial constructs that decrease the likelihood of adverse human health effects.

This talk will give a brief overview of the specific research ongoing within the HERL program, how the research is interrelated, and how the information coming from this program could affect developing risk assessment methods.

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Construction of Noncolonizing E. Coli and P. Aeruginosa

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The wall of the mammalian large intestine consists of an epithelium containing brush border epithelial cells and specialized goblet cells, which secrete a relatively thick (up to 400 μm), viscous mucus covering (1). The mucus layer contains mucin, a 2-MDa gel-forming glycoprotein, and a large number of smaller glycoproteins, proteins, glycolipids, and lipids (2-4). For many years, we have been interested in how *Escherichia coli* and *Salmonella typhimurium* colonize the large intestines of mice and have come to the conclusion that growth in the mucus layer is essential (5). Moreover, when *E. coli* and *S. typhimurium* are grown in intestinal mucus *in vitro*, they synthesize surface proteins that are not synthesized during growth in normal laboratory media (6). These results led us to envision two approaches for obtaining strains of *E. coli* that are perfectly healthy when grown in normal laboratory media but are unable to colonize the large intestines of mice. The first approach is to identify and mutate *E. coli* genes that are necessary for growth or survival in mucus and determine whether such mutants are unable to colonize. The second strategy is to identify major nutrients, for growth of *E. coli* in mucus, isolate mutants unable to utilize these nutrients, and determine whether such mutants are unable to colonize. This report explains how we have been successful in both approaches with *E. coli* and have now obtained strains that are unable to colonize but that are completely healthy in the laboratory. These strains should be as effective as their parents for gene cloning yet more effective for containment of recombinant DNA.

Technological exploitation of modern genetic techniques now holds great promise for use of members of the genus *Pseudomonas* for environmental purposes (e.g., as agricultural biopesticides [7], as detoxifiers of chemicals [8], and in prevention of ice nucleation on plants [9]). For obvious reasons, the strains to be released into the environment must be strong, competitive organisms. Unfortunately, strong, competitive pseudomonads can be opportunistically pathogenic (10,11). Human exposure to these microorganisms may occur in the agricultural or industrial setting during production or application. Because a high concentration of these microorganisms may be found in the air and water, exposure and subsequent disease may occur through inhalation and ingestion. Clearly, strong, competitive *Pseudomonas* strains should be constructed that are unable to colonize the lung tissue or the intestines of humans and animals to minimize the possibility of opportunistic infections resulting in debilitating disease. This report explains our initial attempts at obtaining such strains using the approaches outlined above for E. coli.

Background

E. Coli

E. coli F-18 was isolated from the feces of a healthy human in 1977 and is an excellent colonizer of the streptomycin-treated mouse large intestine. Its serotype is rough:K1:H5. *E. coli* F-18Col$^-$, a poor colonizing derivative of *E. coli* F-18, contains all the *E. coli* F-18 plasmids, and its serotype is also rough:K1:H5. These strains were used in experiments designed to determine why *E. coli* F-18Col$^-$ is a poor colonizer and to identify major nutrients required for successful *E. coli* colonization of the mouse large intestine.

Pseudomonas Aeruginosa

P. aeruginosa AC869 is an environmental strain that has been engineered to utilize 3,5-dichlorobenzoate as the sole source of carbon and energy (11) but which has been found to be pathogenic for mice when administered intranasally (11). This strain was used in experiments to determine changes associated with growth in mouse lung and cecal mucus preparations *in vitro*.

Results

E. Coli

E. coli F-18 DNA was randomly cloned into *E. coli* F-18 Col$^-$ using the plasmid pRLB2. The entire bank was fed to three streptomycin-treated mice, and all three mice

selected the same clone which contained a 6.5 kb insert. This insert increased the colonizing ability of *E. coli* F-18Col$^-$ approximately 1-million-fold. After subcloning and sequencing, we identified the gene responsible for the observed increased colonizing ability: *leu*X, which encodes a leucine tRNA specific for the rare leucine codon UUG. An *E. coli* K-12 derivative, *E. coli* XAc *sup*P, contains a defective *leu*X gene. This strain was found to be unable to colonize the large intestines of streptomycin-treated mice; i.e., mice fed 10^{10} colony forming units (CFU) were essentially free of the strain by Day 11 postfeeding. In contrast, streptomycin-treated mice fed 10^{10} CFU of *E. coli* XAc *sup*P containing the cloned *leu*X gene colonized indefinitely at 10^7 CFU per gram of feces. Here, then, is an *E. coli* K-12 strain that is perfectly healthy when grown in normal laboratory media but is unable to colonize the mouse intestine.

Glucuronate, a major carbohydrate in mouse cecal mucus, i.e., 0.6 percent by dry weight (12), is metabolized in *E. coli* via the Ashwell pathway (13). Mutants unable to grow using glucuronate as the sole source of carbon were isolated after mini-Tn10 mutagenesis. One of the mutants was unable to metabolize glucuronate, gluconate, and galacturonate, suggesting that it was lacking 2-keto-3-deoxy-6-phosphogluconic aldolase (EC 4.2.1.14), an enzyme encoded by the *eda* gene (14). The mutant *eda* gene was transduced into wild-type *E. coli* K-12, and the *E. coli* F-18 *eda*$^-$ strain and the *E. coli* K-12 *eda*$^-$ strain were each fed to streptomycin-treated mice (10^{10} CFU per mouse). Both strains were essentially eliminated from the mouse intestine by Day 9 postfeeding. When the *eda*$^-$ mutants were complemented with the previously cloned *eda*$^+$ gene, both strains colonized indefinitely at between 10^7 CFU and 10^8 CFU per gram of feces. We are presently constructing *E. coli* F-18 and *E. coli* K-12 *sup*P$^-$ *eda*$^-$ double mutants to determine whether such mutants are even more rapidly eliminated from the mouse large intestine.

P. Aeruginosa

Rabbit antisera were raised against *P. aeruginosa* AC869 grown in Luria broth, mouse lung mucus, and mouse cecal mucus. *P. aeruginosa* AC869 grown in these media were subjected to SDS-PAGE and immunoblotting using the three different rabbit antisera as probes. Surprisingly, the major change in *P. aeruginosa* AC869 observed when grown in either mouse lung mucus or cecal mucus was a huge increase in O-side chain containing lipopolysaccharide (LPS). In support of this view, *P. aeruginosa* AC869 grown in Luria broth was found to be untypeable with respect to LPS, whereas the same strain grown in either mouse lung mucus or cecal mucus was typed as O6. [LPS serotyping was kindly performed at the Statens Seruminstitut in Copenhagen, Denmark.] This finding was of great interest, since *P. aeruginosa* strains without O-side chain on their LPS are known to be serum sensitive, i.e., they are killed by normal human serum (15). We are, therefore, presently attempting to isolate mutants of *P. aeruginosa* AC869 that do not make O-side chains when grown in either mouse lung mucus or cecal mucus. It is hoped that such mutants will be perfectly healthy when grown in laboratory media, will remain capable of metabolizing 3,5-dichlorobenzoate, yet will be nonpathogenic when inoculated intranasally into mice.

Summary and Conclusions

The genes *leu*X and *eda* have been shown to be critical for *E. coli* colonization of the streptomycin-treated mouse large intestine. These findings have allowed us to obtain *E. coli* K-12 strains that grow well in normal laboratory media but are unable to colonize the streptomycin-treated mouse large intestine. Moreover, these strains are easily transformed with pBR322-based plasmids containing chromosomal DNA inserts. Developing healthy *E. coli* K-12 strains for recombinant DNA work that will not colonize the human intestine now appears possible.

We have shown that *P. aeruginosa* AC869 synthesizes more O-side chain (O6) when grown in either mouse lung mucus or cecal mucus than in Luria broth. Since *P. aeruginosa* strains that lack O-side chain are serum sensitive, its seems likely that such mutants of *P. aeruginosa* AC869 will be less pathogenic in the lungs of mice. Experiments designed to test this hypothesis are currently in progress.

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Toxicant Generation and Removal During Crude Oil Degradation

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As microorganisms are promoted for environmental bioremediation efforts, the potential risk of adverse effects of pollutant exposure to the microbes must be assessed. Although fungi (1,2) and bacteria (3-5) degrade hydrocarbons, the genotoxic consequences of degradation have not been addressed. Bacterial species use enzyme systems to convert hydrocarbons to metabolites with increased toxicity (6-8) or to mineralize toxic compounds during metabolism (9). This study involves interactive use of microbial culture, analytical chemistry, and mutagenicity bioassays to investigate the genotoxicity of the oil degradation process. Following degradation by two fungi, *Cunninghamella elegans* and *Penicillium zonatum* (10,11), crude oils of low, moderate, and high mutagenicity are tested for their resulting mutagenic activities.

Methods

C. elegans ATCC 36112 or *P. zonatum* ATCC 24353 was inoculated into 500 mL L-Salts medium (12) with 5 mL of crude oil. Flasks were incubated at 30°C for 4 to 30 days; at 2-day intervals, flasks were sacrificed, and crude oil was extracted with methylene chloride by a modification of the method used by Cerniglia (10,13). Oil mass determinations were calculated from oil residue weights. Extracted oils were analyzed for conversion of straight chain hydrocarbons by gas chromatography and for mutagenicity by the spiral *Salmonella* assay (14,15). Controls included "weathered" (uninoculated) oil flasks and fungi grown on 2-percent glucose to test for mutagenic products from fungal growth alone ("fungal mat controls").

Results

Pennsylvania and Cook Inlet Alaska crude oils' mycelial mat weights are directly proportional to biologically linked oil degradation. The fungi consistently form sturdy mats with Pennsylvania crude; the Cook Inlet mat, however, is more fragile. Mat weights are not proportional to West Texas sour crude utilization; sturdy mats are not consistently produced by either organism even though the oil is used as the sole carbon source. The loss of oil mass is evidenced by a significant decrease in C7 to C20 hydrocarbons as incubation time increases. Weathered samples of the three oils do not exhibit changes in mutagenic activity over time. The mutagenicity of the most potent oil, Pennsylvania crude, is significantly reduced following degradation by either fungus (Table 1). The activity of the weakly mutagenic West Texas crude exhibits little change upon treatment (data not shown). The nonmutagenic Cook Inlet Alaska crude oil becomes mutagenic when incubated with either fungus (Table 2).

Conclusion

The fungal species used in this study may convert crude oil hydrocarbons to products more mutagenic than the original compound. Further studies in progress address effects of oxygenation, nitrogen and phosphorus enrichments, and surfactant addition to the experimental system.

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Table 1. Pennsylvania Crude (+++ highly mutagenic)

Organism	Incubation (days)	Mutagenic Response	% Biological Loss*	Mat Weight (g)
C. elegans	2	+++	7%	0
	4	+	9%	0.2
	6	++	17%	0.6
	8	+	23%	0.6
	10	+	42%	1.0
	12	–	26%	0.4
	14	+	32%	0.5
P. zonatum	2	+	8%	0
	4	+	16%	0
	6	–	21%	0.4
	8	–	27%	0.5
	10	–	33%	0.3
	12	–	29%	0.4
	14	–	18%	0.3

*Biological Loss = Amount of oil used by fungus (corrected for procedural nonbiological oil loss)

Table 2. Alaska Crude (- nonmutagenic)

Organism	Incubation (days)	Mutagenic Response	% Biological Loss	Mat Weight (g)
C. elegans	2	–	4%	0
	4	–	4%	0
	6	–	19%	0.1
	8	+	19%	0.1
	10	+	18%	0.1
	12	+	18%	0.1
	14	+	16%	0.1
P. zonatum	2	–	5%	0
	4	+/–	13%	0
	6	+	16%	0.1
	8	+/–	28%	0.2
	10	+/–	24%	0.2
	12	+/–	27%	0.2
	14	+	24%	0.1

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Potentiation of 2,6-Dinitrotoluene Bioactivation by Atrazine in Fischer 344 Rats

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Because of widespread use, pesticides often are found as co-pollutants at hazardous waste sites and other sites contaminated by xenobiotics. The herbicide atrazine is used as a weed control agent during the cultivation of food crops and is found frequently as a ground-water contaminant. To study atrazine as a co-pollutant, this study explored the effect of atrazine treatment on the bioactivation of the promutagen 2,6-dinitrotoluene (2,6-DNT). For 5 weeks, male Fischer 344 rats (21 d) were administered p.o. 50 mg/kg of atrazine. At 1, 3, and 5 weeks, both control and atrazine-pretreated rats were administered 75 mg/kg of 2,6-DNT by gavage and were placed into metabolism cages for urine collection. Following urine concentration, a microsuspension modification of the *Salmonella* assay with and without metabolic activation was used to detect urinary mutagens. No significant change in mutagen excretion was observed in atrazine-pretreated rats. A significant increase, however, was detected in direct-acting urine mutagens from rats receiving atrazine and 2,6-DNT at Week 1 (359 ±68 revertants/mL versus 621 ±96 revertants/mL) and Week 5 (278 ±46 revertants/mL versus 667 ±109 revertants/mL) of treatment. Urinary mutagenicity was accompanied by an increase in small intestinal nitroreductase activity. At Week 5, elevations in large intestine nitroreductase and β-glucuronidase were observed. This study suggests that atrazine potentiates the metabolism and excretion of the mutagenic metabolites of 2,6-DNT by modifying the intestinal enzymes responsible for promutagen bioactivation.

Effects of Lactobacillus reuteri on Intestinal Colonization of Bioremediation Agents

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Lactobacillus reuteri is the predominant heterofermentative species of *Lactobacillus* inhabiting the gastrointestinal (GI) tract of humans, swine, poultry, rodents, and a number of other animals (1). Studies on chicks and poults have shown that oral (probiotic) treatment of flocks at hatch with viable, host-specific *L. reuteri* prior to challenge at Day 1 posthatch with *S. typhimurium* reduces mortality by 50 percent to 75 percent compared with untreated flocks (2). *L. reuteri* is unique among bacteria in its ability to produce and secrete the potent, broad-spectrum antimicrobial agent reuterin when incubated in the presence of glycerol under physiological conditions similar to those which exist in the GI tract (3,4) Reuterin has been purified, chemically characterized, and identified as an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (5,6).

The environmental release of naturally occurring, mutant, and recombinant microorganisms has prompted questions concerning human health and environmental effects (7,8). To date, a variety of microbes have been released into the environment for many uses. Currently, investigators are engineering microorganisms, primarily pseudomonads, for their ability to degrade hazardous environmental contaminants such as pentachlorophenol, 2,4,5-trichlorophenoxyacetate, chlorobenzoates, and trichloroethylene. *Pseudomonas* spp., however, have long been recognized as opportunistic pathogens, readily occurring in serious secondary infections, and they have been linked to major infections in immunosuppressed and leukemia patients as well as those treated with antibiotics (9-11). Because of the clinical significance of *Pseudomonas* spp., their potential health effects have been studied in terms of their ability to compete and survive in a CD-1 mouse model system (12,13). The effects of antibiotics on their survival and translocation to other organs also have been investigated. Results from these studies indicate that environmental pseudomonads can survive in the GI tract for up to 14 days, where they can alter the normal microbiota. Their translocation to the spleen and/or liver also occurs, indicating the potential for a systemic infection (14,15). This research was undertaken to determine if *L. reuteri* prophylaxis could mitigate the pathogenic effects of these *Pseudomonas* spp. in the mouse model system.

Materials and Methods

Bacterial Strains

Three *Pseudomonas aeruginosa* strains were used in this study. Strain BC16 degrades polychlorinated biphenyl, strain AC869 degrades 3,5-dichlorobenzoate, and strain PAO is a clinical isolate. Four mouse-specific *L. reuteri* strains were used.

Animals

Thirty-day-old CD-1 male mice were used in this study. These animals were administered *L. reuteri* (10^9 colony-forming units (CFU)/mL) in sterilized water daily for 5 days prior to *Pseudomonas* administration by gavage (one group 10^8 CFU and the other group 10^9 CFU) and thereafter during the entire experiment. Control mice were given only sterilized water. On Day 2 and Day 7 after the *Pseudomonas* administrations, the animals were sacrificed, and their livers and ceca were analyzed for presence of *L. reuteri* and *Pseudomonas* spp.

Detection of L. reuteri and Pseudomonas spp.

Mice were sacrificed by CO_2 asphyxiation. Ceca and livers were removed aseptically and homogenized in 5 mL PBS buffer. Homogenate dilutions were made in buffer, and duplicate platings were carried out on Lactobacillus selection (LBS) agar and *Pseudomonas* isolation agar (PIA). The LBS medium was used to enumerate the total gut and liver population of lactobacilli. The subpopulation of *L. reuteri* colonies on appropriately diluted plates is identified based on the ability of *L. reuteri* colonies to convert glycerol to reuterin under anaerobic conditions. The PIA plates were used for *Pseudomonas* spp. detection in livers and ceca.

Results and Discussion

Animals that were treated with *P. aeruginosa* strains BC16 and AC869 and *L. reuteri* were cleared of the infectious agent in 7 days. Of animals that were not treated with *L. reuteri*, 55 percent and 33 percent remained infected at that time with *P. aeruginosa* strains BC16 and AC869, respectively. When the mice were given 10^9 cells of *P. aeruginosa* AC869 by Day 7, 83 percent remained infected compared with a 50-percent infection rate in the *L. reuteri* treated group. Animals treated with *P. aeruginosa* PAO (10^9 cells per mouse) in the absence of *L. reuteri* were 75-percent infected by Day 7; those treated with *L. reuteri* were only 50-percent infected.

Some indigenous lactobacilli have been shown to inhibit colonization of pathogenic bacteria, particularly in the small intestine, by means of what has been termed colonization resistance (CR) or competitive exclusion (CE) (16). Neither the mechanism(s) underlying this phenomenon nor the protective effect of *L. reuteri* on the *Pseudomonas* infections described in this report is fully understood. Our research has indicated, however, that 1) *L. reuteri* prophylaxis is beneficial to the host animal's health and 2) this treatment could have applications concerning the protection of animals against *Pseudomonas* spp. Preliminary studies (17) indicate that *L. reuteri's* efficacy in this regard could be based on its ability to stimulate a protective immune response to *P. aeruginosa* infections.

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Section Three
Field Research

Field research is essential for evaluating the performance of full-scale bioremediation processes 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 the introduction of a non-native microorganism. 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 the Moffett Naval Air Station in Mountain View, California, evaluated aerobic cometabolism of trichloroethylene (TCE) and other chlorinated alkenes in ground water at the site. The studies demonstrated the effectiveness of indigenous subsurface microorganisms in degrading contaminants. Efforts to evaluate bioaugmentation at the site are now under way.

Researchers at the Robert S. Kerr Environmental Research Laboratory investigated reductive dechlorination of chloroethenes in subsurface materials from a municipal landfill in Norman, Oklahoma. The goal of the study was to identify the environmental parameters that control the onset and extent of dechlorination activity.

Field site characterization studies of fuel spills performed by the U.S. Air Force demonstrated the usefulness of a laser-induced fluorescence cone penetrometer for taking core samples to define the three-dimensional boundaries of an immiscible oily phase. The studies involved vertical profile core sampling for direct analysis of combustible gas and solvent extractions for total petroleum hydrocarbons (TPH) by infrared spectrometry, or for aromatic hydrocarbons by gas chromatography and mass spectrometry.

EPA has begun to assist in the development of protocols for assessing the effectiveness and safety of putative commercial bioremediation agents (CBAs) for oil spill bioremediation. Supplemental research (in progress) will provide more site-specific information by examining the effects of environmental parameters (e.g., salinity, temperature, water turbulence, increased treatment time or increased CBA application rates) on the effectiveness of the CBAs.

The symposium's poster session included information on the use of *in situ* carbon dioxide measurement to determine bioremediation success, intrinsic bioremediation of JP-4 jet fuel contamination at George Air Force Base, California, and bioavailability factors affecting the aerobic biodegradation of hydrophobic chemicals.

Field-Scale Study of In Situ Bioremediation of TCE-Contaminated Ground Water and Planned Bioaugmentation

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Trichloroethylene (TCE) and other lesser halogenated ethenes are biodegradable through aerobic co-metabolism. Here, microorganisms that possess oxygenases for initiating the oxidation of either aliphatic or aromatic hydrocarbons or ammonia fortuitously can oxidize the chlorinated alkenes to unstable epoxides. The epoxides degrade further to inorganic end products through a combination of chemical and biological transformations. To carry out *in situ* biodegradation of such chlorinated ethenes in ground water, the appropriate aliphatic or aromatic hydrocarbon or ammonia must be added to the ground water as a substrate both to grow a sufficient population of the desired organisms and to supply the energy required for maintaining activity of the oxygenase. Field studies to evaluate the potential of aerobic co-metabolism of TCE and other chlorinated alkenes have been conducted at the Moffett Naval Air Station in Mountain View, California, since 1985 (1-3). Methane, phenol, and toluene now have been added to ground water at this site to determine their effectiveness as primary substrates for chlorinated ethene degradation.

The above studies have shown the effectiveness of microorganisms indigenous to the subsurface environment at Moffett Field for degrading chlorinated alkenes. One potential problem in attempting to translate the results at the Moffett Field site to other field sites is that the same primary substrates may not stimulate the growth of microorganisms with similar effectiveness. Many different microorganisms can grow on the primary substrates found effective for TCE co-metabolism, but their effectiveness for this purpose can vary widely. To better ensure a high degree of effectiveness, an ability to apply bioaugmentation successfully with organisms known to be capable of high rates of biotransformation is highly desirable. In addition, phenol and toluene, substrates found to be highly effective as primary substrates, are also hazardous chemicals. Use of microorganisms that can use less hazardous chemicals as primary substrates while maintaining a high degree of effectiveness is desirable. Efforts to evaluate bioaugmentation at the Moffett Field site now are under way.

A summary of the results from the Moffett Field test site using indigenous organisms is described below, as are plans for *in situ* bioaugmentation.

Moffett Field Test Results

Over the past several years, methane and phenol have been evaluated for their effectiveness in stimulating aerobic co-metabolic degradation of a range of chlorinated alkenes. During this past year, toluene was evaluated as well. The results of these studies are summarized in Table 1. The concentrations of the primary substrates added were based upon their oxygen consuming potential, which was about 20 mg/L. Thus, the added dissolved oxygen concentration, achieved by adding pure oxygen, was maintained somewhat above this, or from 26 mg/L to 30 mg/L. The results indicate that TCE was much more effectively transformed with phenol and toluene than with methane. In addition, both phenol and toluene were much more effective at degrading cis-1,2-dichloroethylene (c-1,2-DCE) than methane, while methane was better at degrading trans-1,2-dichloroethylene (t-1,2-DCE). All primary substrates were highly effective at vinyl chloride (VC) oxidation. The one problem compound here was 1,1-dichloroethylene (1,1-DCE), which was only evaluated with phenol. Here, only 54 percent degradation was achieved, and the presence of this compound was found to be very detrimental to TCE degradation, apparently because of the toxicity of the degradation intermediates. Laboratory studies with methane indicated a similar effect.

One concern with the addition of either phenol or toluene as primary substrates for TCE co-metabolism is the concentration remaining after biodegradation. The Moffett Field studies indicated that within 1 day of travel time from the point of injection, both compounds were reduced by biodegradation from the mg/L range to below

Table 1. Summary of the Effectiveness of Different Primary Substrates for *In Situ* Co-metabolic Biodegradation of Chlorinated Ethenes at the Moffett Field Test Site

	Primary Substrates		
	Methane	Phenol	Toluene
Primary Substrate Concentrations (mg/L)	6.6	12.5	9
Dissolved Oxygen Concentrations (mg/L)	26	30	28
Target Compounds	**Percent Removal**	**Percent Removal**	**Percent Removal**
VC	95	>98	NE
1,1-DCE	NE	54	NE
t-1,2-DCE	92	73	75
c-1,2-DCE	42	92	>98
TCE	19	94	93

NE = Not evaluated

1 µg/L. Here, sufficient oxygen was present for effective oxidation. The EPA maximum contaminant level (MCL) and maximum contaminant level goal (MCLG) for toluene in drinking water is 1,000 µg/L, and the taste and odor threshold is in the range of 20 µg/L to 40 µg/L. Thus, the low levels achieved after addition to ground water in the field suggest that no hazard from toluene addition should remain if sufficient oxygen is present. Phenol, while known to have toxicity similar to that of toluene, has no established MCL value, and so its appropriate safe limits can only be estimated.

Bioaugmentation

A cooperative study is now under way between the EPA Gulf Breeze Environmental Research Laboratory, the Michigan Center for Microbial Ecology at Michigan State University, the University of Western Florida, and the Western Region Hazardous Substance Research Center at Stanford University to evaluate the possibility of bioaugmentation for enhanced *in situ* co-metabolic degradation of TCE. Moffett Field will be used as the test site for this study. The objectives of this study are 1) to evaluate at field scale the potential of bioaugmentation to enhance and improve *in situ* bioremediation of ground water contaminated with TCE; 2) to determine the movement, fate, and effectiveness of introduced microorganisms in an aquifer; 3) to determine and evaluate methods for maintaining dominance of introduced organisms over indigenous organisms; 4) to evaluate environmental and ecological factors that affect organism dominance in aquifers during *in situ* bioremediation; and 5) to evaluate the applicability of molecular tools in the monitoring, operation, and control of *in situ* bioremediation systems.

A new test leg is being constructed at the Moffett Field test site for this evaluation. Soil samples have been collected from this test leg for use in laboratory studies to determine the best approach for carrying out the field bioaugmentation studies and to maintain dominance by the introduced microorganisms. Also, the laboratory studies will be used to develop and evaluate molecular tools for characterizing the phenol and toluene degrading populations present, and the fate of the introduced microorganisms. Possible microorganisms for introduction also are being evaluated in these laboratory studies. These organisms include *Pseudomonas cepacia* G4, an organism that grows on either toluene or phenol and is known for its high effectiveness in degrading TCE, and the PR1 mutant of this organism, which has a constitutive oxygenase that is induced even when it grows on nonhazardous substrates such as lactate.

The laboratory studies will be conducted during the first ongoing year of this study. Field implementation is planned for the second year of study. The different institutions involved in this study will share in the evaluation of the effectiveness of bioaugmentation. The Moffett Field site offers a good opportunity in general for a comparative evaluation of different approaches to *in situ* biodegradation of chlorinated aliphatic compounds, and offers promise for evaluating bioaugmentation as well.

Acknowledgments

The studies reported here were supported by EPA through the Robert S. Kerr and Gulf Breeze Environmental Research Laboratories, the Bic ystems Program, and the Western Region Hazardous Substance Research Center, and by the U.S. Department of Energy. These agencies have not reviewed this publication, and no official endorsements by them should be inferred.

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Geochemistry and Microbial Ecology of Reductive Dechlorination of PCE and TCE in Subsurface Material

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Chloroethenes are among the most common organic contaminants of ground water. In the subsurface and other anaerobic environments, they can be transformed through a biologically mediated, step-wise, reductive removal of chloride ions, known as reductive dechlorination. Potentially this process can lead to nonchlorinated products that are environmentally acceptable. Unfortunately, more mobile and toxic daughter products are intermediates. If the process "stalls," as it often seems to in the subsurface, before reaching nonchlorinated end products, the reductive dechlorination process may increase potential risks to human and environmental health. Thus, the reductive dechlorination process can exacerbate or attenuate the problems created by the release of chloroethenes such as trichloroethylene (TCE) or tetrachloroethylene (PCE) to the subsurface and ground-water environments. In these studies, we have attempted to identify the environmental parameters that control the onset and extent of the dechlorination activity.

Three areas of investigation have been the focus of efforts by Robert S. Kerr Environmental Research Laboratory researchers on the reductive dechlorination of chloroethenes. The first is the effects of alternate electron acceptors, commonly found in the subsurface, on the reductive dechlorination process. The second is to develop a conceptual understanding of microbial populations and interactions that carry out the process. The third is directed toward identifying organic compounds that can serve as sources of reducing equivalents for the dechlorination process under native conditions or as a component of an active biotreatment application.

Results and Discussion

Saturated sandy subsurface sediments from near the municipal landfill in Norman, Oklahoma, were collected and used as the test material in these studies. The subsurface environment from which the material was collected is impacted by landfill leachate and classified as methanogenic. This material has been previously shown to contain microbial populations capable of reductively dechlorinating PCE (1). Figure 1 demonstrates the microorganisms' capacity for complete dechlorination of PCE in long-term batch enrichments.

Alternate Electron Acceptor Studies

Under anaerobic conditions, the oxidation of organic compounds is linked to the reduction of electron acceptors other than oxygen. In the subsurface may be present many different electron acceptors, such as nitrate, ferric iron, sulfate, carbonate, or organic contaminants, such as chloroethenes. If multiple acceptors are present in physiologically acceptable concentrations, then the predominant terminal oxidation process is linked to the acceptor that will yield the most energy. As this acceptor becomes limiting, the acceptor with the next highest energy yield is utilized, and so on, until the acceptor with the lowest energy yield is utilized, which is usually carbonate (methanogenesis). Previous research suggests that in the subsurface, reductive dechlorination may be only a minor fate (less than 10 percent) for the reducing equivalents generated during the anaerobic oxidation reactions (2). Whether this noncompetitiveness is because of the physiological

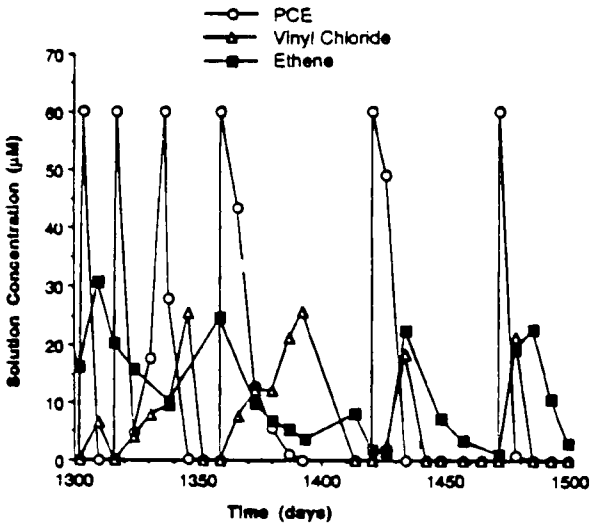

Figure 1. Production of ethene and vinyl chloride from repeated PCE spikes over time in long-term Norman Landfill sediment enrichments. TCE and DCE intermediates are not shown.

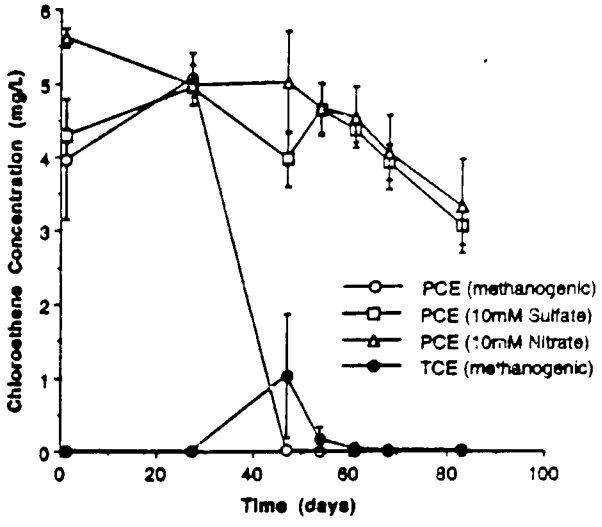

Figure 2. Effects of nitrate and sulfate on the dechlorination of PCE versus time in Norman Landfill microcosms. Values are an average of five replicants. DCE intermediates are not shown.

limitation of the organisms involved, the low potential energy of reactions coupled to reductive dechlorination, or as-yet-unrecognized environmental parameters is unclear.

Laboratory microcosm studies indicated that nitrate was extremely inhibitory to the reductive dechlorination process (Figure 2). In the presence of nitrate, oxidizable organic carbon is quickly utilized by microorganisms in the test material. Whether this was the only mechanism of inhibition was unclear. Sulfate appeared to be partially inhibitory under the conditions tested. Again, competition for electron donor appeared to be the mechanism of inhibition. In experiments with different initial concentrations of sulfate, significant dechlorination activity appeared only after sulfate concentration fell below 400 µM (Figure 3).

Microbial Process Studies

Formation of a conceptual model is the first step in the development of valid mathematical descriptions of *in situ* reductive dechlorination processes. In an effort to define the metabolic processes involved in these reactions and to enhance our understanding of the ecology of the reductive dechlorination process, we have studied the effects of metabolic inhibitors (2-bromoethanesulfonic acid [BESA], molybdate, and vancomycin) on butyrate, ethanol, and formate driven reductive dechlorination of PCE in aquifer microcosms. Molybdate (5 mM) and BESA (1 mM and 10 mM) are used as specific inhibitors of sulfate-reduction and methanogenesis, respectively. Vancomycin (100 ppm) is used as a general eubacterial inhibitor. Molybdate appears to be an effective inhibitor of reductive dechlorination under the conditions tested. BESA completely inhibited dechlorination in microcosms at 10 mM, but only partially inhibited activity at 1 mM (Table 1). The results of experiments, such as those shown in Table 1, suggest that the dechlorinating organisms access the same pool of reducing equivalents as the terminal oxidizing organisms.

Electron Donor Studies

We have shown in the laboratory that the availability of a suitable electron donor is essential for denalogenation of PCE and TCE to occur at appreciable rates in oligotrophic subsurface environments (3,4). We and other groups have identified a wide variety of organic electron donors that can drive biodehalogenation of chloroethenes (2-9). Conceptually, any organic substance capable of being catabolized under anaerobic conditions should be able to support or "drive" reductive dechlorination. At some sites, however, chloroethene plumes are undergoing dechlorination where significant amounts of anthropogenic material is not detected. Physical interactions of chloroethenes with indigenous organic matter in soil, sediment, and aquifer solids are important processes controlling the fate and transport of contaminants in the subsurface (10-12). In many instances, organic carbon concentrations of aquifer solids are assumed to be negligibly low, and in soils they are assumed to decrease exponentially with surface depth. We have tested a working hypothesis that under certain conditions, the release of chlorinated solvents could mobilize soil organic material, which could then serve as an anaerobically metabolizable carbon source that will drive the dechlorination of chloroethenes.

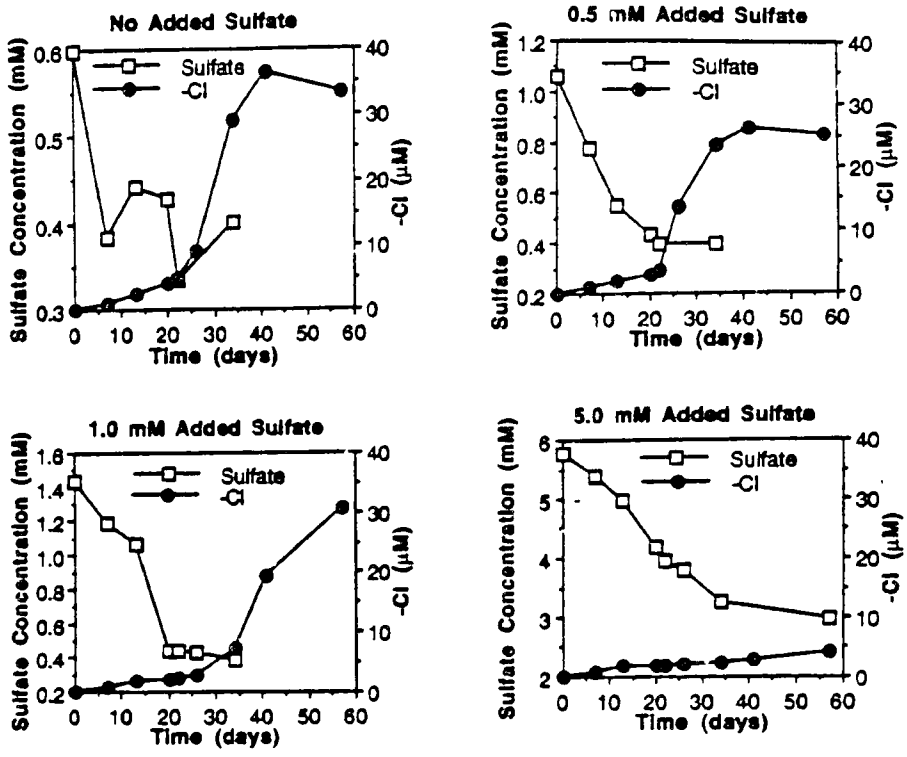

Figure 3. Effects of different initial sulfate concentrations on the onset of reductive dechlorination activity. -Cl is carbon-chloride bonds reduced and is equal to [TCE] + 2[DCE]. Values are an average of five replicants.

Table 1. Effects of Various Inhibitors on Reductive Dechlorination Activity in Norman Landfill Sediments

Donor	Formate		Ethanol		Butyrate	
	RDC	DC	RDC	DC	RDC	DC
Treatment						
BESA (10 mM)	0	+/–	0	–	0	–
BESA (1 mM)	0	+/–	0	–	0	–
Mo (5 mM)	0	–	0	0	–	0
Mo/$SO_4^=$ (5/10 mM)						
	0	–	0	0	0	0
$SO_4^=$ (10 mM)	0	+/–	–	+	0	+
Vancomycin Hydrochloride (100 ppm)						
	0	+/–	–	+/–	–	–

RDC = Reductive dechlorination activity relative to positive control
DC = Electron donor catabolism relative to positive control
Mo = Molybdate ($Na_2MoO_4 \cdot 2H_2O$)
0 = No activity
+/– = No significant change relative to positive control
– = Decreased activity relative to positive control
+ = Increased activity relative to positive control
n = Five each treatment

Organic carbon was extracted from a spodic soil high in humic and fulvic acid concentrations, collected from the vadose zone of the Sleeping Bear site in Michigan. Distilled water and distilled water saturated with TCE were used as extractants. The presence of TCE was observed to improve the extractability of organic compounds (although the specific identity of these compounds is unknown at this time, as is the mechanism of extraction). Experiments were conducted in which microcosms were spiked with the soil carbon extracts in a range of concentrations. The extracted organic material served as the primary carbon/energy source for subsurface microorganisms in the microcosms. The microcosms were monitored over time to determine the ability of the extractable organic carbon to support the dechlorination of PCE. Figure 4 shows the results of the microcosm experiments, which indicate the loss of PCE over time for both types of extracts when present in sufficient concentrations. The dechlorination of PCE in the active experimental treatments correlated with the production of TCE and dichloroethylene (DCE) daughter products (data not shown), indicating that the extracts provide a metabolizable electron donor capable of supporting microbial consortia responsible for reductive dechlorination of PCE.

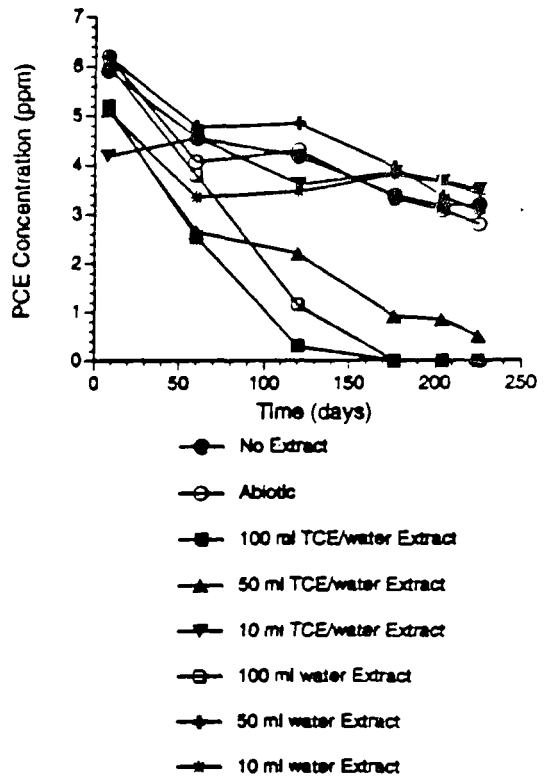

Figure 4. Effects of water and water/TCE extracts on reductive dechlorination of PCE in Norman Landfill microcosms. TCE and DCE intermediates are not shown.

Summary and Conclusions

In situ reductive dechlorination holds significant potential for use in natural (passive) and active *in situ* remediation methods. For reductive biodehalogenation to gain acceptance as a viable alternative to conventional physical and biological treatment methods, however, it must be predictable and well understood. Information and operational experience are needed concerning the environmental parameters, microbial interactions, and metabolic responses that control the initiation, rate, and extent of these degradation processes in the subsurface. An understanding of the controlling mechanisms and the incorporation of such mechanisms into predictive models and operational designs should allow more accurate assessment of the applicability and implementation of anaerobic remediation of chloroethenes at chloroethene-contaminated sites.

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Application of Laser-Induced Fluorescence Implemented Through a Cone Penetrometer to Map the Distribution of an Oil Spill in the Subsurface

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Field monitoring at spill sites usually involves collection and analysis of ground water, soil gas, and/or core material. Applications for soil gas are limited to volatile contaminants in the vadose zone. Groundwater assays are useful but detect only contaminants associated with the aqueous phase. Total contamination of the subsurface, especially by petroleum hydrocarbons, is best measured by vertical profile core sampling and analyses. Our field site characterization studies of fuel spills involve vertical profile core sampling for direct analysis of combustible gas and solvent extractions for total petroleum hydrocarbons (TPH) by infrared spectrometry or for aromatic hydrocarbons by gas chromatography and mass spectrometry.

Objective

The objective of the study was to demonstrate the usefulness of a laser-induced fluorescence cone penetrometer (LIF-CPT) as an inexpensive and rapid alternative to taking core samples for defining the three-dimensional boundaries of an immiscible oily phase. Data are for use in the Bioplume model to determine the amenability of the site to intrinsic bioremediation.

Operative Components

Dakota Technologies, Inc., and Applied Research Associates, Inc., under contract with the U.S. Air Force (Armstrong Laboratory's Environics Directorate), have developed a LIF-CPT tool for mapping the distribution of petroleum hydrocarbons as nonaqueous phase liquids (NAPLs). Principal individuals from the two organizations involved in development and application of the specific LIF-CPT probe used in this study are Wesley L. Bratton, Randy St. Germain, Martin L. Gildea, Greg D. Gillispie, and James O. Shinn. Basic operating components are an optical system to deliver tuneable laser radiation into an optical fiber for transfer downward through a cone penetrometer to a sensor tip equipped with a sapphire window. The subsurface material next to the window fluoresces upon exposure to laser radiation. This fluorescence radiation is transmitted back to the surface, where intensity, fluorescent lifetime, and wavelength are measured.

The LIF-CPT was calibrated for condensed ring aromatic hydrocarbons (specifically, the naphthalene class), which are common constituents of petroleum products. Acquired data were stored on a floppy disk for later processing. Data plots also were displayed on a monitor screen for direct interpretation as the probe moved downward. The LIF-CPT also was used for continuous profiling of soil stratigraphy and collection of soil gas, ground-water, and core samples.

Field Site

The field study site was used extensively as a fire-fighting training area from 1950 to the mid-1980s. Fire training pits were flooded with water, and waste jet fuel mixed with oil and solvents was floated on the water and ignited. The burning oil was extinguished. Any unburned oil infiltrated after these exercises. Pits were constructed in about 70 ft of sand above a confining layer of clay. The lithology is unconsolidated and unifor - glacial outwash sand. The water table is about 30 ft below the ground surface. The groundwater seepage velocity is about 0.4 ft/day.

Less than 3 hours were required to acquire LIF data, recover the tools, decontaminate, and move to the next site. Using the LIF-CPT to collect cores for analyses took 12 hours Samples could not be collected more

than 3 ft below the water table. A conventional hollow stem auger would have required 24 hours to acquire the same samples. The LIF-CPT can detect petroleum hydrocarbons in material below the water table where material cannot be recovered as cores.

Results

Vertical profile LIF-CPT probe responses were obtained at nine locations within the study area. Figure 1 shows probe responses in a longitudinal transect through the fire training area parallel to the direction of ground-water flow. Strip chart displays for each location depict relative fluorescence measurements. Location 84D was within the fire pit. Beginning at 15 feet below the land surface, a LIF-CPT response positive for NAPL was obtained. This response extended another 30 feet downward to a position 5 feet below the water table. A core taken at the water table contained 125,000 mg TPH/kg soil. From combined LIF-CPT and TPH information, an estimated 85 percent of the oily phase is present above the water table. Remediation by vadose zone venting may be able to remove a major fraction of the subsurface contaminated mass.

Test locations 84L and 84F were 100 feet apart and 700 feet downgradient from the fire pit (Figure 1). NAPL was present in the capillary fringe at both locations. Core material collected at the water table depth at location 84F contained 2,050 mg TPH/kg soil. Location 84K, located 100 feet downgradient from 84F, did not have a positive response to LIF-CPT probing. Therefore, the leading edge of the oily-phase plume was concluded to be less than 100 feet beyond 84F.

Figure 2 is a display of the TPH and LIF-CPT results for location 84D and shows a direct relationship with the two parameters. Other information will be presented to show that results obtained for specific fuel aromatic hydrocarbons also show a direct relationship with TPH and LIF-CPT results.

Discussion

The LIF-CPT probe used as an onsite rapid assay tool successfully mapped in three dimensions the oily-phase

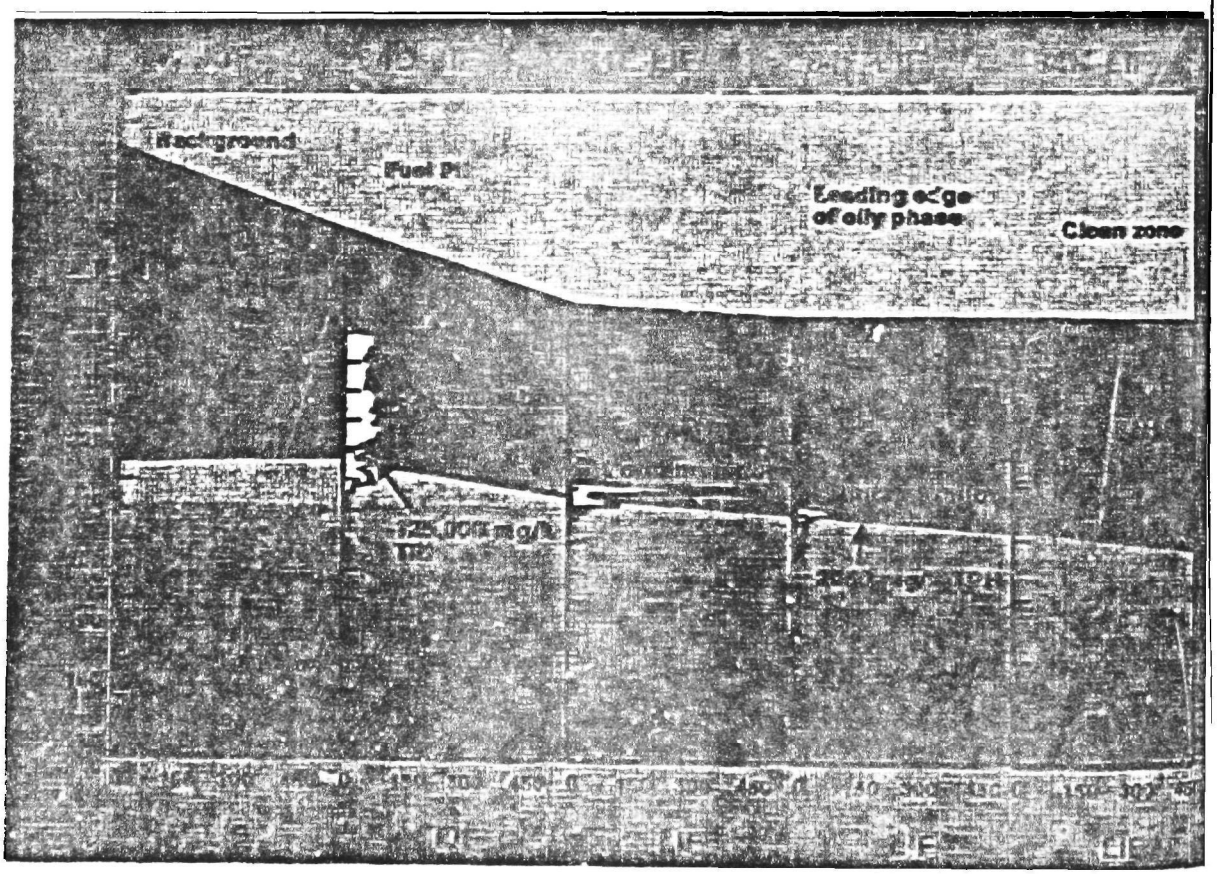

Figure 1. LIF response versus elevation at sampling locations.

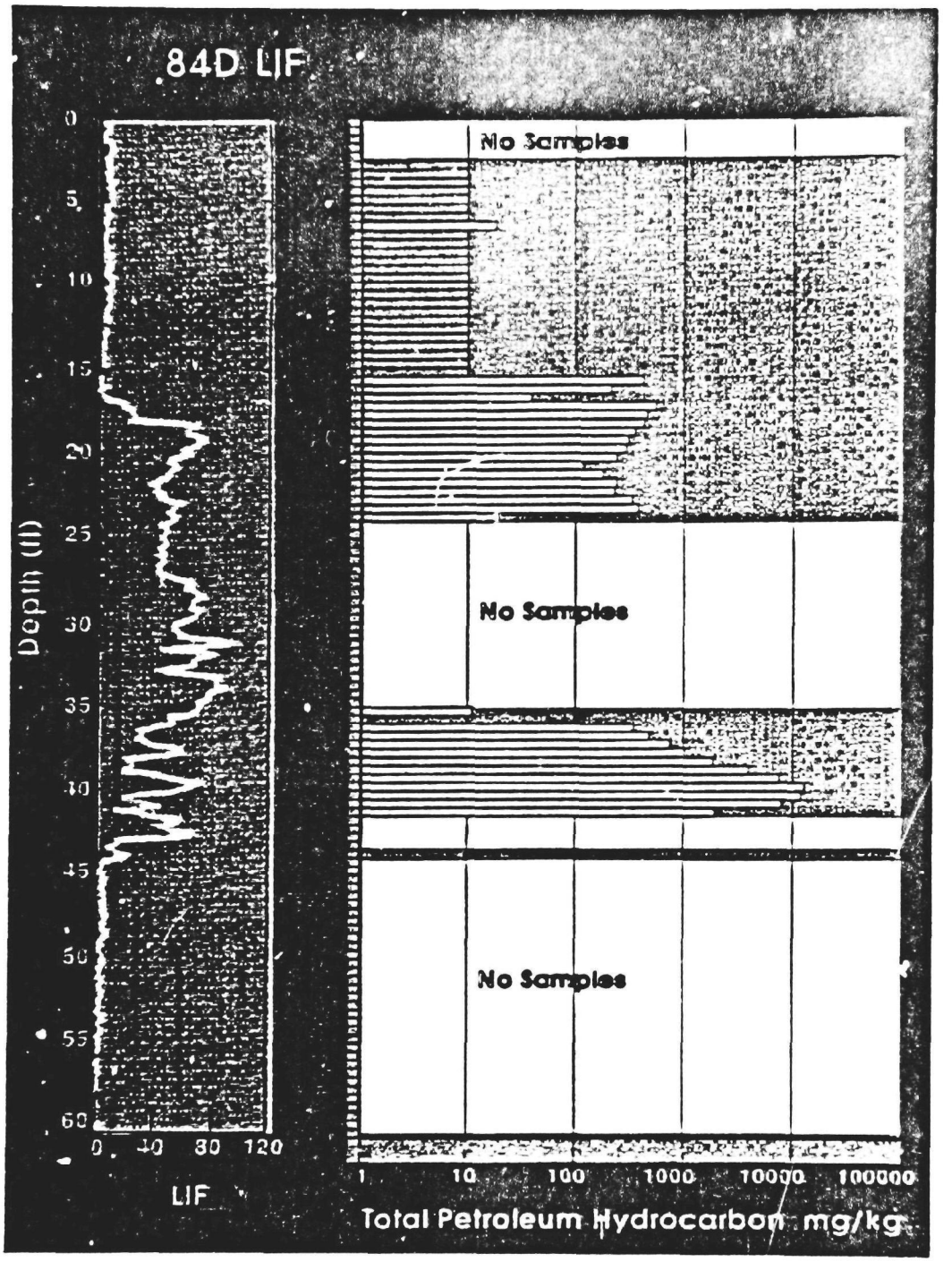

Figure 2. LIF and TPH versus depth at location 84D.

plume studied. Applications of the LIF-CPT technology will be investigated at other field spill sites. We are continuing system development to apply the LIF-CPT method to characterization studies at sites with different classes of hydrocarbons present.

Effectiveness and Safety of Strategies for Oil Spill Bioremediation: Potential and Limitations

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Background

A variety of commercial agents are available for use in oil spill bioremediation. Selection of appropriate bioremediation agents or bioremediation strategies for use in the field, however, has been complicated by the lack of standard tests for assessing agent effectiveness and environmental safety. Acknowledging this problem, EPA began an effort to develop protocols for assessing effectiveness and safety of putative commercial bioremediation agents (CBAs) based on a tiered approach (1,2).

Protocol validation for open-water and beach spill scenarios has progressed using selected CBAs and positive control regimes. CBAs were characterized by vendors as *microbial*, *nutrient*, *enzyme*, *dispersant*, and *other*. Tier I involves the gathering of pertinent information from vendors on potentially hazardous components in the agents, putative mechanism(s) of action, and methods and rates of application. Tier II monitors oil biodegradation in a closed, shake-flask test system in which the oil is physically agitated. Tier III oil spill simulation tests are designed to model field conditions thought to significantly affect CBA effectiveness in open water or on sandy beaches; effluents can be monitored for washed out petroleum hydrocarbons or monitored for toxicity. Tier IV testing will be an actual field evaluation of the protocol test systems, conducted on a controlled release of oil or a "spill of opportunity."

Because of the nature of bioremediation, nutrients are common components of CBAs; however, most forms of inorganic nitrogen exhibit some toxicity to aquatic organisms. The concern for product toxicity is addressed at the Tier III level with two 7-day chronic estimator tests associated with effluent toxicity evaluations that use a crustacean (*Mysidopsis bahia*, mysids) and a fish (*Menidia beryllina*, inland silversides) (3). The mysid test has three endpoints—survival, growth, and fecundity—while the fish test focuses on survival, growth, and development. In addition to evaluation of toxicity of CBA alone, CBA toxicity also is assessed in the presence of a sublethal water soluble fraction of oil to examine potentially detrimental interactions.

This report focuses on results of protocol development for CBA effectiveness and environmental safety using the Tier III open-water and sandy beach test systems.

Tier III Test Systems

The Tier III open-water test system provides an intact, undisturbed oil-on-water slick in a flow-through design (Figure 1). A constant influx of seawater below the oil slick removes CBA microbes and nutrients that do not remain associated with the oil slick, as would be expected at a field site. Test duration is 7 days. Effluent is split: one stream for oil residue analysis and the other for toxicity testing. The slick is analyzed at the end of the test. If a significant amount of oil is mobilized from the slick surface to the water column below (e.g., from biosurfactant production), a subsequent test assesses the biodegradability of the transported oil.

The Tier III oiled beach test system provides a sandy beach substratum, colonized for 1 week by microflora indigenous to seawater. The system models tidal influx and egress. (See Figure 2.) The surface is oiled and 2 days later, a CBA or other bioremediation strategy is applied. Beach test systems run for 28 days, after which the oil residues can be extracted for analysis. Effluents are collected for analytical or toxicological examination.

For the purpose of the Tier III protocol, generic environmental parameters were selected for both the open-water and the beach test systems. The oil was applied to a 0.5-mm thickness, turbulence was standardized,

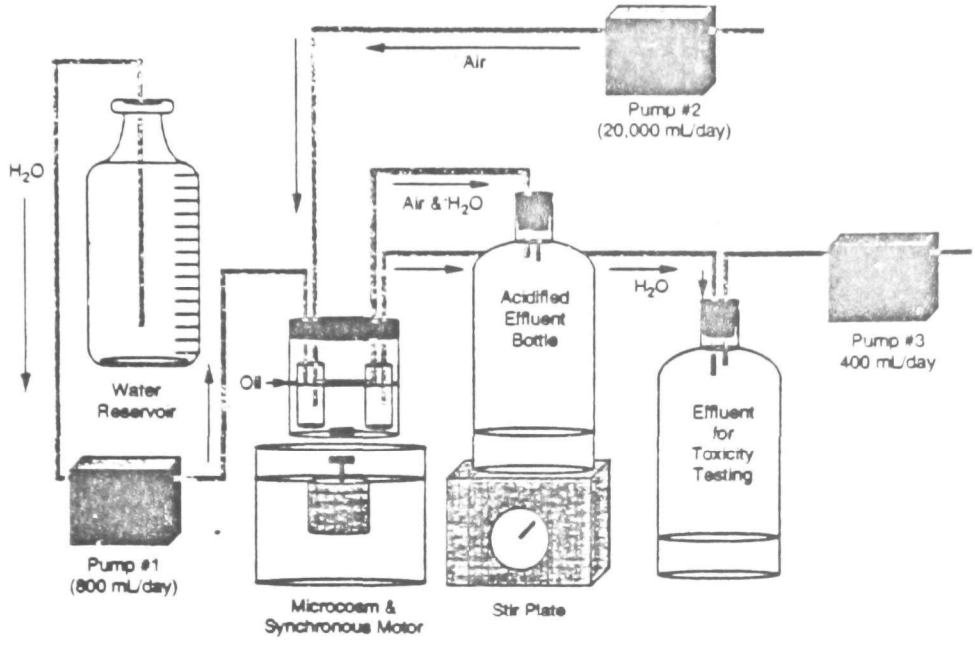

Figure 1. Tier III simulated open-water oil spills test system.

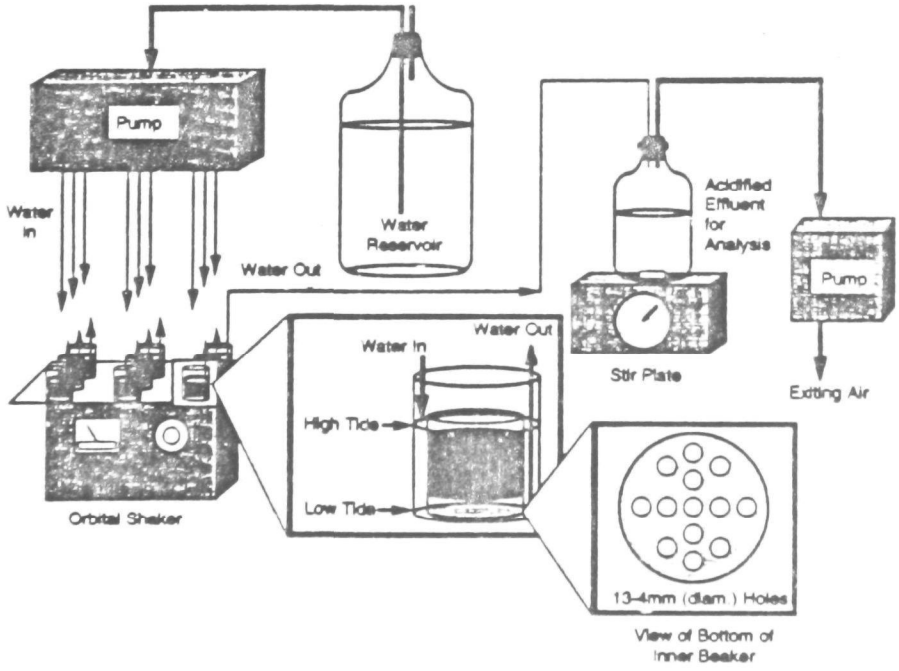

Figure 2. Tier III simulated oiled beach test system.

and temperature was set to 20°C. The oil was artificially weathered (4) to simulate the loss of volatiles expected following a spill and to minimize changes in composition due to loss of volatile components. Gulf of Mexico seawater (30 parts per thousand salinity) provided a source of hydrocarbon-degrading microorganisms capable of responding to increased nutrients in the presence of crude oil as a carbon source.

Two treatments, in three replicates each, are used: 1) a control with oil alone; and 2) a treatment with both oil and CBA. Criteria for evaluating the effectiveness of bioremediation in the Tier III open-water test systems are based on statistically significant ($p \leq 0.05$) reductions in the weight of oil and in the amount of selected gas chromatography/mass spectrometry (GC/MS) analytes remaining in the test vessels and test-system effluent relative to the control vessels and effluents.

Supplemental research (in progress) will examine the effects of environmental parameters (e.g., salinity, temperature, water turbulence, increased treatment time or increased CBA application rates) on the effectiveness of the CBAs to provide more site-specific information.

Results

Validation of Open-Water Test System Using Positive Controls and CBAs

To establish baseline performance for the Tier III open-water test systems, we used positive-control treatments that were surrogates for either nutrient CBAs or microbial CBAs. Three conditions were tested: 1) Gulf of Mexico seawater control, 2) seawater amended with nutrients (to test for the ability of nutrients to enhance the degradation capability of the natural degraders); and 3) nutrient-amended seawater supplemented with a daily inoculation of hydrocarbon-degrading bacteria as a test of competent, high levels of microbial biomass.

The effectiveness of the positive control in the open-water test system is presented in Table 1 as a percent of the oil remaining relative to controls to which neither nutrients nor microbes were supplied. Values represent an average of three replicate test chambers. The number of the GC/MS endpoints out of a total of 70 analytes that were significantly reduced relative to the control for each agent also is tabulated. Nutrients alone failed to stimulate biodegradation by the microbial population indigenous to Gulf of Mexico seawater. Several analyte endpoints, however, were significantly different as the result of action by the hydrocarbon-degrading bacteria in the presence of nutrients.

Table 1 also reports the results of six CBAs selected as representatives of each CBA type. Each was applied to the oil slick in the test systems according to the instructions supplied by the vendor. Of the six, only the nutrient CBA gave a promising result, effecting a change in 18 of the GC/MS analytes and a statistically significant reduction (although only 1 percent) in total oil residue weight. In contrast, the nutrient-amended seawater treatment of the positive control experiment effected a statistically significant change in only one of the GC/MS analytes.

Only in the positive control experiment in which nutrients were supplied continuously and oil degrading bacteria were applied daily did we find effects on a relatively large number of endpoints as well as substantial reduction in the total weight of the oil recovered.

Table 1. Percentage of analyte remaining relative to controls after 7 days of treatment with bioremediation agents or positive control regimes.

	[a]CBA or Positive Control Treatment							
Analyte	**N/M**	**E**	**N**	**N/M**	**M/D**	**D**	**+N**	**+N/M**
C_{18}	97	102	*92	92	103	105	94	**34
C_{30}	101	100	99	96	102	100	99	**57
Phytane	103	103	99	101	102	101	102	95
Pristane	103	99	103	104	101	99	104	98
Fluorene	102	106	96	105	102	107	99	95
Chrysene	103	117	95	107	**90	114	96	95
Phenantrene	102	102	99	102	99	103	102	*97
N-Alkanes	98	105	**92	96	102	106	96	**40
Aromatics	102	105	98	102	102	103	103	97
Total Oil	99	101	*99	103	99	102	102	*93
[b]Endpoints	5	1	18	6	1	0	1	30

[a]Treatment type: E = enzyme, N = nutrient, D = dispersant, M = microbial, +N = nutrient positive control, +N/M = nutrient positive control + microbes.
[b]Number of endpoints showing a statistically significant change at 0.05 or less.
*$p \leq 0.05$; **$p \leq 0.01$

Validation of Oiled Beach Test System Using CBAs

Table 2 shows the percent of oil and oil components remaining in the test systems after 28 days of exposure to four CBAs in Tier III beach test systems. The control treatments, in which seawater flushed the systems in the same tidal regimes as in the CBA chambers, lost substantial amounts of the lower molecular weight polycyclic aromatic hydrocarbons. Positive control experiments and experiments in which we attempted to run sterile control treatments have suggested that the disappearance is biologically mediated, although whether the compounds have been washed intact from the test systems or catabolized is still being investigated.

Environmental Safety of CBAs

An important ecotoxicological consideration for CBAs is the possible production of toxic metabolites. This consideration is addressed at the Tier III level with a mysid 7-day chronic estimator test on the effluent from the open-water and beach test systems. A key assumption is that the test system designs are conservative with respect to dilution; thus, if toxicity is not observed under these mixing scenarios, it is unlikely to occur in a field application. Increased toxicity (compared with the toxicity of effluent from control systems containing only oil) exceeding that of the product alone (from Tier II testing) would suggest the need for further studies that focus on potentially toxic metabolites. Table 3 indicates that the open-water effluent from most CBAs demonstrated low or no toxicity. Safety has not yet been evaluated using the beach test system.

One application of toxicology came as a result of adapting a 10-day amphipod (*Leptocheirus plumulosus*) (5) sediment toxicity test to evaluate potential toxic metabolites associated with the sand of the beach test system after the 28-day CBA efficacy test. We observed that oiled sediment, whether subjected to bioremediation or not, was toxic. Although this phenomenon prevented accurate assessment of potential toxic metabolites in the sediment, it led to research to determine whether toxicity testing could be used as an efficacy endpoint, focusing on the potential of a CBA to render an oiled sediment suitable for amphipod recolonization. The results of preliminary studies will be discussed.

Conclusions

We have completed validation of the open-water and sandy-beach testing systems. Thus far, the CBAs examined during our protocol development work have shown little toxicity and should pose little environmental threat to the organisms tested when applied according to the vendor's suggested regime. Some CBAs effected significant changes in one or more targeted hydrocarbons relative to the control; however, it should be emphasized that the sum of all GC/MS analytes is less than 6 percent of the total oil. Moreover, no substantial decreases in oil residue weights were associated with treatment by CBAs.

By daily addition of microbial biomass and nutrients to the open-water system, however, we were able to demonstrate the greatest biodegradation of oil components within the 7-day period, including a significant weight loss, i.e., significant decreases occurred in 30 of the 70 GC/MS analytes. Thus, we conclude that the test system itself was capable of giving a measurable response, although its accurate modeling of actual site-specific field conditions remains to be evaluated. These results also may indicate that the recommended application rates of CBAs are insufficient to produce substantial changes in oil biodegradation. Daily or more frequent additions may be untenable in some open-water field situations (e.g., large-area spills); however, spills of a

Table 2. Tier III Effectiveness Results of Beach System Tests with CBAs

	Percent Remaining Hydrocarbon Analyte					
CBA Type	Nutrient	Control	Nutrient/ Microbial	Nutrient/ Microbial	Control	Dispersant
C18	**33	90	**20	**25	89	89
Phytane	**86	93	**53	85	89	86
C18/Phyt	**39	97	**37	29	100	100
Fluorene	32	23	29	43	39	50
Dibenzothioph	52	51	52	68	68	81
Phenanthrene	53	48	51	67	68	84
Chrysene	106	106	104	100	99	100
Gravimetric	*92	94	**89	*91	96	94

*Mean of 2 replicates; all others were means of 3 replicates.
*$p \leq 0.05$; **$p \leq 0.01$.

Table 3. Tier III Results of 7-Day Chronic Estimator Tests with *Mysidopsis bahia*

CBA[b]	Max. Effluent Conc. (%)	7-Day LC_{50} (95% C.I.)		Comparison to Oil Control[a] NOEC	LOEC
E	63	> 63	survival	63	NE
			growth	63	NE
			fecundity	63	NE
N	55	> 55	survival	55	NE
			growth	55	NE
			fecundity	55	NE
N/M	66	> 66	survival	66	NE
			growth	66	NE
			fecundity	66	NE
D	10	3.7 (3 - 4.6)	survival	3	10
			growth	NE	NE
			fecundity	3	—[c]

[a]Comparisons were made between the effluent from control systems that contained oil alone and those from systems containing oil and the CBA.
[b]CBA types as defined in the note to Table 1.
[c]Fecundity data at these effluent concentrations greater than 3% are disregarded because no females were found alive.
NOEC = no observed effect concentration; LOEC = lowest observed effect concentration; NE = no effect.

more confined nature may be reasonably treated with higher or more frequent applications.

There are substantial barriers to effective performance of oil-spill CBAs, among them dilution rates, nutrient and biomass limitations, and a limited time in which a CBA can remain in contact with the oil spill. Efficacy indices from analytical chemistry, coupled with assessments of toxicity for CBAs, should provide useful information to an on-scene coordinator. These limitations will be discussed in the light of our experience with the Tier III effectiveness protocol.

Acknowledgments

Validation of the effectiveness protocol for Tier III open-water and beach test systems as well as the ecotoxicology for Tier II and Tier III was performed through a cooperative agreement (CR-818991-01) between the University of West Florida Center for Environmental Diagnostics and the EPA Environmental Research Laboratory at Gulf Breeze. The following people contributed ideas and technical assistance during the development of this project: Wanda Boyd, Mike Bundrick, Peter Chapman, Jim Clark, Carol Daniels, Barbara Frederick, Tim Gibson, Wallace Gilliam, Jeff Kavanaugh, Joanne Konstantopolis, Tony Mellone, Len Mueller, Neve Norton, Jim Patrick, Bob Quarles, Mike Shelton, Scott Spear, Phil Turner, Ling Wan, George Ryan, Vicki Whiting, Diane Yates, and Shiying Zhang.

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The Use of In Situ Carbon Dioxide Measurement To Determine Bioremediation Success

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Monitoring bioremediation success involves complex analytical chemistry and time-consuming microbiology. Potentially, a more valuable tool for the oil spill treatment specialist would be one that enabled the efficacy of a bioremediation strategy to be determined in real time *in situ*. This poster describes preliminary research on a method for making *in situ* measurements of bioremediation efficacy based on the estimation of CO_2 evolution. These studies were conducted in the field near Landevennec, France. The trial involved the oiling of six plots on a beach consisting largely of shale on a clay base. Three plots were amended with a slow-release inorganic nutrient, and three plots remained untreated as controls. Three plots also were delimited on the same beach to act as unoiled controls.

Methods

Two sampling devices were made from stainless steel, consisting of a shallow cylinder (0.2 m high and 1.1 m in diameter) sealed at one end with a base plate. The base plate was pierced with two steel tubes connected to valves on the outside of the device. The samplers were pushed gently into the beach surface, with the base plate facing upward and the valves open to the air. The CO_2 analyzer then was connected to the valves, and air from the sampler was circulated through it, giving an initial CO_2 reading. The CO_2 level was then monitored periodically over the next 5 to 20 min. Measurements were taken at the same coordinates on the oiled controls, the unoiled controls, and the plots treated with oil and fertilizer. Readings were made 26, 116, and 144 days after oiling. Nutrients were applied 11 days after oiling and monthly thereafter.

Results and Discussion

On each sampling day, the rate of CO_2 evolution was enhanced on oiled plots treated with fertilizer in comparison to oiled controls and unoiled controls. The largest difference was noted 15 days after nutrient addition when the rates increased from 3.1 ppm to 4.0 ppm $CO_2.min^{-1}$ on the oiled controls to 12.6 ppm to 22.3 ppm $CO_2.min^{-1}$ on the fertilized plots. The unoiled controls gave values between 2.8 ppm and 4.2 ppm $CO_2.min^{-1}$. These data suggest that nutrient addition stimulated the CO_2 evolution rates when compared with untreated controls. The rates were found to decrease in subsequent measurements of the fertilized plots but were still 1.5 to 2.0 times greater than the controls, suggesting the stimulation in CO_2 production was sustained.

Conclusion

These preliminary data suggest that addition of fertilizer to oiled plots stimulates CO_2 evolution. Whether this stimulation reflects enhanced oil biodegradation, as we suspect, remains to be proven absolutely using gathered chemical samples. Further, although the measured values are, by their nature, relative rates and not absolute indicators of CO_2 production, the results suggest that this technique may provide useful data when examining the efficacy of bioremediation strategies and products on contaminated shorelines. A second field trial conducted in the United Kingdom in the summer of 1994, funded by EPA, will allow a more detailed evaluation of this promising technique.

Intrinsic Bioremediation of JP-4 Jet Fuel Contamination at George AFB, California

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Intrinsic bioremediation is difficult to evaluate from monitoring well data. Depending on the screened interval and the pumping rate, a well may produce water from an uncontaminated part of the aquifer, resulting in a sample that is greatly diluted by clean water. In addition, a well may miss the plume entirely. Both effects give the false impression that *in situ* biological processes are attenuating the contaminants. A rigorous demonstration of intrinsic bioremediation should include 1) information on the use of available electron acceptors; and 2) information on the concentration of a tracer associated with the plume that can be used to correct for dilution.

Ground water at George Air Force Base (AFB) was contaminated by a release of JP-4 jet fuel. Well MW 24 is near the center of the spill. Well MW 25 is 500 feet from well MW 24 in a direction that is perpendicular to ground-water flow. Wells MW 27, 29, and 31 are along a flow path down-gradient of well MW 24. The plume velocity is near 100 ft/yr.

Oxygen and nitrate were depleted downgradient of the spill. The concentration of benzene was reduced more than 300-fold, while the concentration of a more recalcitrant compound, 1,2,3-trimethylbenzene, was only reduced three-fold. After correcting for dilution, benzene concentrations were reduced at least 100-fold due to intrinsic bioremediation.

Table 1. Intrinsic Bioremediation of Benzene and Toluene

	MW 24	MW 25	MW 27	MW 29	MW 31
Location	Center of oil lens	Edge of oil lens	700 ft away	1,200 ft away	1,800 ft away
			(mg/liter)		
Oxygen	< 0.5	8.0	0.6	< 0.5	1.1
Nitrate	0.8	3.7	0.4	0.3	3.1
			(µg/liter)		
Benzene	1,620	194	80	4.8	< 0.5
Toluene	1,500	604	< 0.5	< 0.5	< 0.5
1,2,3-Trimethylbenzene	73	39	56	20	< 0.5

Factors Affecting Delivery of Nutrients and Moisture for Enhanced In Situ Bioremediation in the Unsaturated Zone

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Successful *in situ* bioremediation in the unsaturated zone requires that water, oxygen, and trace nutrients be available in appropriate amounts and correct locations. To enhance degradation rates, some applications may require delivery of moisture, oxygen, or trace nutrients via subsurface or surface application of fluids. Since the exact locations and geometry of contaminated regions are unknown, a practical engineering approach is to design fluid delivery systems to uniformly distribute the fluids to a subsurface region.

This project investigates limitations of engineered systems for delivery of nutrients, either liquid or gas, to contaminated soils in the unsaturated zone. These limitations are derived from two sources: 1) the basic design of fluid delivery systems (e.g., inherent limitations in using vertical wells or surface irrigation systems to uniformly distribute and collect a fluid in an unsaturated subsurface region); and 2) heterogeneity in porous media properties that affect fluid flow in the unsaturated zone (e.g., spatial variability of saturated hydraulic conductivity).

Unfortunately, the design of common fluid delivery systems and the heterogeneity of hydraulic soil properties work against achieving the goal of uniform fluid distribution. Vertical wells and soaker hoses are two means of fluid delivery, but these are essentially point or line sources. Thus, important unanswered questions exist about the proper spacing of these devices to achieve a uniform application rate. A potentially more difficult issue is the significant spatial heterogeneity in the hydraulic properties of natural soils. This heterogeneity creates paths of preferred flow on a variety of spatial scales; only a fraction of the porous media may contribute to fluid flow, and thus, an engineered system designed to deliver moisture, oxygen, or nutrients could fail to achieve a uniform distribution. Thus, the conventional notion of, for example, a well's "region of influence" is less clear and will be critically reexamined through experimental and theoretical approaches.

Work in Progress

This poster presents findings from a review of soil science and *in situ* bioremediation literature, focusing on the potential effects of preferential flow on *in situ* bioremediation effectiveness. This review was initiated at the start of the project in January 1994 and is being used to guide the design of experiments scheduled to begin later this year. Future plans regarding the experimental investigations also will be presented.

Section Four
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. Thus, pilot-scale research is a critical intermediate step in which the success of laboratory experiments are further tested in an expanded but controlled setting.

Pilot-scale evaluations were performed on three alternative biofilter attachment media as part of continuing research on the development of biofiltration for treatment of volatile organic compounds (VOCs). A pelletized medium exhibited the best and most consistent performance of the three media tested. Future work will concentrate on further optimizing the use of the pelletized medium.

Research continued on developing methods for operating land treatment reactors using redox control. Methods will be tested using pentachlorophenol (PCP)-contaminated soil from the American Wood Products site in Lake City, Florida, in pilot-scale soil pan reactors. In a related project, studies continue on the use of combined treatment technologies for remediating contaminated soils from PCP manufacturing facilities and wood preserving sites.

A small-scale field study along the Delaware Bay shoreline is planned to evaluate bioremediation of oil-contaminated beaches. Laboratory and field experiments will be used to test application strategies.

EPA's Testing and Evaluation (T&E) Facility will evaluate the performance of bench- and pilot-scale slurry bioreactors in treating hazardous waste, as part of a general research program on engineering assessment and optimal design. Soil contaminated with creosote constituents from a site in St. Louis Park, Minnesota, will be used to test the reactors. In addition, researchers at EPA's T&E Facility are studying the ability of compost microorganisms to biodegrade polycyclic aromatic hydrocarbons (PAHs) in in-vessel reactors. Soil contaminated with PAHs from the Reilly Tar site in St. Louis Park, Minnesota, will be used to evaluate the performance of this technology.

The symposium's poster session included presentations on a pilot-scale evaluation of nutrient delivery for oil-contaminated beaches; field treatment of benzene, toluene, ethylbenzene, and xylene (BTEX) in vadose soils using extraction or air stripping and biofilters; dechlorination with a biofilm-electrode reactor; the use of sulfur-oxidizing bacteria to remove nitrate from ground water; and an engineering evaluation and optimization of biopiles for treatment of soils contaminated with hazardous waste.

Pilot-Scale Evaluation of Alternative Biofilter Attachment Media for Treatment of VOCs

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

Preliminary investigations (3) were performed on three media: 1) a proprietary compost mixture; 2) a synthetic, monolithic, straight-channeled (channelized) medium; and 3) a synthetic, randomly packed, pelletized medium. These media were selected to offer a wide range of microbial environments and attachment surfaces and different air/water contacting geometries. The results of this preliminary work demonstrated that 95+ percent VOC removal efficiency could be sustained by all three media at a toluene loading of 0.725 kg COD/m^3-d, but at different empty bed residence times (EBRTs). For the pelletized medium, this performance could be achieved at an EBRT of 1 min, for the channelized medium at 4 min, and for the compost medium at 8 min. Both synthetic media developed headloss over time, with the pelletized medium showing a pressure drop in excess of several feet of water after sustained, continuous operation. These results left open the question of which medium could provide the optimum combination of high VOC elimination efficiency at high loading with minimum pressure drop.

This paper discusses the continuing research being performed for development of biofiltration as an efficient, reliable, and cost-effective VOC APC technology. The objectives of the recent research were to conclude the evaluation of the three media and to develop workable strategies for the removal and control of excess biomass from the (ultimately) selected pelletized attachment medium.

Experimental Apparatus

The biofilter apparatus used in this study consists of three independent, parallel biofilter trains, each containing 4 feet of attachment medium: biofilters A, B, and C. A detailed schematic and equipment description is given elsewhere (4). Biofilter A was filled with a proprietary compost mixture, B with a Corning Celcor channelized medium, and C with a Manville Celite pelletized medium. Biofilters A and B are square and have an inner side length of 5.75 in.; biofilter C is round, with an inside diameter of 5.75 in. The air supplied to each biofilter is highly purified for complete removal of oil, water, CO_2, VOCs, and particulates. After purification, the air flow for each biofilter is split off, injected with VOCs, humidified, and fed to the biofilters. The air feed is mass flow controlled, and the VOCs are metered by syringe pumps. The flow direction of the air and nutrient inside each biofilter is downward. Each biofilter is insulated and independently temperature controlled.

Buffered nutrient solutions are fed to biofilters B and C. A detailed description of the nutrient composition is given elsewhere (4). Each of these biofilters independently receives a nutrient solution containing all the necessary macro- and micronutrients, with a sodium bicarbonate buffer. The nutrients required in biofilter A were included as part of the original compost mixture.

Results

Biofilter A

This biofilter run on the compost medium was made to evaluate the effects of temperature and then loading on toluene removal efficiency. Figures 1a and 1b summarize the biofilter performance. The biofilter was started up and, after some operational difficulties, stabilized by Day 10 at 52°F, 50 ppmv toluene, 2 min EBRT, and a removal efficiency of about 58 percent. On Day 17, the temperature was raised to 60°F, resulting in a rise in efficiency to about 75 percent, which decreased after Day 24 into the 60s, and after Day 32 into the 50s. On Day 41, the temperature was increased to 70°F, resulting in a gradual increase in efficiency to about 75 percent by Day 47. On Day 53, the temperature was increased to 80°F, resulting in an increase in efficiency into the low 80s. On Day 61, the temperature was increased to 90°F, resulting in a further increase in efficiency to the mid-90s (Figure 1a). After Day 77, the feed was increased slowly to about 95 ppmv toluene, resulting in a drop in efficiency to about 88 percent. Further increases in the feed concentration to a maximum of 180 ppmv toluene on Day 139 resulted in a further decline in efficiency to about 58 percent (Figure 1b). The run was terminated on Day 215.

Biofilter B

This biofilter run was made on the synthetic channelized medium to evaluate the effects of temperature and then nutrient feed rate on removal efficiency. The biomass in the channels of the medium remaining from the previous run was removed by hydroblasting the eight 6-in. high medium blocks from top and bottom. The corners of these square blocks were filled with grout to provide a "round" active block. This last step was taken to match a round block cross section with the round pattern of the nutrient delivery spray nozzle. Figure 2 shows the biofilter performance as a function of time. The biofilter was started up at 52°F, 50 ppmv toluene, and 2 min EBRT. By Day 36, the removal efficiency had drifted over a range from about 62 to 80 percent. On Day 36, the nutrient feed rate was increased from 30 L/day to 60 L/day, while keeping the mass loading of the nutrients constant. The increased nutrient flow rate effectively doubled the wetting cycle from 20 sec/min to 40 sec/min. An immediate increase in efficiency to 99 percent was observed, which then quickly dropped and ranged by Day 50 between about 30 and 70 percent. On Day 50, the nutrient feed rate was increased to 90 L/day (increasing the wetting cycle to 60 sec/min), but the efficiency dropped from 69 percent and ranged by Day 67 from about 22 percent to 65 percent. On Day 67, the temperature was raised from 52°F to 60°F, and the efficiency increased to 66 percent. By Day 75, the efficiency was 87 percent, and this level was maintained to

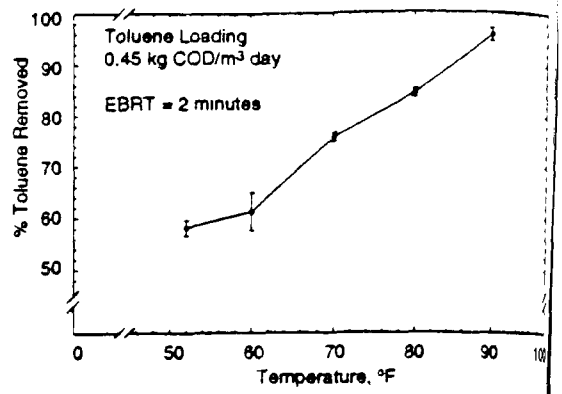

Figure 1a. Effect of temperature on the performance of compost biofilter.

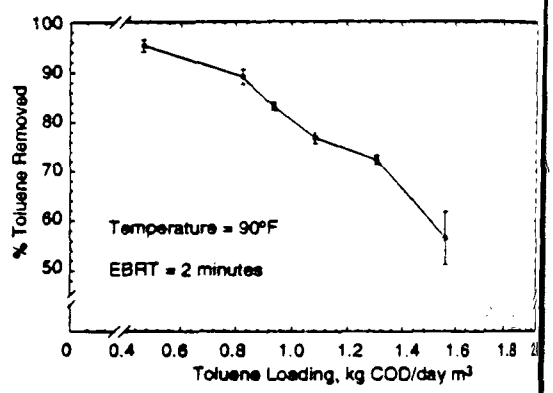

Figure 1b. Effect of toluene loading on the performance of compost biofilter.

Day 83. After Day 83, the temperature was raised 10°F steps to 90°F, but the efficiency did not improve fact, for the rest of the run, at 90°F and 60 L/day efficiency ranged between about 58 percent and percent. The run was terminated on Day 152.

Biofilter C

The first biofilter run on the synthetic pelletized medi was made to evaluate the effects of pressure drop a then temperature on toluene removal efficiency. T biofilter was charged with pellets used in the previo run. These pellets were washed by hand in hot wa (150°F) until the accumulated surface biomass h been removed and the pellets were free flowing. Figu 3 presents the biofilter performance as a function time. The biofilter was started up at 52°F, 50 ppm toluene, and 2 min EBRT. By Day 21, the remov efficiency was 99 percent, and by Day 27, it had reach 100 percent and remained at this level until Day 5 From Day 51 to Day 57, the EBRT was gradua reduced to 1 min, causing the efficiency to drop

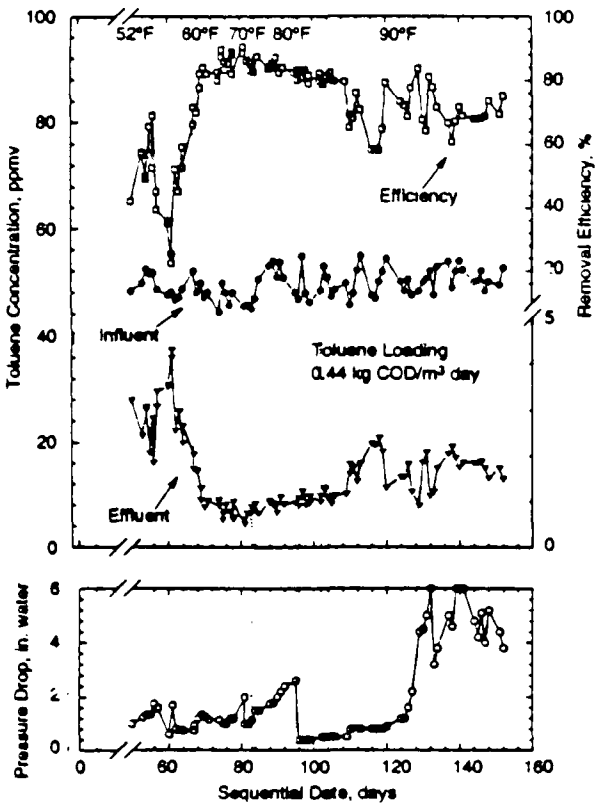

Figure 2. Performance of channelized biofilter with respect to toluene removal of an EBRT of 2 min.

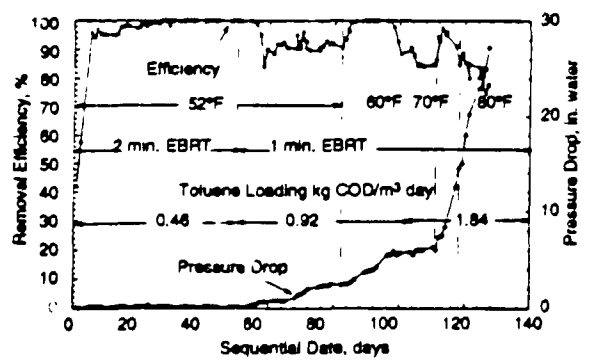

Figure 3. Performance of pelletized biofilter with respect to toluene removal at 1 min and 2 min EBRT without backwashing.

84 percent. Subsequently, the toluene removal efficiency rapidly increased to the low 90s and remained in that range until Day 81. On Day 82, the temperature was raised to 60°F, and the efficiency steadily rose until complete biodegradation of the toluene was reached on Day 89. This essentially 100-percent efficiency in toluene removal was maintained through Day 97. During the period between Day 54 and Day 97, pressure drop across the system increased from 0.2 to 5.5 in. water.

From Day 97 to Day 111, the efficiency dropped steadily from 100 to 86 percent, while the pressure drop increased from 5.5 to 6.0 in. water. On Day 112, the temperature was increased to 70°F, and the efficiency rebounded by Day 113 to a peak value of 97 percent, after which it dropped to 85 percent by Day 188. On Day 119, the temperature was raised to 80°F, and the efficiency rose to about 89 percent by Day 120. During the period from Day 112 to Day 120, the pressure drop increased from 6 in. water to 18 in. By Day 128, the efficiency had steadily dropped from 89 to 77 percent as the pressure drop increased from 18 in. water to 27 in. This pattern of a steady loss of efficiency with a coincident increase in pressure drop suggests the development of short circuiting within the biofilter medium because of biomass accumulation, which results in a significant reduction in actual contact time. The run was terminated on Day 128.

The second biofilter run on this medium was conducted to evaluate routine biomass control by backwashing. The biofilter was charged with a 50:50 mixture of fresh pellets and pellets from the previous run. The used pellets were thoroughly washed by hand in tepid water (90°F) until the accumulated surface biomass had been removed and the pellets were free flowing. Figure 4 shows the biofilter performance as a function of time. The filter was started up at 90°F, 50 ppmv toluene, and 2 min EBRT. By Day 4, the removal efficiency was 100 percent. (Note: This second run, started up with pellets washed in tepid water, contrasts with the slower startup in the first run, where the pellets were washed with hot water.) On Day 8, the feed was increased to 250 ppmv toluene; the efficiency dropped to 97 percent and ranged between 92 and 98 percent until Day 25, when it again reached 99 percent. Subsequently, the efficiency dropped as low as 86 percent before regaining 99 percent on Day 81, after which the efficiency was nearly always 99+ percent. Initially, backwashing was performed once a week by using 100 L of fresh water at a rate of 6 gallons per minute (gpm). After Day 28, the

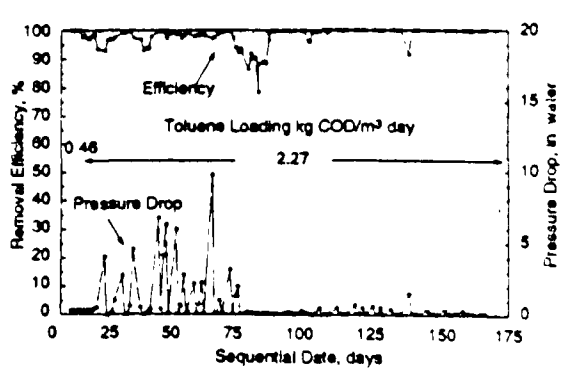

Figure 4. Performance of pelletized biofilter with respect to toluene removal at 2 min EBRT with backwashing.

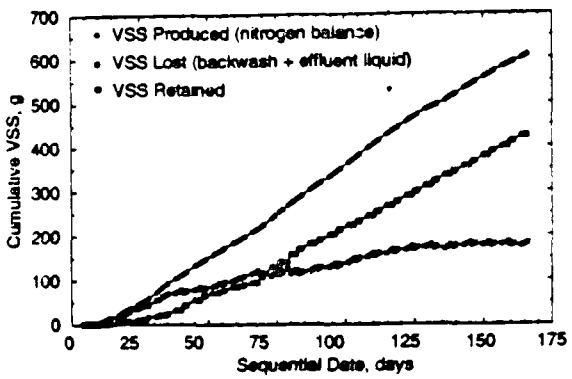

Figure 5. Development of pelletized biofilter with time (VSS closure).

frequency was increased to twice per week, and after Day 38, the volume was increased to 200 L. These changes were made because measurable pressure drop was observed between backwashings. On Day 73, the backwash rate was increased to 15 gpm to induce full fluidization. Although the pressure drop increase was minimal, the efficiency did not improve, suggesting some form of channelizing within the bed. Therefore, on Day 80, the length of the backwash period was increased to 1 hour by recirculating the backwash water. After this final adjustment, the toluene removal efficiency, as mentioned above, achieved and sustained 99+ percent. During this latter period, the total volume of water used per backwash was optimized to 120 L. Of this volume, 70 L were used for the 1-hr backwash recycle, while the remaining 50 L were used to flush the released solids from the reactor. Figure 5 shows the development of biomass with time. After Day 38, the rate of biomass accumulation declined with the increase in the wash volume. After Day 73, the accumulation rate became nearly zero with the implementation of full fluidization. Since then, no change in the backwash procedure has been made, and the accumulation of biomass within the biofilter has leveled off at about 180 g with the pressure drop between backwashings typically under 0.2 in. of water.

Conclusions and Future Work

A marked improvement in toluene removal efficiency with increasing temperature was demonstrated in this study for the compost mixture, the channelized medium, and the pelletized medium. The direct consequence of this finding is that much less medium would be needed for a biofilter operating at 90°F than at 52°F, resulting in a proportional reduction in capital cost. The economic tradeoff with the cost of heating the incoming air should usually favor operation at these warmer conditions.

The modest performance of the compost mixture with respect to increased loading complemented our earlier findings with respect to decreasing EBRT (3). Unfortunately, implicit limitations of the experimental apparatus may have resulted in reduced performance. Specifically, the manufacturers recommended using a width-to-depth ratio of 1:1, rather than 1:8. They also stated that from their experience the only effective means of controlling bed moisture content was to weigh the entire biofilter. Weighing was impossible with the heavy stainless steel unit used here, which was bolted to a support frame. Several moisture measurement and control strategies were attempted, but it was never possible to be certain that the bed moisture content was consistently at the reported optimum range, i.e., between about 50 and 60 percent (5,6). The sometimes erratic performance may have been influenced by variations in bed moisture content. The best removal efficiencies achieved by the compost mixture, however, were better than shown by the channelized media but worse than shown by the pelletized media.

The performance of the channelized medium also confirmed our earlier findings that this medium is distinctly inferior to the pelletized medium (3). The best performance was achieved during the use of new medium blocks. After biomass accumulation within the channels and subsequent removal by hosing, the performance never regained the previous, still modest, levels. Attempts to adjust nutrient flow as a means of testing the effect of the duration of wetting in the nutrient application cycle did not overcome the previously demonstrated efficiency limitations. The more erratic performance of this medium after removal of the biomass suggests that this medium may be unsuitable for sustained efficiency after periodic cycles of biomass removal. This erratic performance, due to suspected random uneven plugging of channels by biomass, coupled with its relatively low overall removal efficiency, difficulty in biomass removal, and intrinsically high medium cost, suggests that this medium may not be a viable option for this application.

The pelletized medium exhibited the best and most consistent performance of the three media tested. It rapidly achieved high removal efficiencies at high toluene loadings. As the first run demonstrated, however, an excessive accumulation of biomass, shown by a rise in the pressure drop across the medium, results in a substantial loss in efficiency, followed by a very rapid rise in pressure drop. This suggested that efficient, sustained performance might be achieved through early and periodic control of biomass accumulation by backwashing. In the second run, the implementation of a suitable backwashing strategy for biomass control was achieved by using full medium fluidization. This strategy permitted sustained operation of the biofilter at high loadings with efficiencies consistently at 99+ percent. According to

mass balance calculations, the biomass retained within the biofilter stabilized at a nearly constant level.

Future work will concentrate on further optimizing the use of the pelletized medium, with the objective of minimizing the medium volume required for a selected APC technology application.

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Biological Treatment of Contaminated Soils and Sediments Using Redox Control: Advanced Land Treatment Techniques

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Soils and sediments contaminated with highly chlorinated aromatic compounds such as polychlorinated biphenyls (PCBs), pentachlorophenol (PCP), hexachlorobenzene (HCB), and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) are found at many of the Superfund sites that have been placed on the National Priority List for cleanup. Bioremediation has been proposed as a means for converting these contaminants into less toxic or nontoxic substances.

The biodegradation rates of many highly chlorinated compounds can be accelerated by controlling the redox potential [or oxidation-reduction potential (ORP)] of the treatment environment. In general, the biochemical pathway providing the highest rate for the initial steps of microbial destruction of highly chlorinated organics is anaerobic reductive dechlorination. Once partially dechlorinated, the resulting compounds typically degrade faster under aerobic, oxidizing conditions. Efficient and complete degradation of highly chlorinated contaminants is possible when the two redox conditions are sequentially applied.

Sequential treatment techniques have been proposed as a means of treating aqueous wastes and slurries containing soils contaminated with highly chlorinated aromatic compounds such as PCBs, PCP, HCB, and DDT, among others (1,2). For example, the *meta* and *para* chlorines of highly chlorinated PCBs are removed by anaerobic reductive dechlorination; however, the *ortho* chlorines are only slowly removed by the same bioprocess. Aerobic organisms remove the *ortho* chlorine and complete the mineralization of the compound relatively quickly. Thus, sequential anaerobic-aerobic treatment should provide relatively rapid destruction of PCBs (3,4). The process applied to PCB-contaminated sediments has been studied by other research groups (1,5) and is currently being demonstrated in the field. Woods et al. (6) suggested that an anaerobic-aerobic sequential treatment strategy would be attractive treatment alternative for highly chlorinated phenols because the anaerobic consortium used in their study was capable of reductively dechlorinating highly chlorinated phenols to monochlorophenols. The monochlorophenols were not reductively dechlorinated further; however, they are known to degrade in aerobic treatment processes. Bench-scale work in our own group has evaluated the applicability of the technology for the treatment of HCB and DDT contamination. Results for DDT degradation in the anaerobic phase have been encouraging. DDT, which usually accumulates as 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) under anaerobic conditions, has been degraded to less chlorinated intermediates such as 2,2-bis(*p*-chlorophenyl)ethanol (DDOH) and dichlorobenzophenone (DBP) using a combination of chemical reducing agents and surfactants in conjunction with anaerobic culture. Aerobic degradation of these intermediate products is under investigation.

A practical means of applying sequential redox control in field-scale remediation is needed. Since land treatment is a well-understood, cost-effective means of conducting *aerobic* biological treatment of soils contaminated with compounds such as petroleum and polycyclic aromatic hydrocarbons (PAHs), we have proposed to extend it to include an anaerobic phase to treat compounds amenable to reductive dechlorination. In this project, methods are being developed for operating

land treatment reactors under *anaerobic* as well as aerobic conditions so that a sequential strategy can be readily applied in the field. Methods of applying multiple cycles of alternating redox conditions to achieve cleanup also are being investigated. During this project year, these methods will be tested using PCP-contaminated soil in pilot-scale soil pan reactors. In subsequent project years, we plan to investigate soils from several types of sites, including sites contaminated with DDT.

Methodology

Reactor operating strategies that deliver adequate anaerobic and aerobic microbial environments are currently being developed using uncontaminated soil in a pilot-scale unit with two pans (reactors). Each pan holds approximately 30 kg of soil. Various methods of maintaining anaerobic conditions in the soil reactor currently are being evaluated, including simply flooding the soil bed, adding an easily degradable organic compound(s) to serve as an oxygen scrubber near the surface, and covering the soil bed with an air-impermeable cover to inhibit the transport of oxygen. Liquid addition and permeate recycle techniques also are being evaluated during the anaerobic phase of operation. Methods for returning the soil bed to aerobic conditions will be investigated when the anaerobic phase is complete. The soil bed will be drained and, if necessary, a vacuum will be applied below the bed to assist in drainage and aeration of the soil. Bulking agent addition may be required to improve aeration of the soil. Hand mixing/tilling methods and sample collection methods will be investigated during both phases.

A source of contaminated soil has been identified, and background information about the site and the range of contaminants and contaminant concentrations has been obtained. Soil samples (courtesy of Wildemere Farms, Inc., Lake City, Florida) from various locations at the American Wood Products site in Lake City, Florida, representing a range of contamination levels have been analyzed for chlorinated phenolics. A comparison of PCP concentrations in these samples found by our group and by an independent laboratory is shown in Table 1.

Trace amounts of less chlorinated intermediates were noted in some of the samples analyzed in our laboratory, but the concentrations were under the method detection limit (~1 mg/kg). Dioxins, low-level contaminants in technical grade PCP, were analyzed by the independent laboratory, the congener with the highest concentration was octochlorinated-dioxin at 18 ppt, and the highest risk congener, 2,3,7,8-tetrachlorodioxin, was nondetectable. For the pilot-scale work at EPA's Test and Evaluation (T&E) Facility, soil will be obtained from two of the sampling points at the site that represent high and low levels of contamination. Approximately 600 kg of soil

Table 1. Soil Analysis for PCP

	PCP in Analyzed Soil Samples*		
Sample	Mean Concentration (mg PCP/kg dry soil)	Standard Deviation (mg PCP/kg dry soil)	Data from Independent Lab (mg PCP/kg soil**)
1	12.2	0.66	16.8
2	37.8	1.2	46.4
3	103	2	64.5
4	109	12	59.7
5	8.66	4.08	3.29
6	3.54	0.19	3.08
7	136	9	115
8	116	7	93.3
9	209	15	178
10	133	7	125
11	445	38	N/A
12	69.2	4.2	N/A
13	4.21	1.00	N/A
14	1.11	0.22	N/A

* Three replicates analyzed per sample
** Dry weight not specifically indicated in report

from each sampling location will be required. The soil will be transported to the T&E Facility, where it will be shredded, sieved, mixed, sampled, characterized, and placed in the pilot-scale units.

Six pilot-scale units with four pans each, a total of 24 pans, will be employed in this study. The experimental design is shown in Table 2. Each treatment will be duplicated in separate reactors. A "clean" soil spiked with PCP will be tested in addition to the two concentrations obtained from the site. The use of recycle for moving the liquid through the soil versus the maintenance of stagnant liquid in the pan will be one of the variables tested. Sterile controls will be run in parallel with each treatment to monitor for abiotic losses. The

Table 2. Experimental Design for Soil Pan Reactors

Treatment		Contamination Level		Spiked Clean Soil
		Low	High	
Biologically Active	No recycle	2*	2	2
	Recycle	2	2	2
Biologically Inactivated	No recycle	2	2	2
	Recycle	2	2	2

*Two reactors per treatment

simplest approach will be tested first. The soil will be flooded with site water, if it can be obtained, or with deionized water (close approximation to rainwater) to create anaerobic conditions.

Specific treatment assignments to specific pans in the six four-pan units have been randomly assigned. Randomization is necessary because this design will be statistically analyzed as a three-factor analysis of variance (ANOVA) with replication. The three factors are biological activity, soil "type," and recycle. The dependent variable that will be used to compare treatments and evaluate treatment effectiveness will be the molar sum of the chlorinated aromatics (parent compound + metabolites) removed per kilogram of dry soil at a set time interval (e.g., after 4 months in anaerobic treatment and after 2 months in aerobic treatment). Molar concentrations will be normalized using the initial concentration in each treatment so that the treatments can be compared statistically using ANOVA techniques.

To supplement the statistical comparison, the pans will be sampled at 2-week interim time points, and the samples will be analyzed for the parent contaminant and chlorinated aromatic metabolites to provide insight into the pattern of removal. Other monitoring will include daily measurement of pH, ORP, and temperature. Total and volatile solids will be determined each time a soil sample is collected so concentration can be calculated on a dry soil basis and so soil moisture can be monitored during the aerobic phase.

Serum bottle experiments using soil from the site will be conducted concurrently with the pilot-scale reactors. In these experiments, alternative treatment strategies including co-substrate and nutrient amendments and inoculation of acclimated organisms will be explored as means of improving treatment rate and extent. Pilot-scale evaluation of alternatives found to be optimal is planned for FY95.

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Research Leading to the Bioremediation of Oil-Contaminated Beaches

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During the summer of 1994, EPA, in cooperation with the Delaware Department of Natural Resources and Environmental Control (DNREC), plans to conduct a small-scale field study on the shoreline along Delaware Bay involving bioremediation of crude oil released in small quantities on 15 identical plots. The goals of this research project are 1) to obtain sufficient statistical evidence to determine if bioremediation with inorganic mineral nutrients and/or microbial inoculation enhances the removal of crude oil contaminating mixed sand and gravel beaches; 2) to compute the rate at which such enhancement takes place; and 3) to establish engineering guidelines on how to bioremediate an oil-contaminated shoreline. Prior to conducting such a study, two important pieces of information need to be defined: 1) the minimum nitrogen concentration enabling the degrading populations to metabolize the oil components at their maximum rate at all times; and 2) the frequency at which the nutrients must be added to maintain such a concentration. The first question is being addressed in the laboratory, the second in the field. This paper discusses the design and conduct of laboratory and field experiments and presents some of the preliminary data answering the two questions posed.

Two nutrient application strategies were tested, one involving a sprinkler system spraying water soluble nutrients on the plot, the other incorporating a trench situated above the high tide line but below the underlying water table (1). In the latter method, tracer is applied through a manifold at the bottom of the trench just before high tide. The underlying ground water carries the tracer to the treatment zone as tides ebb and flow over time.

Methodology

Laboratory Experiment

To determine the minimum nitrogen concentration needed for maximum biodegradation over time, six semicontinuous flow respirometric beach reactors able to mimic tidal flow on a beach (2) were used. A major advantage of this microcosm is its ability to provide continuous, real-time monitoring of oxygen uptake and carbon dioxide evolution without the need for destructive sampling. Each tidal flow reactor measures 75 mm in diameter and 260 mm deep and holds approximately 2 kg beach material. The columns are fed from a 20-L Teflon reservoir containing a flexible inner Teflon bag. Influent seawater contained inside the flexible bag is continuously pumped by a "wave" pump into the top of the reactor through a spray nozzle. The seawater finally returns to the 20-L carboy outside the Teflon bag to maintain separation between influent and effluent. The headspace of the reservoir, the reaeration flasks, and the reactor column are all connected to maintain constant pressure in the system. Oxygen is supplied automatically to the microcosm system from a respirometer whenever a deficit is sensed. The cumulative uptake of oxygen is tracked continuously over time, enabling analysis of reaction kinetics. An experiment was set up in which six different concentrations of nitrate-N (ranging from 0 mg/L to 10 mg/L) were supplied to the reactors, and biodegradation of heptadecane was followed continuously. A mixed culture from the shoreline of Delaware, previously enriched with heptadecane, was used as the inoculum.

Field Experiment

The field study is located on a sandy and slightly gravelly beach south of Slaughter Beach, Delaware. Surface morphology consists of a loose upper 25-mm thick layer of smooth gravel ranging in size from 4.75 mm to 19.1 mm atop coarse sand having a moderately homogenous particle size distribution. Two plots measuring 5 m x 10 m were set up. Two types of wells were situated within and outside the vicinity of each plot: piezometers and sampling wells. The piezometers consisted

of black iron rods about 2.5 m long and 3.2 cm inside diameter (ID). The bottoms were fitted with a specially fritted brass tip that allowed water to enter the well filtered of fine sand or peat particles characterizing the deeper zone of the beach. The piezometers were equipped with pressure transducers connected to a data logger mounted to a wooden post in back of and between the plots. The pressure transducers were used to measure the water head continuously to provide accurate readings of water levels during the tidal cycles.

The sampling wells were constructed of stainless steel and were also about 2.5 m long. Openings of 3.2 mm ID were drilled into the sides of the wells starting at 15 cm from the bottom tip and extending upward at intervals of 15 cm over a total length of 1.8 m. Stainless steel tubing of the same diameter was welded to these openings. The tubing extended inside the wells from the openings to above the tops of the wells, where plastic tygon tubing was attached for collection of water samples via syringe. The openings in the sides of the wells were covered with a fine-mesh stainless steel screen to filter out particulate matter that might clog the tubing. Thus, water samples at each depth interval were totally independent from other water samples, which enabled measurement of tracer concentrations at one depth without influence from tracer concentrations at other depths.

For the sprinkler plot, 20 kg of $LiNO_3$ was dissolved in 800 L of fresh water. For the trench application, 30 kg was dissolved in the 800 L because the trench, being 5 m wider than the plot width, required more tracer for an equivalent amount to reach the desired area of the plot. Two types of samples were collected at each sampling event: subsurface sand and water from the sampling wells. The sand samples were collected with a bulb planter at low tide only, water samples at both low and high tides. Water samples were analyzed for lithium by atomic absorption spectrophotometry (3). Sediment samples were extracted and filtered, and the pore water was measured for lithium by activated alumina (AA).

Results

Laboratory Experiment

Figure 1 summarizes results from two of the six reactors. Space limitations preclude presentation of all the data. Clearly, the reactor fed 10 mg/L NO_3—N exhibited twice the O_2 uptake and CO_2 evolution as the reactor fed 0.5 mg/L. Also, the effluent nitrate levels measured in the reactor fed 10 mg/L were only slightly lower than the influent nitrate levels, whereas effluent nitrate in the reactor fed 0.5 mg/L declined to virtually undetectable levels. Thus, 0.5 mg/L nitrogen appears to limit the biodegradative activity. The next higher concentration used in the experiment was 2.5 mg/L, which gave

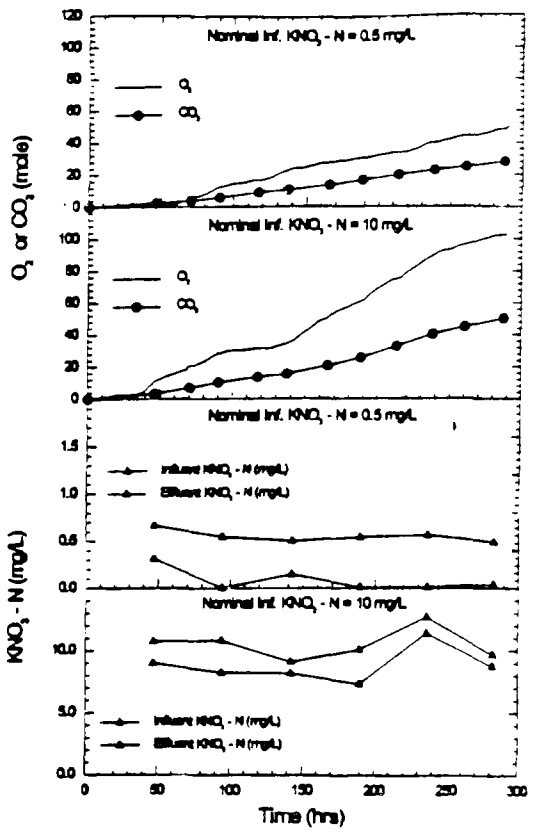

Figure 1. Mineralization of heptadecane in continuous flow microcosms in the presence of 0.5 mg/L and 10 mg/L nitrate-N.

approximately the same results as the 10 mg/L level. Another experiment was designed (results not ready at the time of this writing) to determine more closely the minimum nitrogen level that still provides maximum biodegradation.

Field Experiment

The plots were situated in the high intertidal zone corresponding to where the spring high tide would flood the entire plot. The tide experienced, however, was a neap tide, which means that the high tide did not cover the plot at all during the first few days of the experiment. Figure 2 is a three-dimensional mesh graph summarizing the lithium concentrations measured in the upper 12 cm to 13 cm of sand in the sprinkler plot from time 0 hr to 37 hr after application of tracer, corresponding to six tidal cycles. Immediately after application, the lithium concentration in the sediment pore water ranged from approximately 120 mg/kg to 200 mg/kg sand. Thus, the distribution of the tracer by the sprinkler was not as even as originally hoped. At the next low tide (12 hours later), the lithium had declined about 50 percent and was more evenly distributed over the plot surface. At the next low

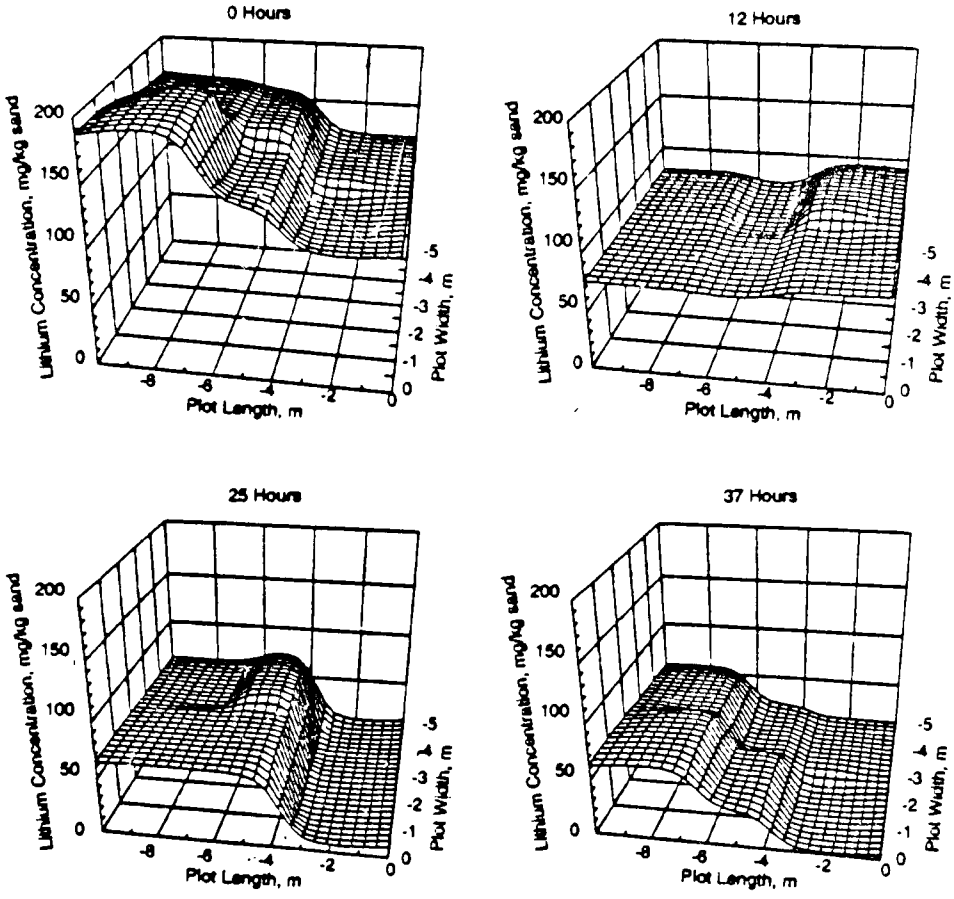

Figure 2. Three-dimensional plot showing behavior of lithium tracer during the first 37 hours after application.

tide (25 hours after application), lithium concentrations at the bottom of the plot had declined to almost undetectable levels. The previous high tide had covered this much of the plot, which explains the low levels of tracer there. Note that the lithium tracer in the upper two-thirds of the intertidal zone, which had not been wetted by the high tide, still persisted at slightly lower levels than the previous low tide. At 37 hours, corresponding to the third full tidal cycle, more of the plot had been covered by the incoming tide as reflected by the lithium concentrations shown in the figure. At the 48-hr mark, a storm had occurred, causing the tidal waters to completely submerge the plot. Lithium levels were undetectable (<1 mg/kg) in the surface sediment from about 55 hours through the remainder of the experiment, which lasted 10 days. Lithium concentrations in the surface sediment of the trench plot were undetectable until after the storm event, when low levels of lithium finally appeared because of underlying water carrying the tracer to the surface.

Tracer levels measured in well water samples from the ground water below the plot (data not shown) persisted for the duration of the experiment. The tracer moved up and down with the tides, which is consistent with observations made by Wise et al. (2) in Alaska.

Conclusions

From the laboratory experiment, the minimum nitrogen concentration needed to stimulate maximum microbial degradation of hydrocarbons is somewhere between 0.5 mg/L and 2.5 mg/L. From the field experiment, it appears that application of fertilizer should be conducted every day when the tide covers the entire contaminated zone. When the tide only covers the lower intertidal zone, nutrient application is not needed, since the nutrients will likely persist for several days. During this period, the microorganisms will be in constant contact with nitrogen and phosphorus, which will allow time for biostimulation to proceed. For the trench method to work, two trenches seem to be needed, one for the spring tide and one for the neap tide.

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Engineering Optimization of Slurry Bioreactors for Treating Hazardous Wastes

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Introduction

Biological treatment of contaminated soil slurries may offer the optimal treatment conditions for soil bioremediation at an economically viable cost. Despite this promise, slurry bioreactor treatment of soils has not achieved the status of a durable, reliable, and cost-effective treatment option. As part of a general program of engineering assessment and optimal design of slurry bioreactors, both bench- and pilot-scale reactors have been developed to address the pressing needs for missing operational data associated with slurry bioreactor use. These reactors are located at the EPA Testing and Evaluation (T&E) Facility located in Cincinnati, Ohio.

Methodology

Application of slurry bioreactors to the treatment of contaminated soil has been conducted with a variety of soil types (1). Case studies and cost comparison are available, but the information associated with these studies is incomplete (2). An EPA best demonstrated available technology (BDAT) study has investigated the application of slurry reactors to creosote-contaminated soil (3).

To systematically evaluate each of the major components of slurry biotreatment, a research program has been organized along the general divisions of physics, biology, and chemistry. Each of these divisions is a major contributor to the slurry biotreatment process. The physics of mixing has been the early focus of the slurry research program. The criteria for optimal mixing for slurries has not received the required attention. Five different criteria have been advanced for the chemical processing industry (4-7): 1) maximum uniformity of suspension; 2) complete off-bottom suspension; 3) complete on-bottom motion of all particles: 4) filleting but no progressive fillet formation; and 5) height of suspension (cumulative particle size distribution, percent solids, percent suspension, weight-percent ultimate suspended solids, and percent ultimate weight-percent settled solids).

For the initial evaluation of the bench-scale reactor (Figure 1), performance was assessed through the correlation of critical factors contributing to the efficiency of mixing (Figures 2 through 6). Solids composition was

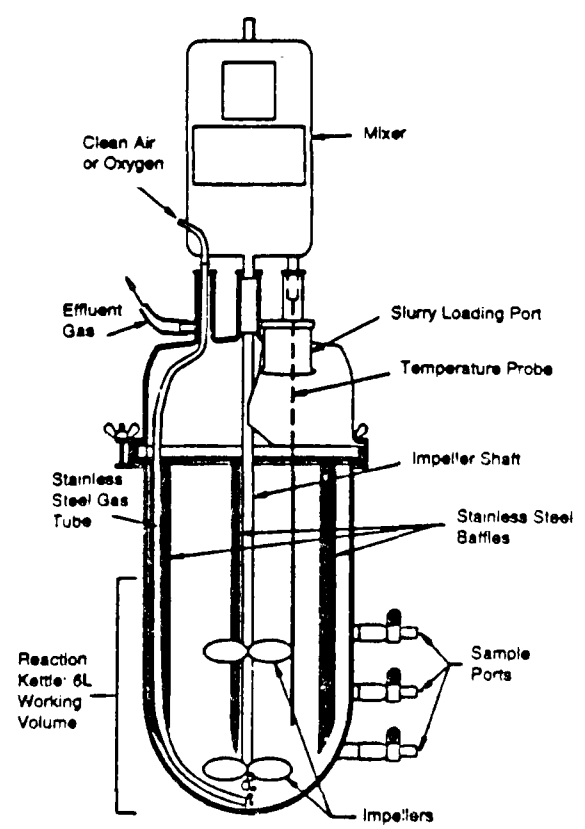

Figure 1. Bench-scale slurry bioreactor.

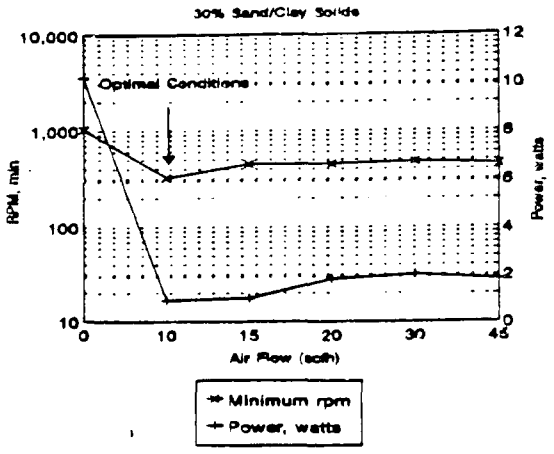

Figure 2. Complete off-bottom suspension (5 in. between impellers, baffle=design 3).

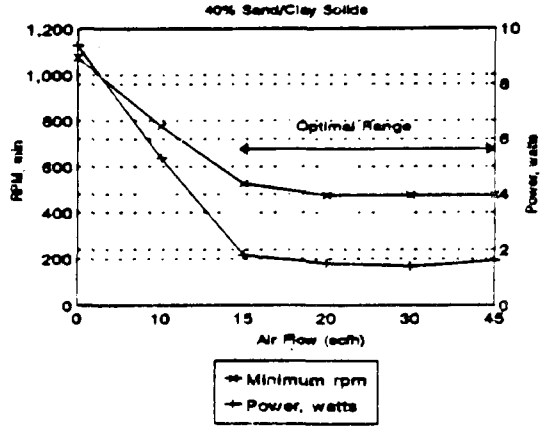

Figure 3. Complete off-bottom suspension (5 in. between impellers, baffle=design 3).

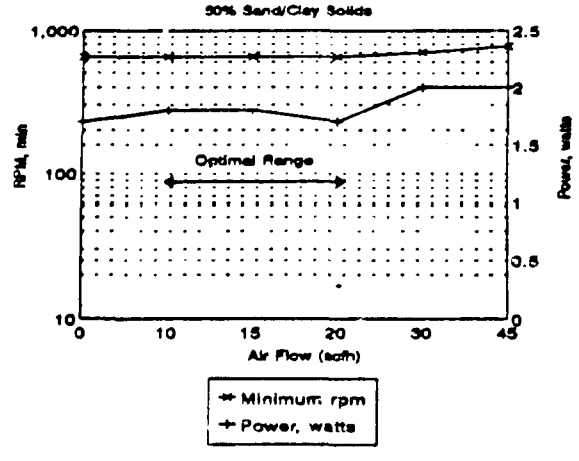

Figure 4. Complete off-bottom suspension (5 in. between impellers, baffle=design 3).

investigated for its influence on power consumption and the rotational speed of the impeller (Figures 2 through 4). Clear optimal ranges for air flow are evident in the recorded data. The optimal operating conditions are found at the point where the lowest power is consumed.

A soil from St. Louis Park, Minnesota, was contaminated with creosote constituents and used to evaluate the performance of bench-scale slurry reactors. The bench-scale bioslurry reactor was constructed from a 8-L glass conventional resin kettle with a four-port cover fitted with standard taper joints. The reactor vessel was fabricated to have three sample ports located 5 cm, 10 cm, and 15 cm vertically from dead center of the reactor bottom. The ports in the reactor cover permitted introduction of the stirring shaft, influent and effluent gas lines, and a thermocouple temperature probe into the soil slurry. Operational slurry volume was 6 L or 75 percent of the total reactor volume.

Ten bench-scale reactors were used to assess the effect of engineering variables on the degradation of polycyclic aromatic hydrocarbon (PAH) constituents over a 10-week treatment period.

The experimental design of the treatability study is outlined in Figure 7. Experimental variables selected for this study were soil loading, rotational speed of the mixing impeller, and dispersant. Soil solids concentrations of 10 percent and 30 percent (dry weight basis) were tested. Two mixing speeds were evaluated. A high mixing rate was selected for complete off-bottom suspension. A low mixing rate was arbitrarily set at 200 rpm lower than the high mixing rate. Effective high mixing rates were found to be 650 rpm and 900 rpm for the 10-percent and 30-percent soil solids, respectively. The dispersant (Westvaco, Reax 100M) was added to test its ability to minimize foam production. Foam formation is an operational problem associated with the application of soil bioslurry technology and is thought to be connected with naturally occurring organics found in certain soils.

Two separate 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 held constant for the reactors:

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

Results

For purposes of convenience, the individual PAH constituents were grouped into two categories: two- and three-ring compounds and four- through six-ring constituents. Initial concentration of total PAHs in the soil prior to treatment were 1,750 ppm in the 10-percent solids loading slurry and 2,047 ppm in the 30-percent slurry, indicating a degree of heterogeneity in the soil slurry. The total PAH concentration was reduced to 408 ppm in the 10-percent slurry (runs 1 through 4) and 419 ppm in the 30-percent slurry (runs 5 through 8) after 7 days of treatment. In the 10-percent slurry runs, the concentrations of two- and three-ring PAH compounds decreased from 709 ppm to 67.4 ppm, and concentrations of four- through six-ring PAHs decreased from 1,041 ppm to 340 ppm; whereas for the 30-percent slurry runs, the concentrations of two- and three-ring PAH compounds decreased from 798 ppm to 45.1 ppm, and concentrations of four- through six-ring PAHs in the 30-percent slurry runs decreased from 1,249 ppm to 374 ppm.

Summary and Conclusions

The total PAH concentration was reduced by 85 to 90 percent after 70 days of treatment. The major decrease in PAH concentrations occurred in the first 7 days, where total PAHs removed ranged from 75 to 82 percent. Soil solids concentrations significantly affected removal rate and the final treatment endpoint (PAH concentration). A maximum removal for the 30-percent solids loading was achieved after 21 days of treatment. Continued treatment after 21 days had little effect on further reduction of PAH concentrations. In the 10-percent solids runs, however, PAH concentrations continued to be reduced between Days 21 and 70. The final concentrations of two- and three-ring and four- through six-ring PAH categories, as well as total PAHs, for the 10-percent solids runs were half the levels in the 30-percent solids conditions.

These results show that removal efficiencies are apparently not as sensitive to complete off-bottom suspension as we had expected. Similarly, removal rates appear to be unaffected by mixing speed ranges. The dispersant additive did not effectively suppress foam formation or enhance PAH removal.

This initial study clearly identifies soil solids composition as a major factor controlling treatment goals. Lower solids compositions and longer treatment duration may favor treatment to lower PAH concentrations in the soil. Because removal rates observed in this work may be specific to the soil matrix selected for study, the generalizations arising from this work can be used for guidance for future applications of soil-slurry bioreactors. Treatability studies are necessary, however, to determine the most effective operating variables for each

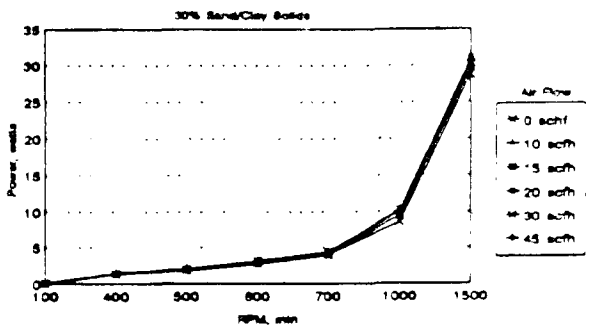

Figure 5. Air flow optimization (5 in. between impellers).

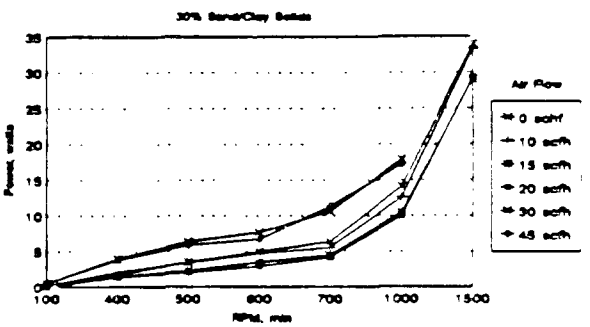

Figure 6. Air flow optimization (6 in. between impellers).

	Variable			
Run	A	B	C	D
1	–	–	–	–
2	–	+	–	–
3	+	–	–	–
4	+	+	–	–
5	–	–	+	–
6	–	+	+	–
7	+	–	+	–
8	+	+	+	–
9	+	+	–	+
10	+	+	+	+

Variable	–	+
Dispersant	0 mg/L	50 mg/L
Mixing Speed	450/700 rpm	650/900 rpm
Soil Solids	0 mg/L	50 mg/L
CH_2O	0 mg/L	50 mg/L

Figure 7. Experimental design (St. Louis Park soil).

waste matrix before embarking on any large-scale treatment. Foaming potential of a contaminated soil should be evaluated prior to treatment to minimize operational problems associated with foam formation at higher solids concentrations.

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Development and Evaluation of Composting Techniques for Treatment of Soils Contaminated with Hazardous Wastes

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Significant progress in optimizing conditions and applying the power of biotechnology to large-scale compost systems requires a working understanding of the processes and mechanisms involved. Prototype bench-scale units have been designed and tested to evaluate composting processes using contaminated soils. Identification of suitable co-compost and bulking agents, appropriate ratios of soil to organic components, and effective aeration strategies and rates have been selected as major factors requiring investigation.

This research program is designed to develop a thorough engineering analysis and optimization of composting as a process to treat soil contaminated with hazardous waste. Bench-scale composters serve as diagnostic tools to estimate the treatment capability of larger systems. Fully enclosed, insulated reactors permit reliable data collection on mechanisms of metabolism and the fate of toxic chemicals during soil composting.

We are currently studying the ability of compost microorganisms to biodegrade polycyclic aromatic hydrocarbons (PAHs) in in-vessel reactors located at the EPA Testing and Evaluation Facility in Cincinnati, Ohio. Soil contaminated with PAHs was obtained from the Reilly Tar Pit Superfund site in St. Louis Park, Minnesota, for use in this study.

Background

Composting holds potential to provide low-cost treatment of hazardous waste with minimal environmental controversy. Commercial compost operations are operated as black-box systems in that optimization is largely approached through trial and error. Treatment of hazardous waste cannot be conducted with suboptimal controls to meet the specified endpoints.

Some proponents of compost treatment have claimed significant success in destruction of hazardous wastes without strong data to support their claims. Disappearance of parent compounds has been used to claim that microorganisms successfully degraded waste chemicals. Some toxic chemicals, however, could potentially adsorb to, or react with, humic substances in the compost and become undetectable by chemical analysis. Such toxicants might later desorb from humus and migrate to the biosphere. This highlights the need for well-controlled studies to rigorously document degradation rates and to identify metabolic products of hazardous chemicals, metabolically active microbial species, and mechanisms of hazardous chemical transformation in compost systems.

The conventional aerobic compost process passes through four major microbiological phases identified by temperature: mesophilic (30°C to 45°C), thermophilic (45°C to 75°C), cooling, and maturation. The greatest microbial diversity has been observed in the mesophilic stage. Microbes found in the thermophilic stage have been spore forming bacteria (*Bacillus* sp.) (1) and thermophilic fungi (2,3). Microbial recolonization during the cooling phase brings the appearance of mesophilic fungi whose spores withstood the high temperatures of the thermophilic stage. In the final compost stage, the maturation phase, most digestible organic matter has been consumed by the microbial population, and the composted material is considered stable.

Reactor Design

Ten 55-gal, insulated stainless steel composters have been constructed to perform closely monitored treatability studies. The units stand upright, and air flows up through the compost mixture. Completely enclosed units permit periodic analysis of volatile organic compounds

(VOCs) and online analysis of oxygen, carbon dioxide, and methane. Cylindrical reactor design permits mixing of reactor contents by rolling each unit on a drum roller at desired intervals.

Each composter houses four thermocouples connected to a central computer for online temperature measurements. Thermocouples reside at three equally spaced locations within the compost mixture, and a fourth thermocouple tracks ambient temperature outside the reaction vessel. One operational scheme permits temperature control by introduction of ambient air through a computer-controlled valving system. If the temperature of a unit exceeds a predetermined value, the computer switches that unit to high air flow to cool the reaction mixture. After the high-temperature unit cools to a specific temperature, the computer switches the unit back to low air flow.

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 recycling of moisture. Otherwise, each unit could lose 10 lb to 15 lb of water daily.

Current Research

Prototype composter evaluation has proceeded through several different designs. The performance of each design was evaluated by conducting a treatability experiment using the St. Louis Park soil. For our design criteria, one particular prototype offered considerable versatility. This design is currently being converted to stainless steel reactor units.

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.

Aeration studies evaluate porosity (air flow) in the compost system and attempt to discover relationships between free air space, forced air flow, and composting rate. Aeration studies also investigate roles of anaerobic and aerobic metabolism in chemical degradation. Anaerobic pockets may benefit the process by initiating degradation of recalcitrant compounds, especially highly chlorinated compounds, via reductive metabolism. After an initial reductive step, aerobic biodegradation of toxicants may proceed more readily. The research program will attempt to identify optimal aeration rates and pile mixing frequency for the most effective combination of anaerobic/aerobic conditions for biodegradation of recalcitrant substrates. These studies will investigate whether forced anaerobiosis and inoculation with a facultative anaerobe prior to development of aerobic compost conditions enhances biodegradation of toxic wastes.

Studies on moisture dynamics measure rates of change in moisture content in different regions of the compost reactor. A compost pile can lose moisture through evaporation and convection. Moisture dynamics are evaluated in terms of aeration, temperature, and compost composition (e.g., soil type and co-compost material).

Heat production may be highly variable throughout the compost reactor. We have devised a method to continually monitor temperature changes (heat production) at various reactor locations. Bench-top composters are insulated to control heat loss, thereby mimicking a large-scale compost pile where heat is lost by ventilation and water evaporation more than by conduction.

Physical properties of the compost mixture include density (g/cm^3), pH changes in various reactor locations, pressure drop across the pile if it is actively aerated, and the fraction of solids, moisture, and organics. These investigations focus on the potential to enhance biodegradation by manipulation of physical and biological parameters that influence the process. These studies also will investigate whether recycling mature compost material into fresh compost enhances biodegradation of contaminants.

Early microbiological studies will focus on characterizing changes in biological activity during the four stages of composting. We will also attempt to identify microbial species responsible for significant biodegradation of PAHs during each compost stage, and look for reappearance of fungi and other mesophiles (e.g., *Actinomycetes*) during the cooling stage.

Future Research

Future investigations will include technical developments necessary to improve composting applications for degradation of hazardous waste. This will involve increased application of pilot-scale compost systems in addition to ongoing research in bench-top composters. Emphasis will be placed on developing techniques for trapping VOCs from pilot-scale systems, determining mass balance of contaminant degradation in the compost, and identifying microbial species responsible for biodegradation of contaminants.

Future studies also will attempt to validate extrapolation of results from bench-top to pilot-scale and field demonstration levels. Maintaining a bench-top system at optimum conditions is relatively easy compared with a large-scale composter, where optimum conditions will not prevail at all times. The degree of variance from optimal conditions requires investigation and approximation in small-scale systems.

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Remediation of Contaminated Soils from Wood Preserving Sites Using Combined Treatment Technologies

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Pentachlorophenol (PCP), a pesticide used as a wood-preserving compound since the 1930s, has been placed on EPA's National Priority List of pollutants (1). The cleanup of contaminated soil from PCP manufacturing facilities and wood preserving sites has been mandated through the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) (2).

Among technologies employing physical, chemical, and biological processes for the removal of PCP from contaminated soils, solvent washing followed by biological treatment of the wash fluid appears to be a viable alternative (3). The selection of the solvent depends on the hydrophobic nature of the pesticide and the soil wetting capability of the solvent (4,5). Mueller et al. (6) found that ethanol effectively removed polycyclic aromatic hydrocarbons (PAHs) from wet contaminated soils. Previously, equal proportions of ethanol and water were found to have the highest removal efficiencies for above-ground batch extractions of PCP from soil at various soil:solvent ratios (7). In addition, 50-percent and 75-percent ethanol solutions achieved higher removal efficiencies at low solvent throughputs in simulated *in situ* soil flushing experiments. Chemically synthesized extracts from the soil washing process were treated using an anaerobic, fluidized-bed granular activated carbon (GAC) bioreactor. The PCP was reduced to an equimolar concentration of monochlorophenol, which caused inhibition of the biological system. Reduction of the feed concentration of PCP to 200 mg/L appeared to alleviate reactor inhibition.

Results and Discussion

Solvent Extraction Studies

The effectiveness of the 50-percent ethanol/water mixture was evaluated for the removal of PCP from soils that had been aged for 3 weeks, 3 months, 6 months, 9 months, and 1 year. The aging of soil spiked with 100 ppm PCP occurred in the absence of natural weathering, i.e., the soil was not exposed to ground and atmospheric influences. The 50-percent ethanol/water solution was used for simulated *in situ* soil flushing of 20 x 40 and 100 x 140 U.S. mesh soils and 20 x 40 U.S. mesh soil conditioned at 60°C. The soil washing batch experiments were conducted on 20 x 40 and 100 x 140 U.S. mesh soils and the clay fraction of the original soil and on 20 x 40 U.S. mesh soil conditioned at 60°C. The *in situ* solvent washing (flushing) of soil was simulated by continuously flushing solvent through a packed bed of soil until the PCP concentration in the effluent did not decrease. The aboveground soil washing was simulated by batch extraction tests conducted on PCP-contaminated soil.

The 50-percent ethanol solution, applied as the flushing solvent, consistently produced higher PCP removal efficiencies at various aging periods from the 100 x 140 U.S. mesh soil than from the 20 x 40 U.S. mesh soil. The higher PCP recovery from the 100 x 140 U.S. mesh soil was due to the larger mass transfer area (contact surface) between the solvent and the soil that the smaller soil particle size provided.

The data in Figure 1 show the results from the batch extraction tests performed on the 100 x 140 U.S. mesh soil. The results indicate that the 50-percent ethanol solution removed more PCP from the soil than did either the 100-percent ethanol solution or deionized water. Similar results were obtained for the other soil fractions. This higher recovery of PCP by the 50-percent ethanol solution was consistent throughout the study. The results also show that PCP recoveries decreased after 9 months of aging. The PCP removal efficiency for deionized water was lower than that for the 100-percent

ethanol solution after 6 months of aging, indicating that the solubility of PCP in the hydrophobic solvent was contributing more to the removal of PCP from the soil than was the superior wetting of soil by water.

In addition to the batch extraction tests with the various ethanol/water mixtures, sonication and soxhlet extractions with methanol/methylene chloride were carried out on the same soil fractions. The results shown in Figure 2 indicate that the PCP recoveries from the sonication and soxhlet extractions of 100 *x* 140 U.S. mesh soil were not superior to those from the batch extraction tests performed with 50-percent ethanol solution. Similar results were obtained for the other soil fractions.

Biological Treatment Studies

Anaerobic, fluidized-bed GAC anaerobic bioreactors were used for the biological treatment of chemically synthesized extracts (spent solvents) from the soil solvent washing process. The synthesized spent solvent solution was fed to GAC bioreactors, where the PCP content of the wash fluid was the biodegradable metabolite and ethanol served as the primary substrate.

The effect of empty bed contact time (EBCT) on the biodegradation of PCP and its degradation products was examined using the GAC bioreactor (8). Throughout the experiments, the influent PCP concentration was maintained constant at 100 mg/L by doubling the mass and hydraulic loadings simultaneously. The EBCTs were based on an effective volume of 7 L (the total volume of the reactor, 10 L, minus the volume due to a 30-percent carbon expansion) divided by the total hydraulic flow rate (Table 1).

Effluent concentrations of PCP and its degradation by-products are shown in Figure 3. Influent and predicted effluent (with no biological activity) PCP concentrations also are shown. In molar units, a relationship between influent PCP and the total monochlorophenol concentration in the effluent indicates nearly complete conversion of the influent PCP to monochlorophenol. PCP concentration was reduced by at least three orders of magnitude (a greater than 99-percent transformation) throughout the study. No biological inhibition because of PCP was observed during any phase, and the EBCT will be further decreased in future work.

Influent chemical oxygen demand (COD) was contributed by PCP, ethanol, and trace salts. As seen in Figure 4, a two-fold increase in the COD loading rate occurred each time the mass and hydraulic loading rates were doubled (see Table 1). Only 5 percent of the influent COD persisted in the effluent COD throughout all phases of the study, while 70 percent was accounted for by the methane produced. The remaining 25 percent of the influent COD was attributed to biomass production.

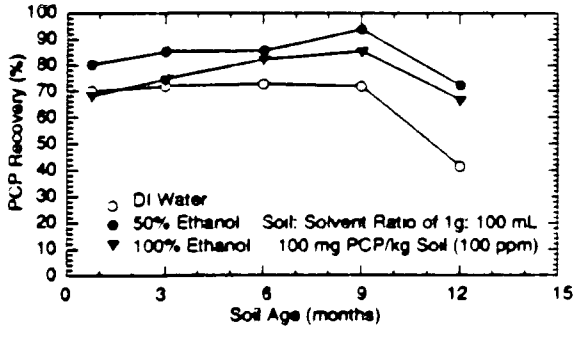

Figure 1. Soil washing batch tests for 100 *x* 140 U.S. mesh soil.

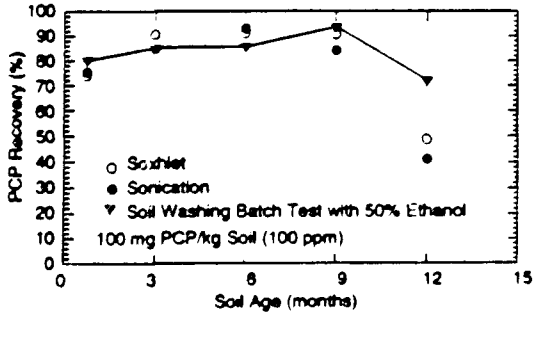

Figure 2. Sonication and soxhlet extractions of 100 *x* 140 U.S. mesh soil.

Table 1. Operation Summary of Bioreactor

Phase	Days of Operation	PCP (g/d)	Ethanol (g/d)	Flow Rate (L/d)	EBCT (hr)
I	480-606	0.60	4.28	6.0	28.01
II	607-824	1.20	8.33	12.0	13.99
III	825-999	2.40	16.66	24.0	7.01

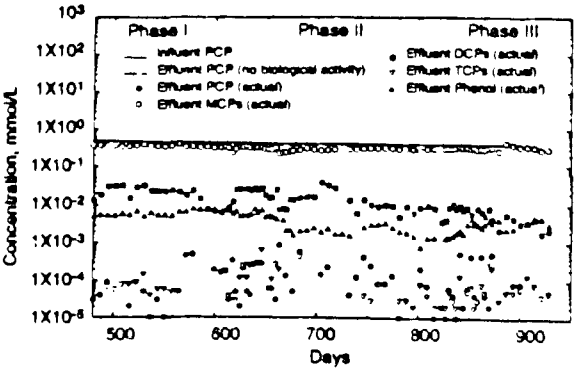

Figure 3. PCP and PCP intermediate effluent concentrations.

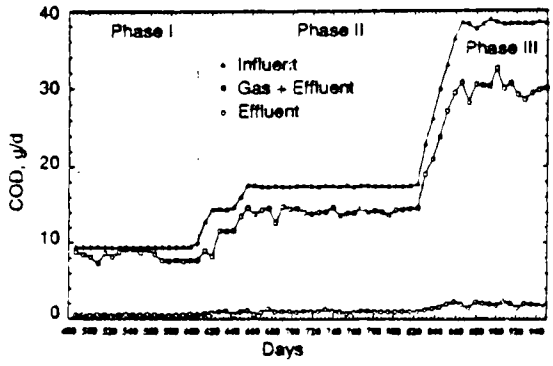

Figure 4. COD balance.

Weekly analysis also was performed on the effluent chloride ion concentrations, volatile fatty acids, and alcohols. The chloride potential is defined as the equimolar amount of chloride from all potential sources (i.e., all chlorinated phenols in the feed). The delta chloride represents the difference between the measured effluent chloride concentration and concentration of chloride in the influent. These analyses confirmed that PCP underwent biological transformation to monochlorophenols through the removal of four chlorine atoms per molecule of the phenol.

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Pilot-Scale Evaluation of Nutrient Delivery for Oil-Contaminated Beaches

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In situ bioremediation is emerging as an efficient and economical strategy for the cleanup of oil-contaminated beaches. The mechanisms and routes of nutrient delivery in the presence of tides, however, are not well understood. The main objective of this project is to investigate these phenomena to identify the best nutrient application technology.

Results and Discussion

For this purpose, a pilot-scale beach simulation unit is being built. This unit will be 8 m long, 0.60 m wide, and 1.8 m tall, and will be equipped with a pneumatic wave generator. The unit is intended to simulate waves that propagate perpendicularly to beaches. The height of the unit was selected to permit investigation of tidal effects. Prior to construction of the pilot-scale unit, a small bench-scale unit was constructed and tested to observe wave generation and beach erosion. The results observed from the bench-scale unit were very encouraging. A periodic wave was generated and sustained over several days.

The initial part of the study will investigate nutrient transport using tracer studies. A distributed computer model will be developed in parallel. The model parameters will be estimated from the results of tracer studies. Subsequently, the model will be evaluated at pilot scale and later on real beaches. The experimental data also will be evaluated against the mathematical model developed by Wise et al. (1).

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Field Treatment of BTEX in Vadose Soils Using Vacuum Extraction or Air Stripping and Biofilters

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Spills of fuels and leaking fuel tanks represent a major source of vadose soil contamination. This contamination, which includes the aromatic hydrocarbons benzene, toluene, ethylbenzene, and the xylenes (BTEX), leaches through vadose soil into ground water. Aromatic hydrocarbons pose health risks when ground water is used as a drinking water supply.

EPA's Risk Reduction Engineering Laboratory (RREL), in cooperation with the University of Cincinnati, is developing engineering systems to bioremediate fuel-contaminated vadose soils or ground water. Vacuum extraction of soils or air stripping of ground water, which transfers the volatile organic compounds (VOCs) from the soils or ground water to air, is combined with air biofiltration to achieve treatment.

Field Demonstration

Two types of air biofilters will be studied: 1) packed beds with ceramic pellets, 6-mm average diameter (Celite, Manville Corporation), as the packing material; and 2) straight-passages ceramic monoliths with 50 square passages per square inch (as shown in Figure 1). A schematic of the experimental system is shown in Figure 2. The aerobic mixed cultures, from an activated sludge treatment plant, are immobilized on the surface of the packing. Nutrient solution, needed for microbial growth, is trickled down through the packed bed, with the contaminated air flowing countercurrent to the nutrient flow. The gas residence time in each biofilter is varied between 1 and 3 minutes. Electricity and water are used to raise the temperature of the extracted air to approximately 30°C and to prehumidify the air. A syringe pump is used during startup to contaminate the air with jet fuel to establish the biofilms in the biofilters.

The biofilters will be constructed at EPA's Test and Evaluation (T&E) Facility in Cincinnati. The system will include gas chromatography for analyses of the influent and effluent gas streams from each biofilter. The biofilm on the support media will be preacclimated to jet fuel

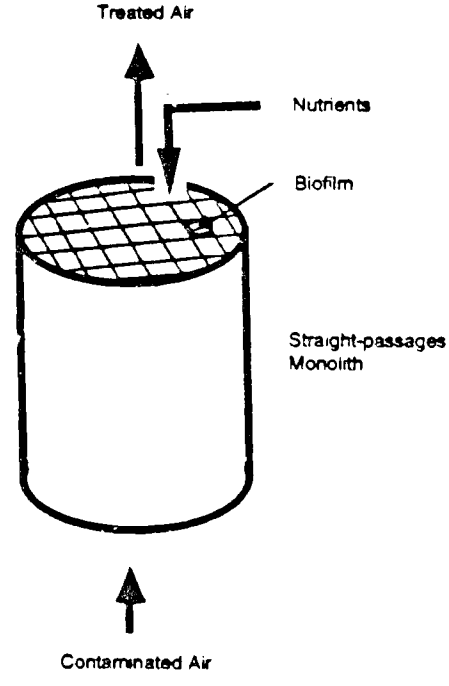

Figure 1. Schematic of the straight-passages monolith media.

(JP-4) hydrocarbons. The skid-mounted biofilters with acclimated biofilms will be transported to the site for connection to the vacuum extraction or air stripping system.

The site for the field demonstration has not yet been selected but is likely to be an air force base in Ohio. The performance of the integrated system will be characterized for approximately 3 months.

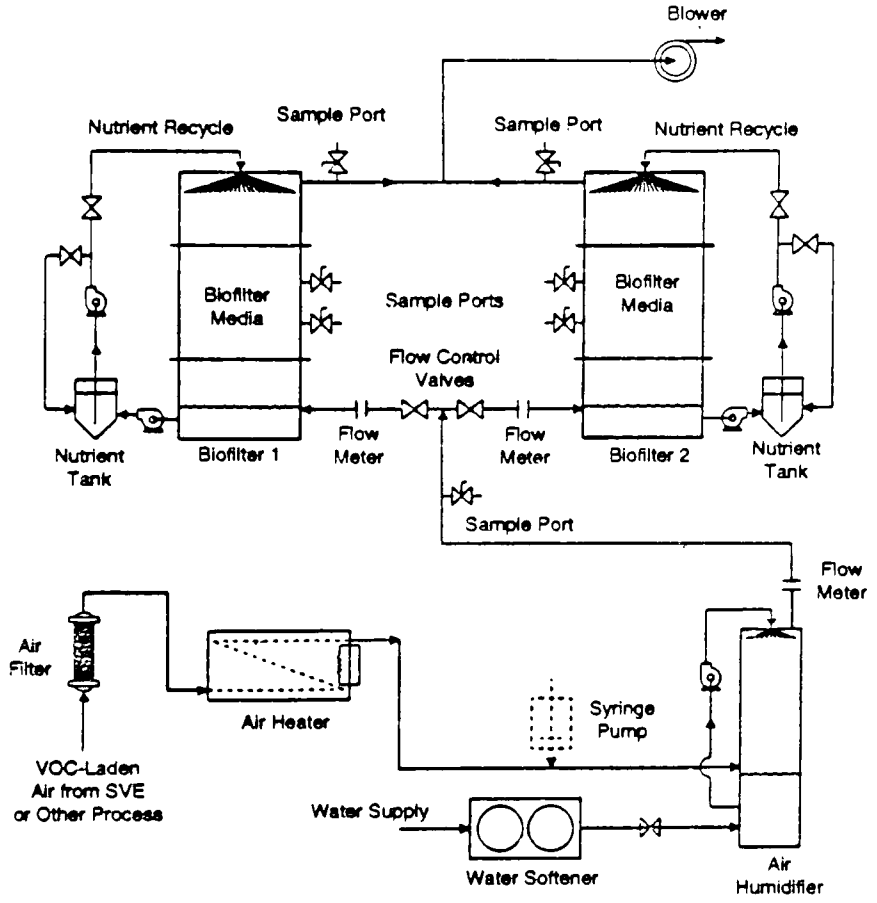

Figure 2. Process flow diagram of the skid-mounted biofilter systems.

Dechlorination with a Biofilm-Electrode Reactor

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Introduction

Pentachlorophenol (PCP) is a pesticide and bactericide that is used widely in the wood and leather preserving industries (1). PCP, however, is a suspected mutagen and carcinogen (2), and, in 1986, EPA set a maximum contaminant level of 0.001 mg/L. Superfund documents have reported PCP levels as high as several hundred milligrams per liter in contaminated ground water.

According to Krumme (3), in systems without a carbon or energy source PCP has been shown to be dechlorinated and mineralized to about 40 percent of the influent concentration (3). In systems using a co-substrate, it has been demonstrated that PCP can be dechlorinated up to 99.9 percent (4). The addition of external carbon and energy sources, however, could pose difficulties in both *in situ* and *ex situ* treatment of contaminated sites. Cell growth is enhanced by the addition of these carbon and energy sources, and the disposal of the resultant sludge can prove to be costly. *In situ* treatment of PCP can also pose problems; the addition of a carbon and energy source into the ground might cause the formation of hazardous soluble compounds. Methods of enhancing microbial activity that could reduce or remove the need to provide external energy and carbon sources should be examined.

Results and Discussion

The objective of this project is to examine the dechlorination and mineralization of PCP under anaerobic conditions using the electrolytic reduction of water to provide an external energy source and hydrogen donor. Researchers have demonstrated that biological processes can be enhanced when subjected to an electric current (5,6). These studies examined the role of electrolytically produced hydrogen in the denitrification of wastewater. Islam et al. (6) found a correlation between the applied current and the removal efficiency of the reactor system and determined the optimum current to be 20 mA, for which the removal efficiency was greater then 98 percent.

The reactor is a fixed-film chemostat with trace salts and nutrients added. PCP dissolved in ethanol is added at two different feed concentrations (5 mg/L and 50 mg/L), with a current of 15.0 mA across the junction. The flow rate is 5 L/day, with a hydraulic detention time of 0.44 days. The reactor was seeded with biomass from an anaerobic, expanded-bed, granular activated carbon (GAC) reactor that had been successfully dechlorinating PCP. The gas production of about 96 mL/day of methane and the intermediates in the effluent indicate the presence of an active growing biofilm.

Good dechlorination of PCP was achieved, with about 0.24 percent of the influent PCP remaining as PCP, 0.1 percent as tetrachlorophenol, 0.87 percent as trichlorophenol, 10.28 percent as dichlorophenol, and about 55 percent as monochlorophenol on a molar basis (Figure 1). The remaining 33.5 percent was presumed to be mineralized to HCl, CO_2, and H_2O. Currently, the feed alcohol concentration is being reduced stepwise as the biofilm stabilizes to the operating concentration.

Work on this project is continuing; new data will be included in the poster presentation.

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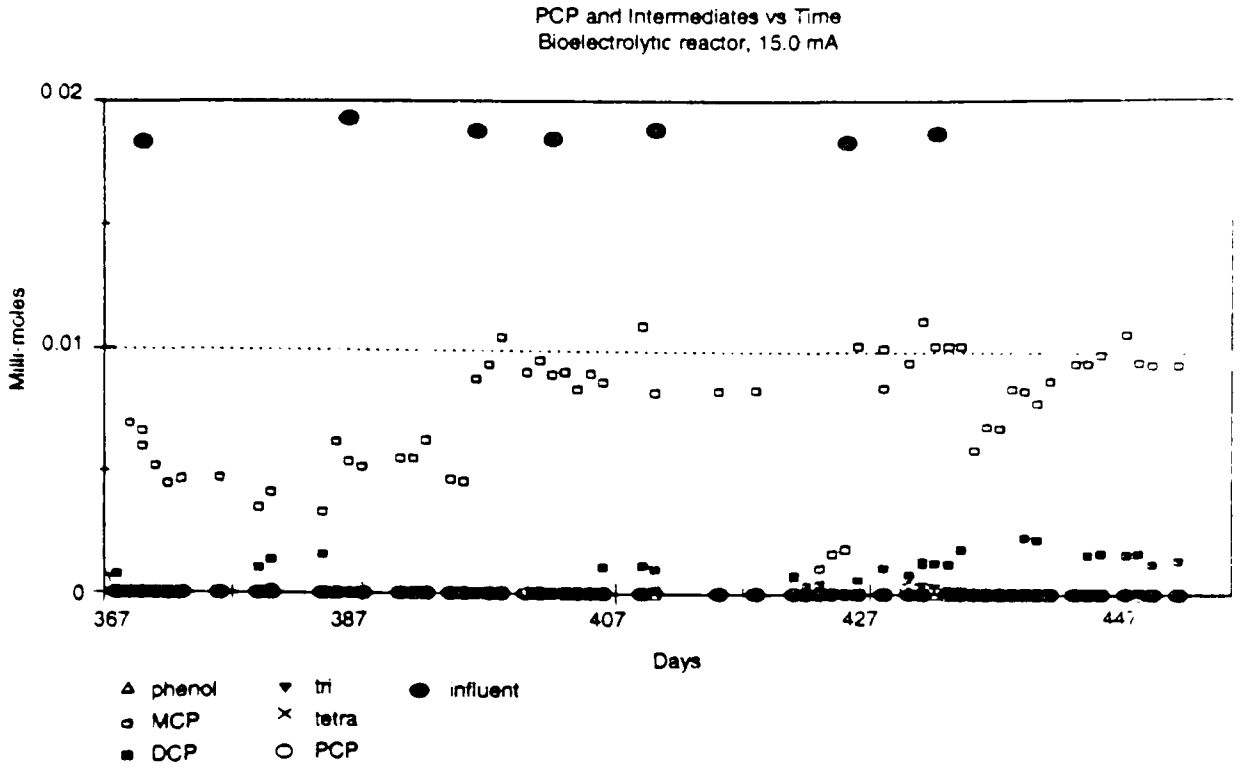

Figure 1. PCP and intermediates versus time (bioelectrolytic reactor, 15.0 mA).

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Use of Sulfur Oxidizing Bacteria To Remove Nitrate from Ground Water

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The chemoautotrophic bacterium *Thiobacillus denitrificans* is capable of effective removal of nitrate from ground water under anoxic conditions. This microorganism is capable of deriving metabolic energy from oxidation of inorganic sulfur compounds including elemental sulfur, hydrogen sulfide, thiosulfate, metabisulfite, tetrathionate, and sulfite. All carbon required for biosynthesis is derived from carbon dioxide, carbonate, and bicarbonate. The primary products of autotrophic denitrification are nitrogen gas, sulfate, water, and biomass. The potential advantages of using elemental sulfur (in powdered, flaked, or prilled form) are as follows: 1) low cost and wide availability of energy source; 2) low toxicity compared with other energy sources (i.e., methanol or ethanol); 3) ease and safety of storage; 4) potential for development of water treatment reactors capable of operating for long periods (months) at a time with little or no maintenance or operator attention; and 5) potential for use *in situ* to remediate nitrate-contaminated aquifers.

A column reactor (3.6 m long x 0.051 m ID) has been operated continuously for more than 1 year outdoors. The reactor was filled initially to a depth of 1.83 m with sulfur granules graded -16/+30 Mesh (U.S. Standard Sieve). Well-water nitrate content could be consistently reduced to less than 0.3 ppm from an influent level of 55 ppm with a reactor feed rate of 0.35 L/min. Increasing flow to 0.45 L/min resulted in an effluent containing nitrate concentrations ranging from less than 0.3 ppm to 5 ppm. Maintenance of constant bed volume for a given flow rate required periodic replenishment of the bed with fresh sulfur granules. As denitrification proceeds, the granules decrease in mass (i.e., are consumed) to the point that their mass is insufficient to remain within the reactor. A novel fluidized bed reactor system has been designed that will permit essentially complete utilization of the smaller particles.

A variety of heterotrophic (organotrophic) bacteria were found to become established in reactors fed only inorganic energy sources (elemental sulfur or sodium thiosulfate). The first survey involved 15 bacterial isolates recovered from a chemostat reactor operated with precipitated sulfur slurry as the energy source and nitrate as the terminal electron acceptor. The isolates were recovered by plating dilutions of water samples on R2A (an organic-based medium) under aerobic conditions. Isolates were purified by restreaking on R2A and were subjected to a proprietary identification system, API-NFT, designed to identify nonfermentative bacteria. Of 15 isolates, one isolate each was identified as *Achromobacter* sp., *Pseudomonas stutzeri*, *Flavobacterium* sp., and *Pseudomonas putrefaciens*. Seven of the isolates were Gram-negative "nonidentifiable." The remaining four isolates were Gram-positive "nonidentifiable." The second survey involved 19 isolates recovered from a chemostat reactor operated with sodium thiosulfate as the energy source and nitrate as the terminal electron acceptor. Of these, one isolate each was identified as *Achromobacter* sp., *Pseudomonas pseudoalcaligenes*, and *Pseudomonas paucimobilis*. Twelve isolates were identified as *Pseudomonas aeruginosa*. Four isolates were Gram-negative "nonidentifiable." The "nonidentifiable" designation refers to isolates that gave biochemical reactions profiles uncharacteristic of the API-NFT database collection. Work in progress should result in identification to the genus level.

Sodium thiosulfate was tested as an energy source in a small, prototype fluidized bed reactor. The Pyrex column (40 cm long x 2.54 cm ID) contained a 16-cm deep bed of 0.10-mm diameter silica spheres (settled bed depth under zero flow conditions). In this reactor configuration, the silica spheres serve only as an inert support matrix. Sodium thiosulfate is highly soluble in water and can be supplied in correct proportion with the aid of a metering pump. The degree of bed expansion was easily controllable between 0 and 100 percent. The reactor demonstration involved recirculation of 14 liters of a defined mineral salts solution containing 1,227 ppm nitrate and 2,252 ppm thiosulfate through the column. Following inoculation, flow was set at 30 mL/min (equal to 25 percent bed expansion). Approximately 7 percent of the

nitrate was removed by Day 7. Nitrate removal had increased to nearly 35 percent by Day 11. Runs conducted with varying concentrations of nitrate relative to thiosulfate revealed that acceptable denitrification efficiency required careful control of the relative proportions of the two reactants. While technically feasible, the level of control required to reliably produce denitrified water on a practical scale might prove difficult. Thiosulfate also suffers from the disadvantage of higher cost per unit of nitrate removed in comparison to elemental sulfur.

Respirometric experiments were conducted using pure cultures of *Thiobacillus denitrificans*. Washed cells obtained from aerobic cultures with either thiosulfate or tetrathionate as the energy source were unable to denitrify in short-term experiments. This demonstrates that, as is the case with heterotrophic bacteria, denitrification is an inducible rather than a constitutive metabolic capability. However, anoxically grown cells could tolerate exposure to oxygen without immediate deterioration or loss of denitrification activity. On a practical level, this suggests that a biological denitrification reactor could readily withstand periodic ingress of oxygen resulting from periodic air-scour or high flow backwash procedures, as might be required to control formation of excess biomass deposits. Rapid recovery of denitrification activity following such treatments would be a decided advantage.

In conclusion, sulfur-mediated biological denitrification of ground water appears to be technically feasible. A fluidized bed reactor containing granular sulfur has been operated for more than 1 year. Autotrophic sulfur bacteria and nonautotrophic (organotrophic) bacteria appear to coexist stably. The nature of their relationship (possibly syntrophic or mutualistic) is under further study. The use of readily soluble sulfosalts as thiosulfate or tetrathionate in reactors containing an inert support material is less certain. This approach will require additional basic research to determine the relationship between nitrate concentration and energy-yielding substrate and their overall effect on denitrification rates and efficiency.

Engineering Evaluation and Optimization of Biopiles for Treatment of Soils Contaminated with Hazardous Waste

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Biopile systems offer the potential for low-cost treatment of hazardous waste in soil. Biopiles provide favorable environments for naturally occurring microorganisms to degrade soil contaminants. The microbial environment can be manipulated to promote aerobic or anaerobic metabolism. Air is supplied to the system by a plumbing network that forces air through the pile by applying either pressure or vacuum.

Biopiles differ from compost piles in that bulking agents necessary for composting are not added to biopiles. Some nutrients and exogenous microorganisms, however, may be added to a biopile in the form of manure or other nutrient-rich material. Biopiles will normally produce less heat than compost piles because less organic substrate is added, although significant aerobic microbial activity will produce some heat. While heat production is often desired in compost piles, we may wish to limit heat production in biopiles to avoid killoff of mesophilic organisms involved in biodegradation of soil contaminants.

The goal of this project is to evaluate the potential of biopile systems to remediate soils contaminated with hazardous chemicals. Pilot-scale reactors with a volume of 2 yd^3 to 3 yd^3 each are being constructed at EPA's Test and Evaluation (T&E) Facility in Cincinnati. Contaminated field soil from selected sites will be brought to the T&E Facility for this research. Depending on availability of soil, contaminants may include any or all of the following: pentachlorophenol, creosote, munitions, and petroleum hydrocarbons.

Short-term work will focus on designing and constructing pilot-scale biopile reactors and defining suitable operating conditions. Pilot-scale operations may permit collection of reliable data to develop effective aeration strategies, document degradation rates and metabolic products of hazardous chemicals, and identify metabolically active microbial species. Physical and chemical data to be collected include heat production; density (g/cm^3); fractions of solids, moisture, and organics; pressure drop across sections of aerated biopiles; and pH changes in various reactor locations. Subsequent studies will emphasize treatability of contaminated soils.

Future investigations will focus on the potential to enhance biodegradation by manipulation of physical and biological parameters. For example, anaerobic treatment may be necessary to initiate degradation of recalcitrant compounds via reductive metabolism. Following reductive metabolism, toxicants may be amenable to aerobic biodegradation. Research may identify the most effective combination of anaerobic/aerobic conditions for biodegradation of recalcitrant substrates in biopile systems.

Section Five
Process Research

Process research involves isolating and identifying microorganisms that carry out biodegradation processes and the environmental factors affecting these processes. In this way, researchers establish the building blocks of new biosystems for treatment of environmental pollutants in surface waters, sediments, soils, and subsurface materials.Thorough evaluation is critical at this level of research, since a firm scientific basis can facilitate the scaling up of a promising bioremediation method or technology. Process research is being conducted on a number of environmental pollutants.

Several research projects are focusing on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) and creosote. Specific areas of study include the metabolic and ecological factors affecting the bioremediation of PAH- and creosote-contaminated soil and water; the environmental factors affecting creosote degradation by catabolically competent microflora, such as *Spingomonas paucimobilis* strain EPA505; and a comparison of sulfur and nitrogen heterocyclic compound transport in creosote-contaminated aquifer material.

Research also is being conducted on phenols, including a study on the modeling of steady-state methanogenic degradation of phenols in ground water at an abandoned wood treatment facility in Pensacola, Florida; and a study demonstrating the conversion of pentachlorophenol (PCP) to phenol in sediment slurries inoculated with cells from a 4-bromophenol (4-BP) dehalogenating enrichment culture.

Two other projects focused on the dechlorination of polychlorinated biphenyls (PCBs). One study examined limiting factors in order to develop effective methods for stimulating microbial dechlorination of PCBs. Another study focused on the addition of single congeners of chloro- and bromophenyls for enhanced dechlorination of PCBs in contaminated sediments.

One project investigated the kinetics of anaerobic biotransformation of munitions wastes. Two others focused on the degradation of hydrocarbons, specifically the effect of heavy metal availability and toxicity on anaerobic transformations of aromatic hydrocarbons and the biodegradation of petroleum hydrocarbons in wetlands microcosms, including constraints on natural and engineered remediation.

Another major focus of process research was the biodegradation of chlorinated solvents, particularly trichloroethylene (TCE). One study focused on the characterization of bacteria in a TCE degrading biofilter. Another study provided a risk analysis for inoculation strategies in the bioremediation of TCE. Related research was conducted on the aerobic/anaerobic degradation of recalcitrant volatile chlorinated chemicals in a hydrogel encapsulated biomass biofilter.

Other process research projects were the use of 5-chlorovanillate as a model substrate for the anaerobic bioremediation of paper-milling waste; the effect of surfactants on microbial degradation of organic contaminants; and reaction

mechanisms and development of remediation schemes related to the covalent bonding of aromatic amines to natural organic matter.

The symposium's poster session included presentations on metabolites of oil biodegradation and their toxicity, the alteration of a plasmid bacterial strain for TCE degradation; degradation of a mixture of high molecular-weight PAHs by a mycobacterium species; and factors affecting the delivery of nutrients and moisture for enhanced in situ bioremediation in the unsaturated zone.

Metabolic and Ecological Factors Affecting the Bioremediation of PAH- and Creosote-Contaminated Soil and Water

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Polycyclic aromatic hydrocarbons (PAHs) are a class of potentially hazardous chemicals whose natural presence in the environment is attributable to a number of petrogenic and phytogenic sources (1,2). Environments contaminated with large amounts of these chemicals (e.g., creosote waste, coal tar processing sites) are considered hazardous owing to potential carcinogenic, mutagenic, and teratogenic effects of specific PAHs (3). Generally, high molecular-weight (HMW) PAHs, containing four or more fused rings, present the greatest potential hazard to both the environment and human health (4). Consequently, much interest exists in developing remedial methods, such as bioremediation, to selectively remove these chemicals from contaminated environmental materials.

When environmental conditions (e.g., waste load, nutrients, oxygen, pH) are suitable, biodegradation of low molecular-weight PAHs by indigenous microorganisms readily occurs (5-7). Under the same conditions, however, biotransformation of HMW PAHs is less likely. Although bacteria have been isolated in pure culture that grow on HMW PAHs, such as fluoranthene and pyrene (7-9), strategies for stimulating this activity, as well as the degradation of other HMW PAHs, in contaminated soils are not readily available in part because of a poor understanding of the biodegradation ecology of complex mixtures of hydrophobic chemicals in the environment. How, for example, do microorganisms interact during a degradation process to promote the degradation of these complex mixtures? Can this interaction be enhanced through population management of microbial communities or adjustment of specific environmental conditions? And, have microbial communities in contaminated soils adapted (genetically and/or physiologically) to utilize hydrophobic PAHs more effectively? An improvement of our understanding of biodegradation ecology for PAHs and creosote could, therefore, lead to new and effective strategies for bioremediation of these contaminants. This paper provides a summary of our research efforts in this area, with specific attention given to co-metabolic processes, bioavailability, inoculation, and microbial community adaptation.

Results and Discussion

Co-metabolism

The process of co-metabolism in bioremediation generally refers to the transformation (not necessarily mineralization) of a hazardous waste chemical(s) as an indirect or fortuitous consequence of the metabolism of another chemical that a bacterium uses as a source of carbon and energy (growth substrate). Co-metabolism, an intriguing consequence of broad enzyme specificity, is one of the important elements in the recent emergence of new bioremediation strategies. Unfortunately, however, its occurrence in natural microbial communities is neither well documented nor understood, and the process is difficult to control in the field. In addition, concerns exist regarding the fate and environmental impact of the partial oxidation products that are thought to be produced. Successful degradation of HMW PAHs has been argued to involve extensive co-metabolic reactions (6); that is, enzymes used by specific bacteria in a microbial community to degrade one type of PAH fortuitously oxidize other PAHs. Biochemical evidence for this type of reaction is provided in the paper by Chapman et al.

The importance of co-metabolism in PAH degradation is illustrated by studies in which a bacterium *(Sphingomonas paucimoblis* strain EPA505) that used fluoranthene (an HMW PAH containing four fused rings and a major constituent of most creosote and coal tar wastes) as a sole source of carbon and energy was found to biotransform many PAHs that were not growth substrates (10). This included fluorene, pyrene, chrysene, and benzo(a)pyrene. If this bacterium and other PAH degraders are exposed to the PAH fraction of creosote in a standard shake flask assay (8) for 10 days and the creosote fraction is monitored by extraction and gas chromatographic analysis, considerable loss of most of the PAHs occurs even though only a few of the PAHs are used as growth substrates. A comparison of results from strain EPA505 and strain N2P5, a bacterium also isolated from creosote-contaminated soil, is shown in Table 1. Strain N2P5 grew only on two- and three-ring PAHs, such as phenanthrene, and had far less capacity for this co-metabolic phenotype. A variety of isolates are currently being studied to more fully characterize this co-metabolic capability. The resulting partially oxidized degradation products from this co-metabolism have not been specifically identified but are likely to be more soluble and possibly more biodegradable than the parent compound, perhaps leading to further degradation or metabolism by other members of a microbial community.

Other bacteria in nature may behave like these PAH degraders studied in the laboratory, thereby giving microbial communities the capability of co-metabolism. Few experimental results are available, however, to show that this is indeed the case. We are conducting experiments to specifically relate pure culture studies to PAH degradation patterns in natural microbial communities. At a bioremediation site, where environmental conditions are established to promote PAH degradation by the indigenous microflora (aeration, inorganic

Table 1. Degradation of Creosote PAHs by Selected Bacterial Isolates

Compound (mg/L)	Uninoculated (sd)	EPA 505 (sd)	% Reduction	N2P5 (sd)	% Reduction
Naphthalene	39.33 (2.47)	0.04 (0.01)	100	0.10 (0.06)	100
Thianaphthene	1.41 (0.08)	0.11 (0.03)	92	0.79 (0.17)	44
2-Methylnaphthalene	18.88 (0.79)	0.07 (0.02)	100	0.17 (0.03)	99
1-Methylnaphthalene	6.23 (0.18)	0.04 (0.01)	99	0.79 (0.12)	87
Biphenyl	3.30 (0.16)	bdl	100	0.62 (0.07)	81
2,6-Dimethylnaphthalene	2.85 (0.19)	0.10 (0.03)	96	0.50 (0.05)	82
2,3-Dimethylnaphthalene	0.67 (0.04)	0.06 (0.03)	91	0.37 (0.09)	45
Acenaphthylene	0.55 (0.03)	0.21 (0.07)	62	0.48 (0.17)	13
Acenaphthene	22.46 (1.20)	bdl	100	10.06 (1.30)	55
Dibenzofuran	16.01 (0.84)	0.12 (0.02)	99	0.08 (0.01)	100
Fluorene	19.83 (1.22)	0.15 (0.05)	99	0.11 (0.06)	99
Dibenzothiophene	6.85 (0.58)	0.28 (0.15)	96	7.42 (0.56)	0
Phenanthrene	55.22 (3.00)	bdl	100	0.14 (0.01)	100
Anthracene	2.80 (0.16)	0.48 (0.10)	83	1.09 (0.05)	61
Carbazole	2.94 (0.28)	0.35 (0.12)	88	0.43 (0.11)	85
2-Methylanthracene	1.02 (0.55)	0.21 (0.11)	79	1.58 (0.06)	0
Anthraquinone	5.07 (0.76)	1.12 (0.21)	78	4.43 (0.57)	13
Fluoranthene	26.53 (2.31)	bdl	100	28.46 (4.09)	0
Pyrene	15.92 (1.40)	8.39 (0.75)	47	16.01 (5.90)	0
Benzo(b)fluorene	2.85 (0.20)	0.76 (0.08)	73	2.66 (0.11)	7
Benzo(a)anthracene	5.94 (2.49)	5.98 (1.00)	0	6.02 (0.09)	0
Chrysene	2.42 (1.12)	1.77 (0.32)	27	2.31 (0.09)	0
Benzo(b/k)fluoranthene	1.64 (0.20)	1.19 (0.21)	27	2.34 (0.16)	0
Benzo(a)pyrene	0.60 (0.02)	0.49 (0.06)	18	0.94 (0.11)	0
TOTAL	261.32	21.92		88.46	

nutrient amendment, moisture control, etc.), however, co-metabolism may not have its maximum effect because the PAHs serving as inducers of the enzymatic processes responsible for co-metabolism are not maintained at sufficient concentrations. As a consequence, it may be reasonable to add a specific PAH in low concentrations to stimulate microbial communities to co-metabolically degrade HMW PAHs, thereby more easily bringing PAH concentrations to stipulated cleanup levels. Clearly, for any long-term bioremediation treatment involving co-metabolism, more ecological and biochemical research is required.

Bioavailability

Because of their strongly hydrophobic nature, HMW PAHs usually occur as contaminants in natural ecosystems and waste treatment systems at mass levels that exceed their water solubility. In addition, equilibria strongly favor particle-bound chemicals (e.g., sorbed to soils). These characteristics largely account for the slow biodegradation of HMW PAHs (11). Therefore, understanding treatment conditions and environmental factors that can be manipulated to enhance bioavailability and consequently biodegradation is critical to bioremediation considerations.

It has been suggested that pure cultures of bacteria can use PAH compounds only in the dissolved state (12-14). Therefore, the dissolution of PAHs may be a prerequisite for initial oxidation and mineralization. Dissolution rates are usually determined by the solid-liquid contact surface area and the equilibrium concentration of the PAH compound (11,12,15). Surfactants can enhance PAH solubilization and dissolution, thus increasing the equilibrium concentration of the compound in the aqueous phase (16) which should lead to faster degradation rates. The use of surfactants at high concentrations, however, reduced or inhibited biodegradation (17,18) because of surfactant toxicity to the bacteria used in the study.

On the contrary, Teichm has shown that a variety of nonionic surfactants are nontoxic to a *Mycobacterium* sp. that is able to grow on fluoranthene and pyrene and consequently increase rates of PAH biodegradation (19). Likewise, we have studied the mineralization of ^{14}C-radiolabeled fluoranthene by *S. paucimobilis* strain EPA505, an organism that grows on this PAH as a carbon and energy source, and initial rates of mineralization were enhanced by the presence of the surfactant Triton X-100. An example of this response is shown in Figure 1 (top). For this experiment, cells were grown in complex medium, washed several times in buffer, and suspended to a final cell density of 8×10^{10} cells/mL in minimal salts medium containing 20 mg of unlabeled fluoranthene, approximately 60,000 dpm of ^{14}C-fluoranthene, and various concentrations of Triton X-100. The

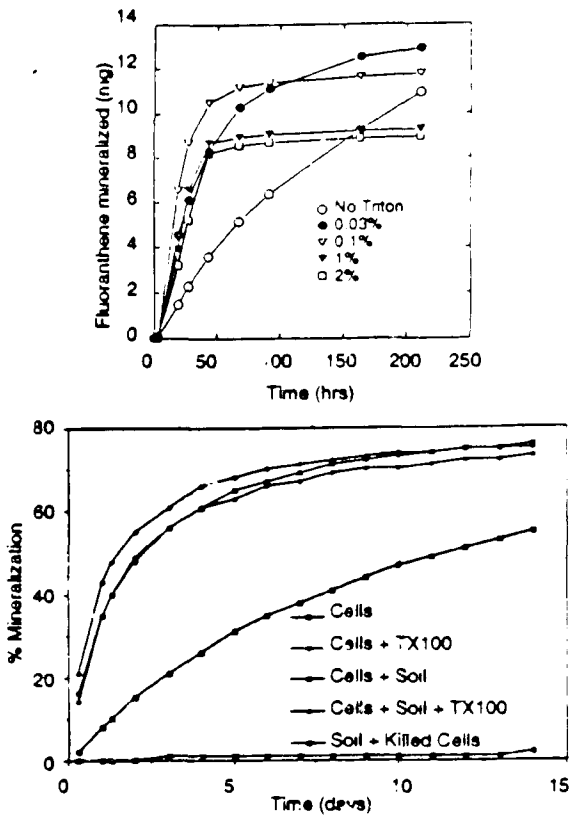

Figure 1. Mineralization profiles of ^{14}C-fluoranthene by strain EPA505 in minimal salts medium with various concentrations of Triton X-100 (top) and various combinations of cells, soil particles, and surfactant (bottom). Inoculum concentration = 8×10^{10} cells/mL; fluoranthene concentration = 0.4 mg/mL; particle concentration = 30 mg/L.

surfactant concentrations tested were all above the critical micelle concentration for this surfactant. Initial rates of mineralization were clearly enhanced by all concentrations of the surfactant. The reduced extent of mineralization at the two highest surfactant concentrations may have resulted from the sequestering of fluoranthene degradation intermediates (e.g., leaching from the cells), making them unavailable for mineralization. The bacterium clearly was able to tolerate high surfactant concentrations, thus emphasizing the importance of properly selecting PAH-degrading microorganisms that are not inhibited by surfactants or selecting surfactants that are nontoxic.

Dissolution of a chemical is also a problem in soil slurry systems, where the presence of soil particles may decrease the aqueous concentration of a PAH compound due to the sorption effects (20); reduced aqueous concentrations would decrease the rate of biodegradation. The presence of soil particles in a solution, however, can provide a higher solid-liquid contact surface area, thereby enhancing the solid-liquid mass transfer. Slow

degradation rates, in essence, are counterbalanced by greater chemical turnover as is true in the case of fluoranthene degradation. As shown in Figure 1 (bottom), an aqueous suspension of soil particles (30 mg/mL) and fluoranthene crystals (20 mg) together resulted in greater mineralization rates than suspensions with only fluoranthene crystals, apparently the effect of higher solid-liquid contact. Although increases in biomass or in the activity of the biomass as a result of exposure to soil particles also may explain the effect, this explanation is unlikely since the biomass (10^8 cells/mL) and the mineralization rates were initially high. Note that the effect of soil particles was equivalent to that of adding surfactant, a further indication of increased dissolution by either material.

In contaminated soils, fluoranthene and other HMW PAHs will likely exist at concentrations far in excess of their aqueous solubility. Given that undissolved PAHs will not exist as crystals in the environment, it is important to know if they exist in a form in which soil particles provide higher solid-liquid contact or in which surfactants can promote greater dissolution, or both.

Research needs to be accelerated in this area because the use of surfactants will almost assuredly play a significant role in future bioremediation procedures. Also, engineering strategies for using surfactants or other means of increasing mass transport in the field must be developed. This should include consideration of how to remove the bioavailability-enhancing chemical from the field after it has done its job, and how to protect against a negative effect on contaminant distribution in the field (e.g., seepage into uncontaminated areas).

Bioaugmentation

If we define bioaugmentation as the process of introducing microorganisms of sufficient biomass into a site in a manner in which it can be documented that the inoculated organism(s) survives to a point of significantly affecting the fate of a target chemical(s), then very few scientifically documented examples exist where this process has been successful on a significant scale. Yet, many possible situations can occur in which bioaugmentation of chemically contaminated sites with microorganisms possessing unique and specialized metabolic capabilities could potentially be a feasible bioremediation approach. With more careful attention to selection and application of the inoculants, it is quite reasonable that bioaugmentation could become a major and effective component of biological cleanup methods.

Many recognizable limitations to the use of bioaugmentation in bioremediation exist. Only a few limitations have been systematically addressed in an experimental sense (20-23). These include the inability to support the growth and/or activity of the introduced organism because of competition by the indigenous microflora. Success, however, can be realized by employing specialized techniques to reduce competition and to maintain a biomass high enough to effect efficient degradation of the target chemicals. In addition, the contaminated environment almost certainly will have to be physically modified, perhaps over an extended period, to optimize the bioaugmentation process. This modification generally means establishing conditions in which the availability of oxygen, inorganic nutrients, temperature, degradable substrate, moisture content, etc., are optimized.

Bioaugmentation using microorganisms with requisite metabolic capacities is one suggested approach for enhancing biodegradation of these HMW PAHs (6). Although biodegradation of HMW PAHs by identified microorganisms has been reported, suitable strategies for using these microorganisms as inocula in the field need to be further developed. We have been experimenting with the concept of introducing immobilized cells using different encapsulation procedures (24). For example, polyurethane polymer (PU) has been used to immobilize *S. paucimobilis* strain EPA505. The immobilized cells were tested for their ability to mineralize fluoranthene under these conditions. As shown in Figure 2 (top), no significant difference in fluoranthene mineralization profiles by the PU-immobilized cells of strain EPA505 occurred when compared to nonimmobilized cells. Since the same inoculation size was used in all flasks during this experiment, the results suggest that the immobilization process does not significantly affect microbial activity. Cells immobilized in the PU polymer remain active for months when stored at 4°C.

Active immobilized cells then offer several additional possibilities for further enhancing biodegradation and environmental control. For example, inclusion of adsorbents in the immobilization matrix can result in a more rapid uptake of toxic compounds from the environment, thereby potentially providing greater accessibility of the adsorbed chemical to the immobilized bacteria. Two issues need to be addressed, however, when using co-immobilized adsorbents: 1) Is microbial activity affected by co-immobilization with adsorbents? and 2) Is availability of the adsorbed chemical to the immobilized cells maximal? To study these questions, diatomaceous earth and powdered activated carbon were co-immobilized with strain EPA505 in the polyurethane matrix. In Figure 2 (top), the degrading activity of the cells co-immobilized with the adsorbents was the same as the nonimmobilized cells, indicating that the degradation of the adsorbed fluoranthene was complete.

Another possibility involves *in situ* bioremediation situations, where direct addition of nitrogen and phosphorous into soil or water may have a negative consequence because of enhancement of the activity of undesired indigenous microflora and/or the leaching of

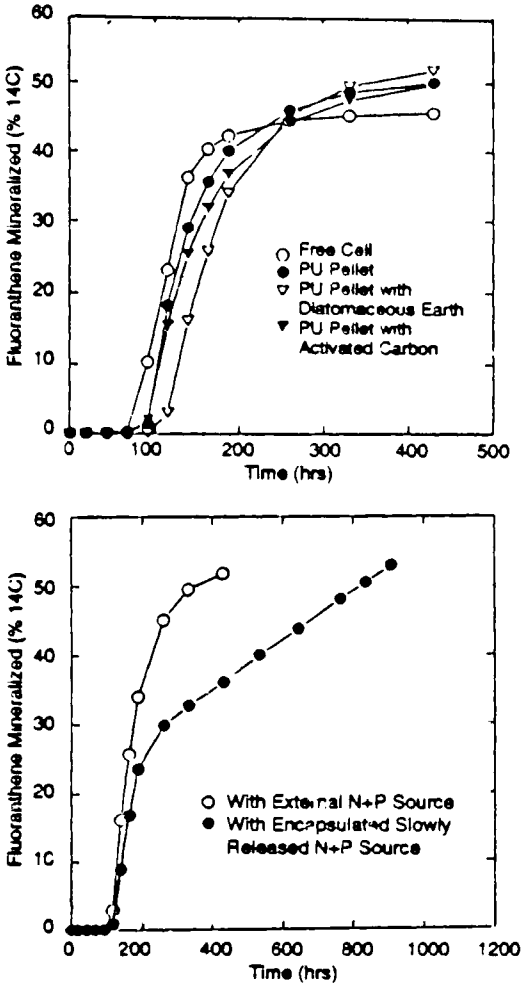

Figure 2. Mineralization profiles of ^{14}C-fluoranthene by nonimmobilized and polyurethane-immobilized cells of strain EPA505 with different adsorbents (top) and external and encapsulated nitrogen and phosphorus sources (bottom) in minimal salts medium. Inoculum concentration = 8 x 10^8 cells/mL; fluoranthene concentration = 0.4 mg/mL; Triton X-100 concentration = 0.1 percent; particle concentration = 30 g/mL.

the nutrients into ground water. By co-immobilizing slow-release formulations of nutrients in the polymer matrix, a major part of the nutrients can be provided to the immobilized cells with considerably less available for leaching into the environment. In our experiments, slow-release formulations of nitrogen and phosphorus were co-immobilized with EPA505 in the polyurethane matrix, then tested in buffer for fluoranthene degradation. As a positive control, the immobilized cells with external sources of nitrogen and phosphorus (solution of inorganic salts) also were used. As can be seen in Figure 2 (bottom), co-immobilized nitrogen and phosphorous supported extensive biodegradation, although the biodegradation rate was slower than with externally supplied nutrients. Further studies on the effect of release rates of the co-immobilized nutrients may provide more information for optimizing this approach to bioaugmentation.

Adaptation

We have been characterizing a variety of different fluoranthene-degrading bacteria from around the world and have shown that the degradation capacity for this PAH is common and distributed among a variety of bacteria. To date, however, only soils and sediments polluted with PAHs and creosote have produced fluoranthene degraders. Phenanthrene degraders can be readily isolated from any type of soil. Thus, the ability to utilize and grow on HMW PAHs represents an ability to deal with very low available substrate concentrations. What is the source of these fluoranthene degraders? Are they present in many different environments but only enriched to the point of detection in polluted soils? Or is gene recruitment actually occurring in natural microbial communities that in essence "creates" this metabolic capability? A clearer understanding of the origins of these organisms has significant implications in bioremediation, for it may be possible to ultimately adjust environmental or ecological conditions in the field to accelerate this adaptation process and therefore more readily affect the outcome of a bioremediation treatment for PAHs.

To this end, we have been characterizing the genetics and physiology of our isolates. As has been documented for many other catabolic functions for xenobiotic chemicals in bacteria, one of these organisms harbors the fluoranthene degradative genes on a plasmid. Dr. Tom Lessie, in our laboratory, has shown that it also contains three mega-plasmids or multiple replicons. These replicons are quite large, 3,400, 2,300, and 1,200 kilobases in size. The presence of these mega-plasmids has been reported for other species of *Pseudomonas* (25), as well as other genera of bacteria. The physiological and genetic functions of these mega-plasmids are unknown, but they may be related to the large and broad metabolic capability that these organisms possess and perhaps even to the ability to degrade fluoranthene. By understanding more about this genetic makeup, it may eventually be possible to manipulate adaptation in the field in a time frame that could accelerate or increase the extent of bioremediation.

Summary and Conclusions

The successful bioremediation of PAH-contaminated soils and sediments requires a clear understanding of the metabolic and ecological factors that can be manipulated to increase the rate and extent of PAH biodegradation. We provide evidence in this report suggesting that 1) co-metabolism may be a potential mechanism for

degradation of HMW PAHs; 2) bioavailability of PAHs may be improved through the application of surfactants; and 3) the success of bioaugmentation may be increased by the use of procedures that immobilize PAH-degrading microorganisms, adsorbents, and/or nutrients. In addition, the knowledge of how microbial communities become adapted for enhanced PAH biodegradation may play an important role in developing future strategies for bioremediation.

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Metabolic Pathways Involved in the Biodegradation of PAHs

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The principal sources of polycyclic aromatic hydrocarbons (PAHs) in the environment are the use and spillage of fossil fuel-related materials, either petroleum- or coal-derived. Both sources contain complex mixtures of PAHs but differ in amount and composition. Coal-based materials such as creosote and coal tar are rich in PAHs, with relatively little alkyl substitution. Petroleum, on the other hand, generally contains a smaller fraction of PAHs composed of a wide array of alkyl-substituted homologues. Knowledge of the aerobic biodegradation of PAHs derives largely from studies of pure bacterial cultures isolated for their ability to utilize for growth single, unsubstituted aromatic hydrocarbons such as naphthalene, biphenyl, and phenanthrene (1). In all cases studied, catabolism is initiated by oxygen adding reactions usually forming *cis*-dihydrodiols on arene rings. While biological methods for removal of PAH-containing environmental contaminants are now seriously considered options for remediation, details of the processes involved are little understood. For example, little is known of the extent to which biotransformation (co-metabolism) is involved in the removal of higher molecular weight PAHs in complex mixtures and the organisms and growth substrates required. Are products of biotransformation accumulated? What are their environmental effects?

Some recent findings relevant to these questions are summarized below.

Naphthalene Degradation: New Insights

Investigation of reactions of naphthalene degradation catalyzed by enzymes encoded by the NAH7 plasmid was undertaken using a molecular biological approach involving cloning and subcloning of pathway genes (2). As a result, a collection of strains of *Pseudomonas aeruginosa* was obtained containing key genetic sequences of the plasmid encoding for the degradative pathway extending various distances from naphthalene. Such strains were used to accumulate, under physiological conditions, catabolites of naphthalene otherwise difficult to isolate and characterize. As a result, *trans*-2-hydroxy benzylidene pyruvate was identified as a metabolite of 2-hydroxy chromene-2-carboxylic acid and a new reaction was recognized as responsible for formation of salicylaldehyde and pyruvate by means of a novel hydratase-aldolase enzyme.

Degradation of Creosote PAHs

For studies of the bacterial degradation of creosote PAHs, an aromatic hydrocarbon fraction free of polars, resins, and phenols, with little if any N-heterocyclic material was obtained by column chromatography. Enrichments employed this fraction in mineral salts medium to establish cultures (from creosote-contaminated soils). These were incubated with shaking at 20°C to 24°C in the dark, with transfers biweekly. Amounts of remaining PAHs, determined by gas chromatography/flame ionization detector (GC-FID) after methylene chloride extraction, showed extensive losses of low molecular weight PAHs not accounted for by abiotic losses. Fluoranthene, pyrene, and PAHs with higher retention times were recovered essentially unchanged, being associated with insoluble black resinous material accumulated in cultures. Column chromatography and thin-layer

chromatography has shown this material contains both low molecular weight neutral products and complex polymeric material. Among the neutral products identified were acenaphthenone, fluorenone, and other ketones formed from naphtheno-aromatics. Certain of these products previously have been shown to result from the action of bacterial reductive dioxygenases (3).

Naphthalene Dioxygenase Action on Naphtheno-Aromatic Hydrocarbons

With the cloned genes of naphthalene dioxygenase available in a strain of *P. aeruginosa* (2), it was possible to investigate the action of a reductive oxygenase on simple naphtheno-aromatic hydrocarbons and related compounds (4). Induced cells were incubated in buffer with fluorene, acenaphthene, acenaphthylene, and other hydrocarbons having benzylic functions; products were extracted for characterization. Fluorenone was identified as a product of fluorene oxidation, with acenaphthenone formed from acenaphthene and acenaphthylene together with a *cis*-dihydrodiol and acenaphthenequinone in the latter case (Figure 1).

Evidently the first formed secondary alcohols are acted on by broad-specificity cellular dehydrogenases to give ketonic end products. Apparently anomalous oxidations at benzylic positions, such as observed here, may be expected in situations where biodegradation of mixtures of aromatic and naphtheno-aromatic hydrocarbons occurs.

Bacterial Utilization of a Naphtheno-Aromatic: Fluorene

Given that oxidation of benzylic functional groups may be unavoidable when arene dioxygenases are confronted by naphtheno-aromatics, it was of interest to examine whether such reactions are involved when bacteria utilize naphtheno-aromatics as growth substrates. Accordingly, the reactions employed in the utilization of fluorene by a *Pseudomonas* isolate were investigated. An earlier study with a different strain (5) suggested that the productive route of catabolism involved initial aromatic-ring dioxygenation and cleavage and that fluorenone was a dead-end metabolite. By contrast, the pathway established for the *Pseudomonas* isolate is initiated by benzylic oxidation leading to fluorenone formation. Subsequent reactions include formation of a novel angular diol (6) before opening the central five-membered ring to generate a dihydroxylated biphenyl carboxylic acid (Figure 2). This route (7,8) represents a significant difference from earlier characterized routes initiated by conversion of arenes to *cis*-dihydrodiols in that the naphthenic ring is first oxidized and then opened, thereby accommodating both fluorene and fluorenone.

Organisms possessing this biochemistry, therefore, are equipped to channel products of anomalous

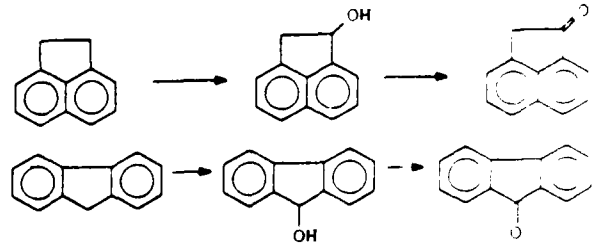

Figure 1. Transformation of naphtheno-aromatics by naphthalene dioxygenase.

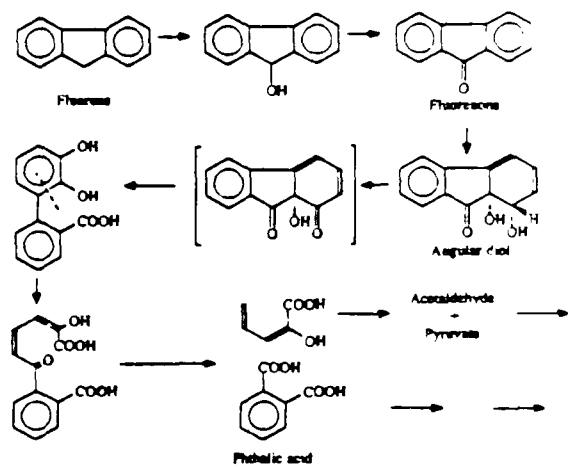

Figure 2. Route of fluorene degradation in *Pseudomonas* F274

oxidation by arene dioxygenases into productive catabolicpathways.

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Environmental Factors Affecting Creosote Degradation by Sphingomonas paucimobilis Strain EPA505

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The presence of polycyclic aromatic hydrocarbons (PAHs) in soil and ground water is recognized by EPA as a priority environmental problem. Because of inadequacies intrinsic to the design and operation of wood treatment facilities of the past, coal tar creosote represents one of the major anthropogenic sources of excessive PAH concentrations in the environment (1). Coal tar residues from coal gasification and creosote distillation processes represent another major source of environmental PAH contamination.

Of the hundreds of locations potentially affected by PAHs from industrial operations, most have been thoroughly assessed and characterized. In cases where remedial actions to restore soil and ground water have been prescribed, a variety of treatment alternatives have been evaluated. Unfortunately, many of the more conventional approaches have proven ineffective and/or prohibitively expensive. For example, ground-water pump-and-treat approaches have proven ineffective for PAH-contaminated aquifers (EPA Office of Solid Waste and Emergency Response [OSWER] memorandum, May 27, 1992). For soils, excavation followed by secondary treatment (e.g., soil washing followed by slurry-phase biotreatment) is of such a scale that costs and practicability have become prohibitive. In addition, from an end-user's perspective, many conventional remedial technologies are unacceptable because of regulatory problems and technical feasibility.

Of the alternative remedial options available for creosote-contaminated soil, bioremediation may represent a technology of choice. Despite the many potential advantages of bioremediation, the reported effectiveness of PAH biodegradation in contaminated media has varied (2). This variability is due to a number of recognized factors, including the presence of free product as dense nonaqueous phase liquid (DNAPL) and/or light nonaqueous phase liquid (LNAPL), the heterogenous nature of soil and subsurface matrices, and the use of ineffective delivery and implementation strategies. From a biological perspective, effective biodegradation is influenced, in part, by the presence of catabolically competent microflora at a contaminated site and by certain environmental factors that enhance the activity of this microflora, including availability and concentration of electron acceptors, inorganic nutrients, and the target chemical(s). The ability to control and regulate these factors is the foundation for bioremediation application to PAH/creosote-contaminated soils.

In an effort to enhance the biodegradation of PAHs in the environment, we have recently focused on several environmental and toxicological factors influencing the ability of *Sphingomonas (Pseudomonas) paucimobilis* strain EPA505 to mineralize PAHs individually and in complex mixtures (e.g., creosote). We believe that more effective management of natural microbial community activities, through control of these factors, may lead to more efficient bioremediation of soil and water contaminated by PAHs. Additionally, these studies should help inoculant microorganisms be employed more effectively for site restoration.

Materials and Methods

Evaluation of Temperature and pH Effects

Biometer flasks (3) containing minimal salts medium, radiolabeled fluoranthene or phenanthrene, and cells of strain EPA505 were used to monitor $^{14}CO_2$ evolution over a range of pH and temperature. A mixture of unlabeled (10 mg PAH) and ^{14}C-labeled PAH (approximately 41,000 dpm) was added to 250-mL biometer flasks from acetone stock solutions, and the solvent was evaporated. To each flask was added 50 mL of Bushnell-Haas,

and the contents were sonicated. The pH of the medium was adjusted with HCl or NaOH. The buffering capacity of Bushnell-Haas was such that the pH was stabilized over the course of 2 days at the target pH. For temperature studies, the medium was adjusted to pH 7.1, and flasks were equilibrated at various temperatures for about an hour prior to inoculation. All flasks were maintained at a selected temperature over the course of the studies.

To initiate studies, 1.0 mL of 2N NaOH was added to each sidearm of the biometer flasks to trap $^{14}CO_2$. The inoculum was prepared from a cell concentrate (48-hr growth on complex medium LB, harvested, washed, and resuspended in 0.05 M phosphate buffer) and added to obtain an initial optical density of 0.5 at 600 nm (about 3 to 5 x 10^8 cells/mL). Flasks were run in duplicate, and killed-cell controls also were used. Flasks were shaken at 120 rpm at 30°C in darkness for up to 8 days. NaOH samples were collected intermittently and analyzed by liquid scintillation the same day.

Identification of Inhibitory Creosote Constituents

Biometer flasks again were used to monitor $^{14}CO_2$ evolution from ^{14}C-PAH in the presence of various concentrations of creosote and its acid-, neutral-, and base-extractable fractions to study the effect of phenols, PAHs, and neutrally extractable heterocycles (carbazole, dibenzothiophene, dibenzofuran, and thianaphthene) and other N-, S-, and O-containing heterocycles, respectively (4,5). Synthetic mixtures of each of these fractions were prepared as defined in Table 1 to more accurately evaluate the effect of each of these mixtures (6). An "artificially weathered" (heating the neutral fraction at 65°C ± 5°C for 24 hours), creosote-neutral fraction also was analyzed to examine the effect of low molecular-weight PAHs (i.e., those containing two fused rings). A killed-cell control was run for each different substrate, and a positive mineralization control (no creosote) was run with each set of incubations.

The incubation medium was prepared as described above. Bushnell-Haas, however, was supplemented with 0.03-percent Triton X-100 to facilitate study of constituents at concentrations above their natural water solubilities. For consistency, Triton X-100 was added to each flask. The appropriate amount of creosote, or some fraction thereof, was added via glass gas-tight syringe.

Flasks were shaken 120 rpm at 30°C in darkness for 10 days. NaOH samples were collected daily and analyzed by liquid scintillation the same day. At the

Table 1. Composition[a] of Synthetic Mixtures of Creosote Constituents[b] Used in Mineralization Inhibition Studies

Neutral Fraction (PAHs)	Acidic Fraction (Phenolics)	Basic Fraction (Heterocyclics)
Naphthalene	Phenol	Quinoline
2-Methylnaphthalene	o-Cresol	Isoquinoline
1-Methylnaphthalene	m-Cresol	Carbazole
Biphenyl	p-Cresol	Acridine
2,3-Dimethylnaphthalene	2,5-Xylenol	2-Methylquinoline
2,6-Dimethylnaphthalene	3,5-Xylenol	4-Methylquinoline
Acenaphthene	2,3-Xylenol	Dibenzothiophene
Acenaphthylene	2,4-Xylenol	Dibenzofuran
Fluorene	2,6-Xylenol	
Phenanthrene	3,4-Xylenol	
Anthracene	2,3,5-Trimethylphenol	
2-Methylanthracene		
Anthraquinone		
Fluoranthene		
Pyrene		
Benzo[a]anthracene		
Chrysene		
2,3-Benzofluorene		
Benzo[a]pyrene		

[a]Composition of fractions based on data reported by Mueller et al. (6)
[b]Compounds listed in order of elution during gas chromatography according to methods previously described (4,5)

conclusion of these studies, flasks exhibiting inhibition were cultured for the determination of viable cells. The remaining contents of each flask subsequently were extracted and analyzed for the concentration of creosote constituents by a gas chromatography/flame ionization detector (GC-FID) (4,5).

Results and Discussion

Average (n=2) percent release of $^{14}CO_2$ from ^{14}C-fluoranthene by strain EPA505 was essentially identical for pH values of 6, 7, 8, and 9 (Figure 1). In these flasks, postincubation pH was lowered by 0.5 to 1 pH unit. The pH-5 flasks quickly reached a plateau, after which mineralization ceased. This plateau was not characteristic of any of the other pH treatments. The postinoculation pH of this flask was 4.6. Absence of extensive mineralization in the pH-4 and pH-10 flasks correlated with the absence of the characteristic color change (colored degradation intermediates) normally associated with fluoranthene mineralization by this bacterium (1,7,8).

Strain EPA505 was active at all temperature ranges tested to date (Figure 2), although rates and extents of mineralization decreased with decreasing temperature. At the 25°C incubation temperature, mineralization extent was reduced compared with 30°C and 37°C but might eventually reach that seen with the higher temperatures given incubation times beyond 200 hours. At 18°C, mineralization rates appeared to be leveling off at values below those seen at higher temperatures, and it does not appear that continued incubation beyond 200 hours will increase mineralization much further. We currently are evaluating activity of this strain at a wider range of temperature and incubation times. The effects of pH and temperature on the mineralization of ^{14}C-phenanthrene by strain CRE-7, a low molecular-weight PAH degrader, are currently under study.

Of the creosote fractions assessed, the acid-extractable (phenolic) and base-extractable (heterocyclic) fractions were the most inhibitory to the activity of strain EPA505. At 50 mg/L, the phenolics fraction slowed the onset of mineralization; at 70 mg/L, no mineralization was observed (Figure 3). The base-extractable fraction (mostly heterocycles) was inhibitory at 35 mg/L (data not shown). Whole creosote was inhibitory at 200 mg/L. The neutrally extracted fraction and the weathered neutral fractions were not inhibitory at any concentration tested (210 mg/L).

The basis of this inhibition is not known but could be the result of direct toxicity to the cells or isotope dilution caused by the use of more readily degradable substrates, or could be an effect of decreased availability of the radiolabeled substrate. Studies are currently in progress using synthetic mixtures of all fractions to decipher the inhibitory mechanism and more accurately identify inhibitory constituents and concentrations.

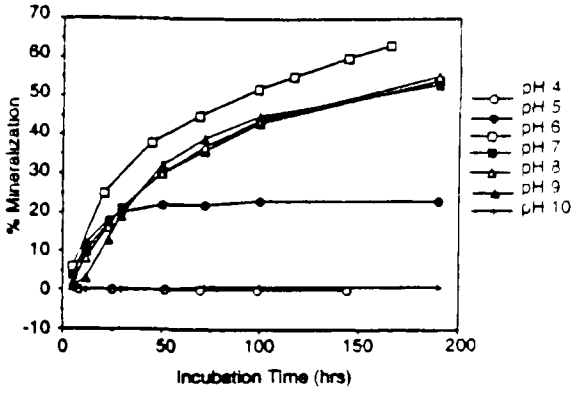

Figure 1. Effect of media pH on ^{14}C-fluoranthene mineralization by strain EPA505.

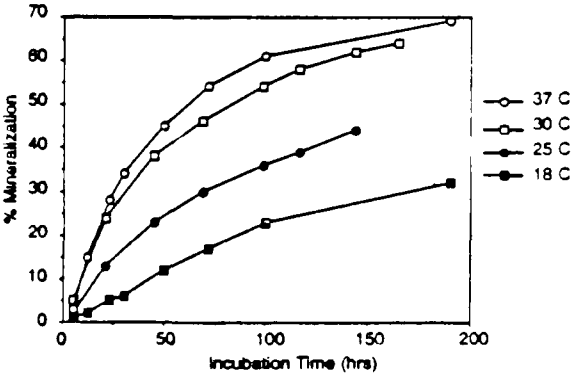

Figure 2. Effect of incubation temperature on ^{14}C-fluoranthene mineralization by strain EPA505.

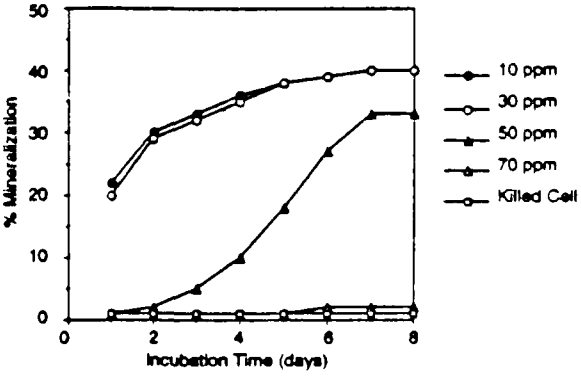

Figure 3. Mineralization of ^{14}C-fluoranthene by strain EPA505 in the presence of the acid-extractable fraction of creosote (phenolics).

These studies also will identify individual creosote constituents most inhibitory to this strain. Similar studies with strain CRE-7 are in progress. In addition, the results of pure culture studies will be compared with results

from studies using natural microbial communities that have been enriched to degrade creosote.

Summary and Conclusions

If the isolated strains of bacteria under study represent the potential activities of bacteria in contaminated site material, then environmental conditions may have to be manipulated, in some cases, to provide optimal activity. Where low temperature and pH extremes are encountered in the field, substantial effects on PAH mineralization can be expected. In addition, if bioaugmentation is considered as a biotreatment strategy, inoculants may have to be carefully selected to be effective under these suboptimal conditions.

These data further support implementation of creosote bioremediation via a two-stage process (patent pending) employing co-inoculation (e.g., bacterial strain to degrade the "toxic" phenolic and heterocyclic fractions) and secondary biotreatment of more recalcitrant constituents (e.g., strain EPA505 to treat high molecular-weight PAHs) (9).

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Molecular Genetic Approaches to the Study of the Biodegradation of Polycyclic Aromatic Chemicals

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Petroleum, coal, and their derivatives are composed of a variety of chemicals, including polycyclic aromatic hydrocarbons (PAHs), heterocyclics, and alkyl-substituted aromatics. As these chemicals increase in size and complexity, bacteria have more difficulty metabolizing them. In addition, their catabolic pathways are lengthy and often branched, making it more difficult to study them.

The approach that we are taking to study the biodegradation of individual xenobiotic chemicals involves a variety of strategies; foremost among these are molecular genetic techniques such as 1) cloning genes that encode enzymes that catalyze reactions of interest; and 2) isolating transposon-induced mutants that lack enzymes of a metabolic pathway. These approaches allow an individual enzyme-catalyzed reaction or set of reactions to be studied in the absence of other reactions that complicate analysis. This approach obviously allows the simultaneous study of both the enzymes and the genes that confer on an organism its metabolic capabilities.

Naphthalene and benzothiophene are simple, fused-ring compounds that can serve as models for more complex polycyclic aromatic chemicals (PACs) in biodegradation studies. The pathway for the bacterial metabolism of naphthalene (Figure 1) was characterized (1,2) using recombinant bacteria containing genes cloned from the naphthalene catabolic plasmid NAH7 (Figure 2). Bacteria carrying the plasmid, pRE657, which contains a 10-kb *Eco*RI-*Cla*I fragment on which the genes *nahA*, *nahB*, and *nahC* are located, converted naphthalene (Figure 1, I) to a mixture of two chemicals, 2-hydroxychromene-2-carboxylate (HCCA, Figure 1, VI) and *trans-o*-hydroxybenzylidenepyruvate (tHBPA, Figure 1, VII). The initial product, HCCA, and tHBPA spontaneously isomerize in aqueous solution to form an equilibrium mixture of the two compounds, making their identification difficult. Separation was possible, however, using column chromatography on Sephadex G-25 with water as solvent; this allowed the rigorous identification of these compounds using ^{1}H- and ^{13}C-NMR spectroscopy and gas chromatography/mass

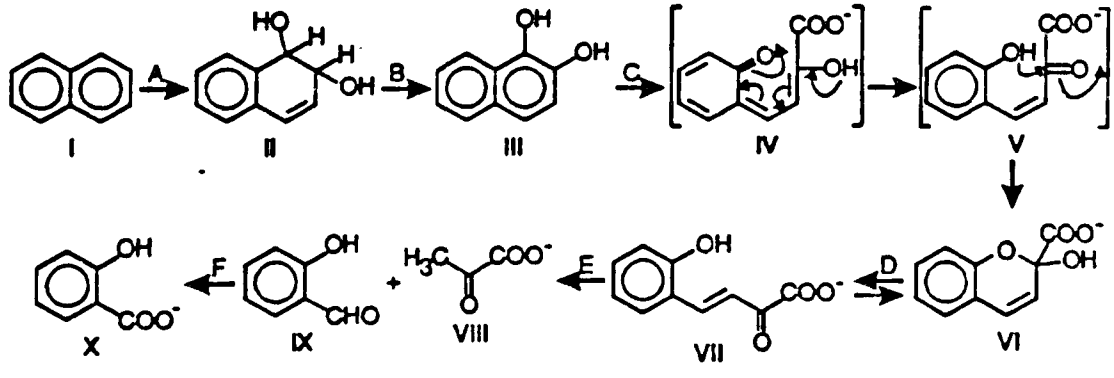

Figure 1. Pathway for the bacterial metabolism of naphthalene to salicylate.

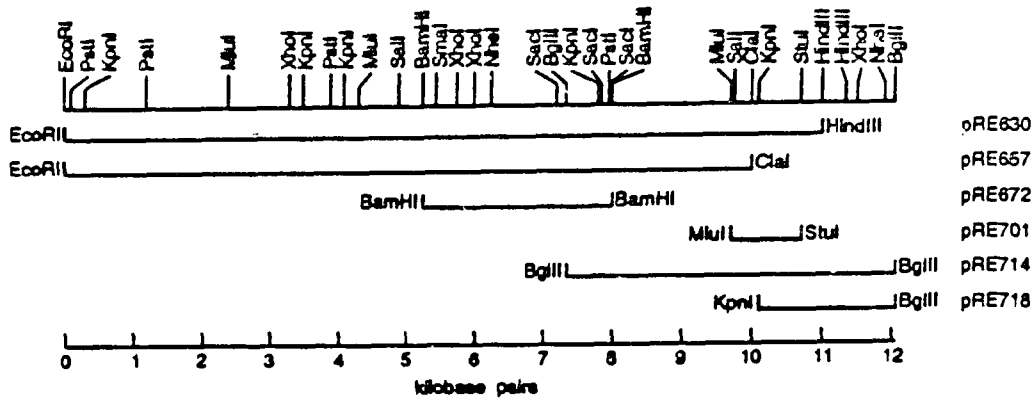

Figure 2. Genetic map of the region of plasmid NAH7 that encodes the metabolism of naphthalene to salicylate.

spectrometry (GC/MS). Subclones pRE701 and pRE718 were obtained that encode the enzymes tHBPA hydratase-aldolase (Figure 1, E) and HCCA isomerase (Figure 1, D), respectively, and act on these intermediates. These two intermediates, and the enzymes that degrade them, are characteristic of pathways for the degradation of aromatic compounds with two or more rings. The genes that encode these enzymes (*nahE* and *nahD*) thus may have value as specific probes for environmental microorganisms that degrade PACs, serving as part of the justification for the recently completed sequencing of these genes (3).

The sulfur-containing heterocycle benzothiophene is transformed by isopropylbenzene-degrading bacteria to a mixture of products. One of these strains, *Pseudomonas putida* RE204, and its Tn*5*-generated mutant derivatives (4) were used to study these biotransformations (5). Three products were formed from benzothiophene by the isopropylbenzene-induced wild-type strain RE204: *trans*-4-(3-hydroxy-2-thienyl)-2-oxobut-3-enoate (Figure 3, XIII), 2-mercap-tophenylglyoxalate (Figure 3, XV), and 2′-mercaptomandelaldehyde (Figure 3, XVI). The latter was identified following its conversion to its isomer, *trans*-2,3-dihydroxy-2,3-dihydrobenzothiophene. Once again, chromatography on Sephadex G-25 was employed to separate these relatively unstable chemicals, which subsequently were identified by 1H- and ^{13}C-NMR spectroscopy and GC/MS. A 2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase (Figure 3, enzyme B′) deficient mutant strain, RE213, converted benzothiophene to *cis*-4,5-dihydroxy-4,5-dihydrobenzothiophene (Figure 3, XII) and 2′-mercaptomandelaldehyde, presumably formed by the spontaneous opening of the thiohemiacetal *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene (Figure 3, XVI). Neither XIII nor XV was formed by this mutant; apparently the dihydrodiol dehydrogenase is required for the formation of these compounds. The complex mixture of products formed by the fortuitous metabolism of the relatively

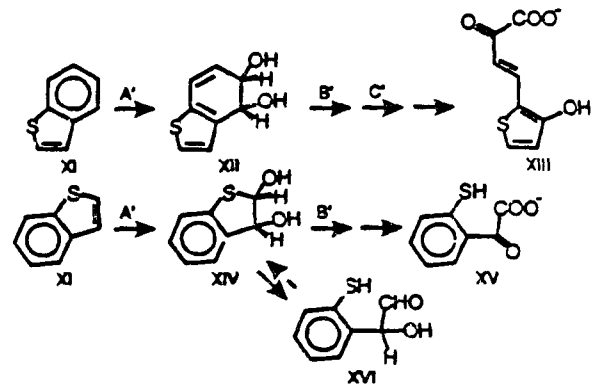

Figure 3. Biotransformations of benzothiophene.

simple chemical benzothiophene illustrates the problems that can occur in any bioremediation treatment in which co-metabolism occurs. While substrates disappear, products that often are not targeted by the analytical method employed may form and accumulate.

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Comparison of Sulfur and Nitrogen Heterocyclic Compound Transport in Creosote-Contaminated Aquifer Material

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Commonly, ground-water solute transport model inputs are generated from chemical and ground-water properties that are not comparable with those at the site of contamination. Care must be taken when assuming that chemicals with similar molecular structures or characteristics possess equivalent transport properties. In addition, ground-water characteristics, such as pH, must be compared with ionization constants (pK_a) to determine the influence of the sediments ion exchange capacity. Simulated transport will not be accurate if the parameter determined at one pH differs from that of the ground water.

In this paper, we compare the values of partition coefficients and retardation factors for the sulfur and nitrogen heterocyclic compounds benzothiophene, dibenzothiophene, quinoline, 2(1H)-quinolinone, acridine, and carbazole on low organic carbon content, low ion exchange capacity aquifer material. Column breakthrough curves (BTCs) were modeled using the local equilibrium assumption (LEA) for compounds with a log octanol-water partition coefficient (log K_{ow}) of less than 2.5 and the nonequilibrium assumption (NEA) for compounds with a log K_{ow} greater than 2.5.

Background

The column material is taken from sediment 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 (PCP). For more than 80 years, a large but unknown quantity of waste water, consisting of extracted moisture from the poles, cellular debris, creosote, PCP, and diesel fuel from the treatment processes, was discharged to unlined surface impoundments that were in direct hydraulic contact with the sand-and-gravel aquifer. The ground water, at a pH of 5.9 and moving at approximately 1 m/d, is continually dissolving the more soluble compounds found in creosote, creating an extended contamination plume. The aquifer material for the laboratory columns consisted of a low organic carbon content (0.024 percent organic carbon), low ion exchange capacity (2 meq/ 100 g) claylike sand from the approximate centroid of the plume (Table 1).

BTCs of the water-soluble heterocyclic compounds in laboratory columns can be described by the convection-dispersion equation using the LEA as proposed by Hashimoto et al. (2),

$$(\theta+\rho_b K_d)\frac{\partial C}{\partial t} = \theta D\frac{\partial^2 C}{\partial x^2} - \theta v\frac{\partial C}{\partial x},$$

where θ is the porosity (-), ρ_b is the bulk density of the aquifer material (g/m^3), K_d is the partition coefficient (m^3/g), C is the aqueous concentration (g/m^3), t is the time (d), D is the dispersion coefficient (n^2/d), x is the distance (m), and v is the linear velocity (m/d).

Transport of hydrophobic chemicals commonly must be modeled using the NEA as proposed by van Genuchten and Wierenga (3), which accounts for a readily mobile

Table 1. Aquifer Material and Column Characteristics

Median Particle Diameter (m)	0.000375
Percent Organic Carbon (-)	.024
Cation Exchange Capacity (meq/100 g)	1.6
Column	
Length (m)	0.354
Diameter (m)	.025
Porosity (-)	.449
Bulk Density (g/m^3 x 10^{-6})	1.361
Flow Rate (m^3/d x 10^6)	140

fraction and a stagnant or immobile fraction of water in the aquifer matrix (subscripts *m* and *im*, respectively),

$$(\theta_m + f\rho_b K_d)\frac{\partial C_m}{\partial t} + [\theta_{im} + (1-f)\rho_b K_d]\frac{\partial C_{im}}{\partial t} = \theta_m D\frac{\partial^2 C_m}{\partial x^2} - \theta_m v_m \frac{\partial C_m}{\partial x} \quad (2)$$

$$[\theta_{im} + (1-f)\rho_b K_d]\frac{\partial C_{im}}{\partial t} = \alpha (C_m - C_{im}), \quad (3)$$

where f is the fraction of sorption sites in the mobile region (-) and α defines the transfer rate of the solute between mobile and immobile water (d^{-1}). As described by van Genuchten (4), the variables, f and α, from equations 2 and 3, can be related to two fitted, dimensionless parameters, respectively: β, the fraction of the sites in the mobile region where sorption is instantaneous, and ω, the ratio of hydrodynamic residence time to characteristic time of sorption (5). The NEA model is based on the assumption that convection and dispersion govern transport in the mobile water, and that diffusion controls the transfer of contaminant between mobile and immobile water.

Both models assume a linear isotherm. Retardation factors, R, which describe the movement of contaminants relative to a conservative tracer, can be related to partition coefficients, bulk densities, and porosity by

$$R = 1 + \frac{\rho_b K_d}{\theta} \quad (4)$$

Parameters were fit to BTCs using nonlinear regression analysis by the computer programs HASHPE (6), to determine R for LEA, and CFITIM (4), to determine R, β, and ω for NEA. The dispersion parameter for all model simulations was determined from $CaCl_2$ breakthrough.

Brusseau and Rao (7) suggest that, for values of 3 less than approximately 10, the NEA should be used instead of the LEA to account for the observed tailing. The values of 3 for benzothiophene, dibenzothiophene, carbazole, and acridine (compounds with log K_{ow} >2.5) are well below 10 (Table 2), justifying the use of the NEA model. The NEA model determined that the values of 3 were much greater than 10 for quinoline and 2(1H)-quinolinone (compounds with log K_{ow} <2.5). Thus, the LEA model was used to determine breakthrough parameters for these compounds.

Results and Discussion

Fitted parameters and original coefficients for benzothiophene, dibenzothiophene, quinoline, 2(1H)-quinolinone, carbazole, and acridine using the models are given in Table 2. The chemical structures are shown in Figure 1. The retardation factors for benzothiophene, quinoline, 2(1H)-quinolinone are quite similar to each other. 2(1H)-Quinolinone, with a pK_a of 5.29, is approximately 20-percent ionized, and quinoline, with a pK_a of 4.9, is approximately 9.1-percent ionized. Zachara et al. (8) have shown that sorption of quinoline is dominated by ion exchange up to 2 pH units above its pK_a. 2(1H)-Quinolinone, like quinoline, should be retained by both ion exchange and organic sorption. Benzothiophene, however, is nonionic and subject to organic sorption alone.

The values of β for the sulfur heterocycles agree with each other but are greater than those for the nitrogen heterocycles, suggesting a larger percentage of sites at which instantaneous sorption for the sulfur heterocycles occurs. The value of ω for the sulfur heterocycles is much less than that for the nitrogen heterocycles, indicating that the characteristic time of sorption contributes more to the retardation of nitrogen heterocycles, and to acridine transport in particular.

The retardation of acridine is much greater than that of dibenzothiophene and carbazole, despite the fact that all have two benzene rings fused to a sulfur or nitrogen heterocyclic ring (Figure 1 and Table 2) and have similar log K_{ow}; dibenzothiophene and carbazole, however, are

Table 2. pK_a, log K_{ow}, Partition Coefficients, Retardation Factors, and Nonequilibrium Assumption Parameter Values for Benzothiophene, Dibenzothiophene, Quinoline, 2(1H)-Quinolinone, Carbazole, and Acridine

	pK_a	log K_{ow}	Partition Coefficient $m^3/g \times 10^6$	Retardation Factor	β	ω
Benzothiophene		3.12	0.184	1.74	0.90	0.48
Dibenzothiophene		4.38	0.789	3.84	0.93	0.23
Quinoline	4.90	2.03	0.133	1.32	0.0	160
2(1H)-Quinolinone	5.29	1.26	0.231	1.54	0.0	1300
Carbazole	-5.70	3.29	1.01	4.34	0.60	2.6
Acridine	5.60	3.40	4.56	39.6	0.61	1.2

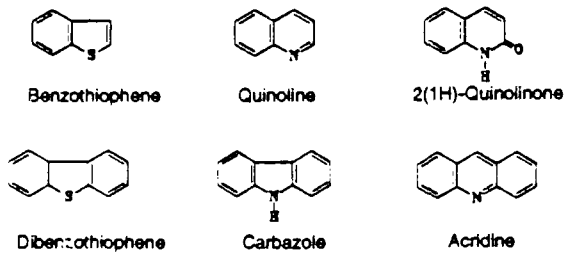

Figure 1. Chemical structures of benzothiophene, dibenzothiophene, quinoline, 2(1H)-quinolinone, carbazole, and acridine.

subject to organic sorption alone, whereas acridine is subject to both organic sorption and ion exchange. The pK_a of acridine is 5.6 and of carbazole is -5.7 (Table 2). Thus, at pH 5.9, the ionized-fraction of acridine is 0.33, but carbazole is completely un-ionized. The degree of affinity (the selectivity) of acridine to charged functional groups on the aquifer material and the extent of ionization as well as the sediments cation-exchange capacity contributes to the retention capacity. With an acridine concentration of 18 g/m^3 (0.10 meq/L), the column capacity due to ion exchange is 160. The column capacity is based on the assumption of total sorption of the ionized fraction of acridine to the aquifer material and complete displacement of calcium ions.

Transport of organic chemicals in ground water must be modeled using parameters similar to those at the site of interest. Assumptions about solute transport based on chemical and physical properties of similar but not identical compounds, aquifer sediments, and ground water are not always valid. Field conditions, such as pH, flow velocity, and chemical properties (such as selectivity and pK_a), must be taken into consideration to effectively model solute transport.

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Modeling Steady-State Methanogenic Degradation of Phenols in Ground Water at Pensacola, Florida

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The study site is an abandoned wood treatment facility in the extreme western end of the Florida Panhandle within the city of Pensacola. For about 80 years, creosote-derived contaminants and pentachlorophenol from unlined waste-disposal ponds entered the ground water in the underlying sand and gravel aquifer. Concentrations of phenol and 2-, 3-, and 4-methylphenol have been monitored at the study site for more than 12 years. The data indicate that a nonaqueous-phase source below the ponds provides a constant input of dissolved phenols that then are degraded within 200 m downgradient. Figure 1 is a generalized geologic section along a flow line down the axis of the plume together with contours of total phenolic compound concentration. The degradation process appears to be at steady state because the concentration profile has not changed over the last 12 years. The aquifer consists of approximately 90 m of poorly sorted fine to coarse grained deltaic sand deposits interrupted by discontinuous silts and clays. Ground-water flow is generally horizontal and southward toward Pensacola Bay. Flow velocities range from 0.3 m/d to 1.2 m/d (1).

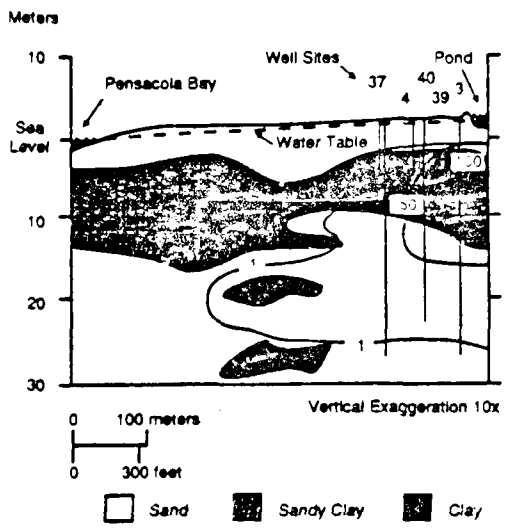

Figure 1. Generalized geologic section along a flow line down the center of the plume. Contours of total phenols are shown in mg/l.

Model Description

Godsy et al. (2) determined methanogenic utilization rates for four phenolic compounds in microcosms containing aquifer sediments. They fit the change in concentration with time and the associated microbial growth to the equations for Monod growth and substrate utilization. Their results, given in Table 1, were used in a model describing transport and degradation at the field site.

The modeled profile is 6 m below the surface in the methanogenic part of the contaminated zone, below the depth at which recharge and floating hydrocarbon at the water table affect concentrations and above the clay lenses. A one-dimensional model was used because the flow direction is primarily horizontal and perpendicular to a wide contaminant source. Acridine orange direct counts (AODC) indicate that the bacteria population is spatially uniform and low (5×10^3 to 7.6×10^7 AODC/g dry weight of sediment) relative to subsurface enumerations at other sites (3). The existence of a steady-state degradation profile of each substrate, together with a low, uniform bacteria density, indicates that the bacterial population is exhibiting no net growth (4). Thus, the bacteria concentration in the model is held constant in time and uniform in space.

We assume that the substrate profile at a depth of 6 m satisfies the one-dimensional transport equation with a Monod reaction term:

$$\frac{\partial S}{\partial t} = D\frac{\partial^2 s}{\partial x^2} - v\frac{\partial s}{\partial x} - \frac{\mu_m}{Y}\frac{B}{\theta}\frac{S}{K_s+s} \qquad (1)$$

where S is the substrate concentration (mg/L); t is time (d); x is distance downgradient from the first observation well (m); D is the dispersion coefficient (m^2/d); v is

Table 1. Kinetic Constants from Microcosm Studies for Each of the Phenolic Compounds Tested (2)*

Compound	Growth Rate μ_m (d^{-1})	Half Saturation K_s (mg/L)	Yield Y (mg/mg)
Phenol	0.111 ± 0.005	1.33 ± 0.07	0.013
2-Methylphenol	.044 ± 0.001	.25 ± 0.82	022
3-Methylphenol	.103 ± 0.078	.55 ± 6.67	.026
4-Methylphenol	.099 ± 0.110	3.34 ± 11.1	.025

*Yield values were obtained from protein determinations before and after substrate utilization.

average linear velocity (m/d); μ_m is maximum growth rate (d^{-1}); Y is yield (mg bacteria per mg S); B is the concentration of the active degrading bacteria (mg/L); θ is porosity; and K_s is the half-saturation constant (mg/L). This equation was solved using a computer code described by Kindred and Celia (5), with boundary and initial conditions given by:

$$S(0,t) = S_0 ; \left.\frac{\partial S}{\partial x}\right|_{x=250} = 0 ; S(x,0) = S_0 ; \qquad (2)$$

where S_0 is the contaminant concentration 6 m below the ground surface at Site 3, the closest site to the source.

Model Results

Two predicted steady-state substrate profiles, along with the measured phenolic-compound concentrations at 6 m below land surface at each sample site, are shown in Figure 2. The computed profiles are steady-state solutions to a one-dimensional advective-dispersive equation with a biological reaction term (Equation 1). The upper curve predicts the field profile that would result from the phenol degradation rate that was measured in the lab, whereas the lower curve corresponds to the rate measured for 2-methylphenol. These two rates were used because they have the smallest associated errors and bracket the rates for the other two compounds (Table 1). The values for bacteria concentration were varied to obtain the best match to the data. The parameters used in the solution for phenol and 3-methylphenol were: S_0 = 26.0 mg/L, μ_m = 0.111 d^{-1}, Y = 0.013, K_s = 1.33 mg/L, B = 1.5 × 10^{-2} mg/L, v = 1.0 m/d, D = 1.0 m^2/d. For 2- and 4-methylphenol, the values used were: S_0 = 13.5 mg/L, μ_m = 0.044 d^{-1}, Y = 0.022, K_s = 0.25 mg/L, B = 3.0 × 10^{-2} mg/L, v = 1.0 m/d, D = 1.0 m^2/d. A steady-state solution was obtained for all $t \geq$ 1,000 days.

The model profiles indicate that the rates measured in the microcosm simulations accurately represent the degradation process taking place in the field. The validity of the Monod kinetics expression for the degradation rate is apparent from the field data because the rate of decrease in the phenol concentration changes dramatically around Site 40 (located about 90 m downgradient from Site 3). When the substrate concentration is high (upgradient of Site 40), the degradation kinetics can be approximated by a zero-order reaction term consistent with the low values of K_s observed in the lab studies. When the substrate concentration is close to the value of K_s, the degradation rate drops as predicted by Monod kinetics. The fitted bacteria concentration for the upper curve is twice that for the lower curve, because the yield value for phenol is half that for the other compounds.

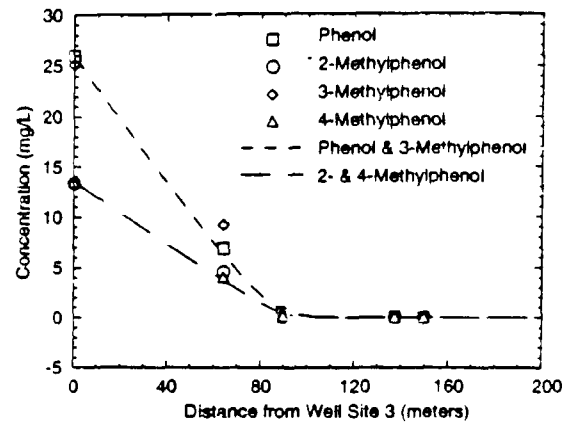

Figure 2. Computed concentration profiles (lines) and measured concentrations (points) of the four phenol solutes plotted against distance downgradient from Site 3. The measured data values are from 6 m below land surface at Sites 3, 39, 40, 4, and 37, from left to right, respectively. (Site locations are shown in Figure 1.)

Recall that to obtain a steady-state solution for the concentrations, it was necessary to assume no net growth for the bacteria. To investigate how this may happen, we used the values in Table 1 and the computed phenol concentrations from Figure 2 to predict the growth rate that is consistent with the observed degradation rate. Figure 3 shows predicted bacterial growth rates with and without the effect of toxic inhibition. In the curve with no inhibition, the peak growth rate is roughly 0.1 day^{-1}. To maintain zero net growth, the plotted growth rate must be balanced by an equivalent decay rate. A decay rate of 0.0192 day^{-1} for methanogens was found by Suidan et al. (6) in a continuous reactor. This value, shown as a horizontal line in Figure 3, is almost an order of magnitude too low to balance the predicted

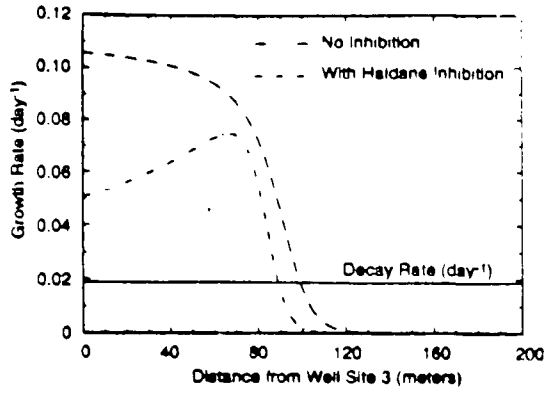

Figure 3. Theoretical growth rate computed from the phenol concentration, the Monod growth expression, and the growth parameters measured in the microcosm simulations. The two curves are computed with and without the effect of Haldane inhibition.

growth. Furthermore, in theory, the functional form of the positive growth curve cannot be balanced by a constant decay rate. When the toxicity of phenol is accounted for using a Haldane (7) inhibition model, the predicted growth is about 50 percent lower but still much higher than the published decay rate.

Summary and Conclusions

We have created a model of methanogenic degradation of phenolic compounds for a sand and gravel aquifer at Pensacola, Florida. The model verifies that field disappearance rates of four phenols match those determined in batch microcosm studies performed by Godsy et al. (2). The degradation process appears to be at steady state because a sustained influx of contaminants over several decades has been continuously disappearing within 150 m downgradient of the source. Goerlitz et al. (8) concluded that sorption was insufficient to explain the observed loss. The existence of a steady-state degradation profile of each substrate, together with a low bacteria density in the aquifer, indicates that the bacterial population is exhibiting no net growth possibly because of the oligotrophic nature of the bacteria population indicated by the low value for K_s. A low K_s causes growth and utilization to be approximately independent of the phenolic-compound concentration for most of the concentration range. Thus, a roughly constant bacteria growth rate should exist over much of the contaminated area. This growth could be balanced by an unusually high decay or maintenance rate caused by hostile conditions or predation. Alternatively, the loss of bacteria by transport downgradient is being investigated with column studies.

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Anaerobic Biodegradation of 5-Chlorovanillate as a Model Substrate for the Bioremediation of Paper-Milling Waste

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5-Chlorovanillate (5CV; 5-chloro-4-hydroxy-3-methoxybenzoic acid) was selected as a model compound for studying the biodegradation of paper mill effluents. This compound contains the methoxy-, chloro-, and carboxyl side groups often present on aromatic chlorinated compounds released in paper mill effluents. The major pathway of 5CV degradation previously was determined to be stepwise demethoxylation to 5-chloroprotocatechuate (5CP; 5-chloro-3,4-dihydroxybenzoic acid), decarboxylation to 3-chlorocatechol (3CC; 3-chloro-1,2-dihydro-xybenzene), and dechlorination to catechol, which was completely degraded (Figure 1). The current research further investigates the anaerobic bacterial species responsible for the individual transformation steps. Once obtained in pure culture, studies can be performed investigating individual transformation steps with reduction in toxicity of paper mill waste.

Selective media containing guaiacol (2-methoxyphenol), protocatechuate (dihydroxybenzoic acid) and catechol as the sole energy source were inoculated with the original 5CV culture. Transformation of target compounds in these enrichment cultures was followed using high performance liquid chromatography analyses. Immediately upon completing the transformation of interest, the cultures were passed to fresh medium. The guaiacol, protocatechuate, and catechol cultures were sequentially transferred through their respective media several times, followed by several refeedings of the target compound to enrich for the bacterial species of interest. These enrichments then were diluted in the respective media to obtain bacterial cultures responsible for demethoxylation (Figure 2), decarboxylation (Figure 3), and catechol (Figure 4) degradation. The data indicate that the demethoxylating and decarboxylating bacterial species were more numerous by three orders of magnitude than the catechol-degrading bacterial species. The transforming and degrading activity in these cultures has been sustained for several months and through several transfers, indicating that the activity is stable—a condition necessary for bioremediation applications. The demethoxylating and decarboxylating cultures continued to transform guaiacol and protocatechuate in the presence of fairly high concentrations of catechol. Demethoxylation rates begin to decline above 3 mM catechol (Figure 2B), while decarboxylation rates did not decline significantly at 10 mM catechol (Figure 3B). Because paper mill waste contains other phenolic compounds, applied bacterial cultures must tolerate other toxics while performing the desired

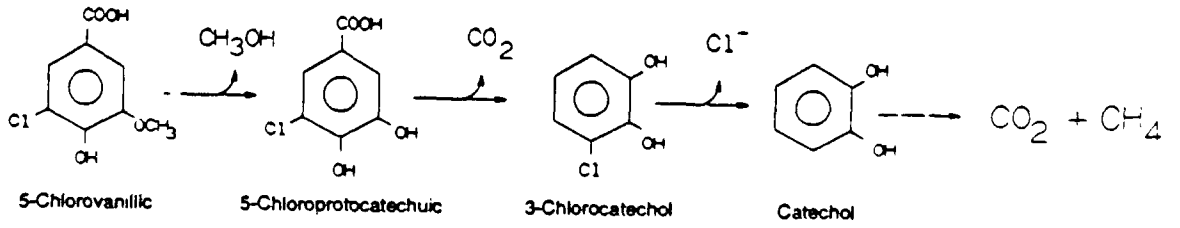

Figure 1. Pathway for the complete degradation of 5-chlorovanillic acid.

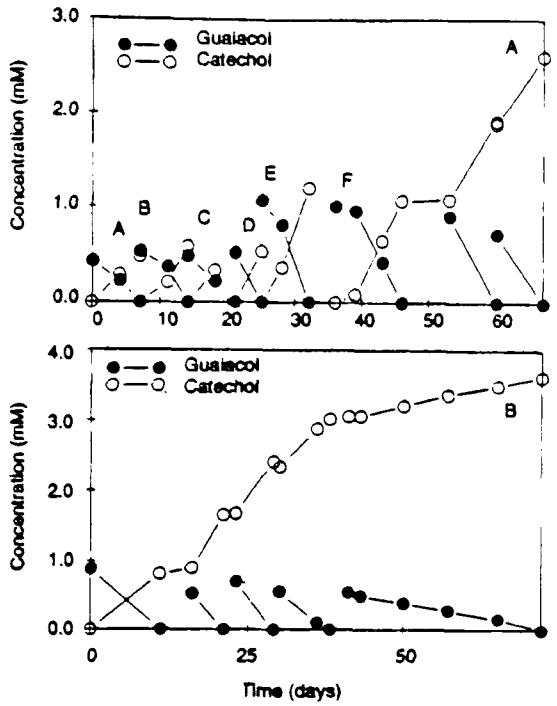

Figure 2. Enrichment for demethoxylating anaerobic bacterial species (A) and demethoxylating activity in highest active (10^{-7}) dilution of a demethoxylating (B) anaerobic bacterial consortium.

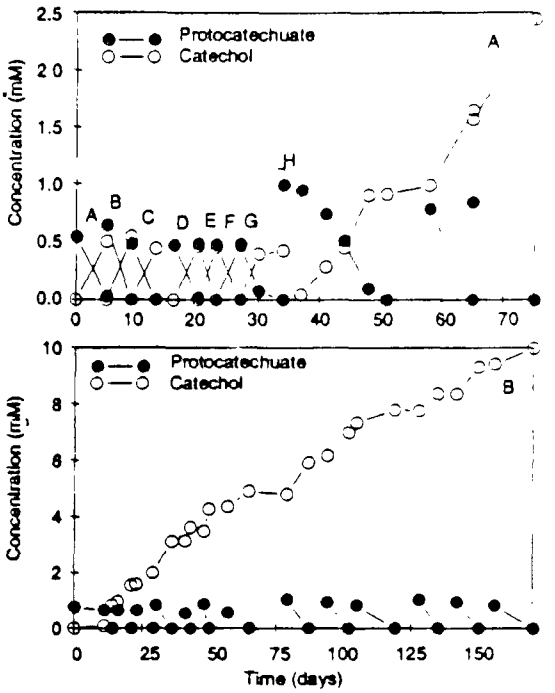

Figure 3. Enrichment for decarboxylating anaerobic bacterial species (A) and decarboxylating activity in highest (10^{-7}) active dilution (B) of decarboxylating anaerobic consortium.

transformation. Photomicrographs of these cultures show apparently pure cultures. Purity of these cultures currently is being confirmed.

The initial dechlorination of 5CV was investigated using a 3-chlorobenzoate-dechlorinating anaerobic co-culture, which dechlorinated 5CV to vanillate and then demethoxylated vanillate to protocatechuate. Protocatechuate was not further metabolized. A sulfate-reducing bacterium was isolated from this co-culture and identified as a new bacterial species, *Desulfomicrobium escambium* (1). Initial investigations with the pure culture of *D. escambium* showed a decline in the concentration of 3-chlorobenzoate (3CB) in defined pyruvate/3CB medium, which depended upon the presence of pyruvate. Because reductive dechlorination has been shown to be very specific for halogen position (2,3), and 3CB and 5CV are both *meta*-chlorinated, the basis for the decline in 3CB by *D. escambium* was further investigated.

Further studies indicated that *D. escambium* transformed not only 3CB but 3-bromobenzoate (3BB) and benzoate as well (Figure 5). Again, the decline was dependent upon the presence of pyruvate. Lactate, formate, ethanol, and hydrogen, which are used by *D. escambium* as electron donors for sulfate reduction, did not support the transformation of these three compounds. The similarity in transformation rates

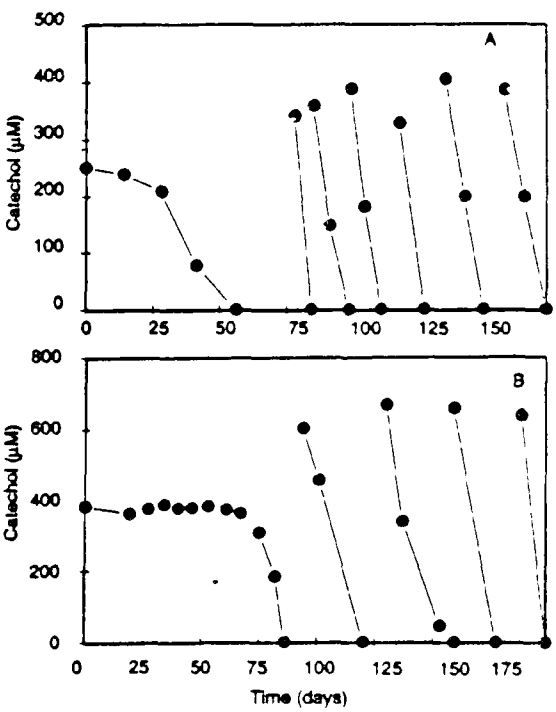

Figure 4. Enrichment for catechol-degrading anaerobic bacterial species (A) and catechol-degrading activity in highest (10^{-4}) active dilution (B) of a catechol-degrading anaerobic consortium.

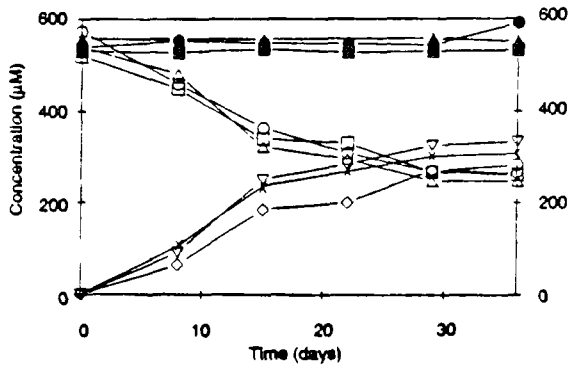

Figure 5. Reduction of 3CB (●), 3BB (▲), and benzoate (■) to 3-chloro- (▼),,3-bromo- (✖), and benzyl alcohol (◆) by *desulfomicrobium escambium* strain ESC1. Symbols: Open, 0.2-percent pyruvate; closed, minus pyruvate.

between benzoate and the two halogenated benzoates suggested that the transformation being observed was not dehalogenation. After derivitization, gas chromatography analysis revealed the presence of two unknown compounds in each culture. Further investigation using gas chromatography/mass spectrometry (GC/MS) analysis indicated that 3CB, 3BB, and benzoate were being reduced to their respective alcohols without dehalogenation (Figure 5).

During GC/MS analysis, the second unknown peak was identified as succinate. Under anaerobic conditions, succinate can result from the carboxylation of pyruvate. A followup study showed that benzoate was not reduced in medium containing a gas phase of 100-percent nitrogen. The requirement for both pyruvate and carbon dioxide indicates that the reduction of the benzoate compounds to their respective alcohols by *D. escambium* is dependent upon carboxylation of pyruvate to succinate. If sulfate is added to the pyruvate/benzoate medium, sulfate is reduced, benzoate does not decline, and pyruvate is degraded to acetate and carbon dioxide. Apparently, the reducing equivalents in this case are diverted from the reduction of benzoate to the reduction of sulfate, energetically a more favorable reduction. If reductive dechlorination competes similarly for reducing equivalents, the presence of sulfate would be unfavorable for detoxification of paper mill waste.

Because *D. escambium* reduces but does not dechlorinate 3CB in pure culture, attempts are currently under way to isolate the second member of the 3CB-dechlorinating co-culture. This bacterial species may be responsible for dechlorination of 3CB and 5CV by the co-culture or may provide a factor that enables *D. escambium* to divert reducing equivalents to the dechlorination of 3CB or 5CV.

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Characterization of a 4-Bromophenol Dehalogenating Enrichment Culture: Conversion of Pentachlorophenol to Phenol by Sediment Augmentation

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Pentachlorophenol (PCP), a carcinogen and ionophore (energy transfer inhibitor), is included on EPA's list of priority pollutants. Reductive dechlorination was found to be a significant reaction mechanism for the anaerobic degradation of PCP. The sequential removal of chlorines from PCP and its intermediate products may lead to less toxic products. In this abstract, we present data to demonstrate PCP transformation to phenol in sediment slurries inoculated with cells from a 4-bromophenol (4-BP) dehalogenating enrichment culture. We also describe partial characterization of the 4-BP-dehalogenating enrichment.

Methods

Sediment samples were collected from a freshwater pond in Cherokee Trailer Park, near Athens, Georgia. Sediment slurries were adapted to degrade 3,4-dichlorophenol (3,4-DCP) by the sequential addition of 3,4-DCP (61 µM) to the slurries immediately following the disappearance of the previous addition of 3,4-DCP (every 2 to 3 weeks). After 12 months, the 3,4-DCP-adapted sediment slurry was transferred (1:1 vol/vol) to a mineral medium containing 0.1 percent yeast extract, 0.2 mM 3,4-DCP, and 50-percent (vol/vol) site water according to Zhang and Wiegel (1). The pH of the medium was adjusted to pH 7.2 to 7.3 with HCl. Transfers were made when the 3,4-DCP was reductively dechlorinated to at least 3-chlorophenol (3-CP). The 3,4-DCP dechlorination activity also could be maintained by substituting 4-BP for 3,4-DCP. The 4-BP (0.5 mM to 0.8 mM) maintained culture was used in subsequent experiments to examine the dechlorination of PCP and its intermediate products. These experiments were performed using 1) 4-BP inoculated cultures in yeast extract-containing mineral medium; 2) washed cell suspensions prepared from cells grown with 4-BP in the mineral medium; and 3) sediment slurries amended with the 4-BP washed cell suspension.

Results and Discussion

2,3-DCP, 2,4-DCP, or 3,4-DCP, added to mineral medium and inoculated (20 percent v/v log phase culture) with cells grown on 4-BP, were dechlorinated to monochlorophenols (MCPs). Under the same conditions, PCP (18.8 µM to 37.5 µM) was not dechlorinated. 4-BP was dehalogenated to phenol in the control culture (plus 4-BP grown cells) supplemented with only 4-BP but not in the culture supplemented with both 4-BP and PCP, indicating that PCP inhibited growth and/or activity of the dehalogenating culture.

4-BP grown cells that were harvested from a late log culture, washed, and resuspended in phosphate buffer to concentrate cells 40- to 100-fold, exhibited dehalogenating activity in the presence of pyruvate. All chlorophenols tested (19 congeners), except the three MCPs, were dechlorinated at *ortho*, *meta*, or *para* positions in the presence of chloramphenicol, which inhibited any further production of dehalogenating enzymes. As examples, 2,4-DCP was dechlorinated to 2-CP and 4-CP, and 3,4-DCP was dechlorinated to 3-CP, which was not further transformed. These results are consistent with a previous observation that all six dichlorophenol isomers were dechlorinated in 3,4-DCP-adapted sediments (2).

Although PCP (300 µM) was preferentially dechlorinated at the *ortho* position by the 4-BP grown cell suspension (concentrated 40-fold), dechlorination of *meta* and *para* chlorines also was observed. 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachlorophenol (TetCP) were identified as intermediate products using a combination of high performance liquid chromatography, gas chromatography, and gas chromatography/mass spectrometry analyses. Addition of either hydrogen, formate, or ethanol did not stimulate the dechlorination activity. Heat-treated

(10 min at 90°C) or solvent-permeated (toluene-treatment) cells lost dehalogenating activity. Sulfite, thiosulfate, and sulfide inhibited the *ortho* and *para* dechlorination of 2,4-DCP. The addition of sulfate or sodium chloride had no effect.

In a 4-BP grown cell suspension assay prepared in 99.9-percent deuterium oxide, 2,3,4-trichlorophenol (2,3,4-TCP) was transformed to DCPs and MCPs containing one and two deuterium atoms, respectively. This transformation verified the identity of the proton source (water) for the dechlorination of 2,3,4-TCP and its intermediates. This phenomenon also has been observed for the reductive dechlorination of 2,5-dichlorobenzoate and 2,3,4,5,6-pentachlorobiphenyl (3,4).

PCP (28 µM) was dechlorinated to phenol (about 90-percent stoichiometric conversion) in 5 days in sterilized (autoclaved) and nonsterilized freshwater sediment slurries inoculated (equivalent to 8-percent inoculation) with a washed cell suspension prepared from a 4-BP dehalogenating enrichment culture. 2,3,4,5-TetCP, 3,4,5-TCP, 3,5-DCP, and 3-CP were detected as transient intermediates (Figure 1). In addition, small peaks with retention times similar to those found for 2,3,4,6-TetCP and 2,3,5,6-TetCP also were detected. In sterilized and in nonsterilized, noninoculated control slurries, PCP was not transformed. The PCP transformation pathway identified in this study was somewhat different than the pathway reported by Bryant et al. (2) for 3,4-DCP adapted sediment slurries (or a combination of 2,4-DCP- and 3,4-DCP-adapted sediments) prepared from the same site. 2,3,5,6-TetCP and 2,3,4,5-TetCP, either alone or together, have been detected as products of PCP transformation in samples from other ecosystems (5).

Specific experimental conditions were modified to identify factors affecting PCP transformation in nonsterilized sediment slurries inoculated with the 4-BP enrichment culture. In these studies, the PCP transformation rate was dependent on the concentration of added 4-BP grown cells, pH, and temperature. Addition of potential electron donors, including pyruvate, formate, and yeast extract, did not stimulate the transformation of PCP, suggesting that the concentration of electron donor in the sediment slurry was not a rate-limiting factor for PCP transformation. The presence or absence of 4-BP (0.15 mM) in these experiments did not significantly affect PCP transformation. The rate of PCP transformation in an estuarine sediment slurry amended with 4-BP grown cells was 25 percent of the rate observed in the freshwater sediment slurry.

In a previous study, Mikesell and Boyd (6) demonstrated that by inoculating PCP-adapted sewage sludge into soil, PCP was dechlorinated to TCPs, DCPs, and MCPs in 28 to 35 days. In our study, PCP was converted to phenol (90-percent recovery) within 5 days when a cell

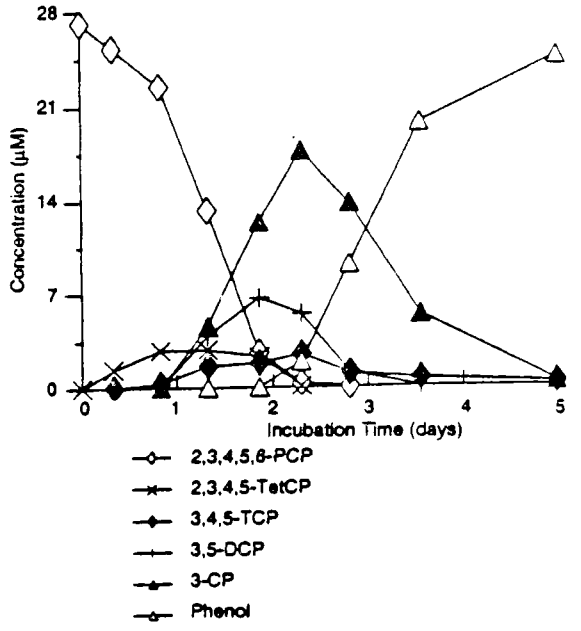

Figure 1. Dechlorination of PCP to phenol in a nonsterile and unadapted sediment slurry inoculated with cells harvested from a 4-BP dehalogenating enrichment culture.

suspension of the 4-BP dehalogenating enrichment culture was added to freshwater sediment slurries. Taken together, these results suggest that bioaugmentation (and possibly induction) of microbial populations may provide an alternative method of bioremediating PCP-contaminated soils and sediments.

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Stimulating the Microbial Dechlorination of PCBs: Overcoming Limiting Factors

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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 co-metabolized 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, how to stimulate more rapid and complete PCB dechlorination in areas where the natural rate and/or extent of dechlorination is limited is important to determine. The general approach we have taken is to identify site-specific factors limiting *in situ* PCB dechlorination, then to apply treatments to alleviate the limitation(s). During the first year of this project, our research focused on enhancing the dechlorination of PCBs in soil and in sediments from the River Raisin in Michigan.

Drag Strip Soil Experiment

Factors most likely limiting PCB dechlorination in soils are a high redox potential, lack of organic carbon availability, and absence of PCB-dechlorinating microorganisms. To determine how to alleviate limitations because of these three factors, we conducted an experiment with PCB-contaminated drag strip soil from Glens Falls, New York. Alternate means tested for achieving low redox conditions were to use a chemical reductant (Na_2S) or to provide carbon so that microbial activity would consume all oxygen present. The effectiveness of defined and complex carbon sources were compared. Methanol was chosen as a defined carbon source because it has been shown to enhance microbial dechlorination of PCBs (5). Trypticase soy broth (TSB) was used because it is a complex carbon source used for the general culture of anaerobic microorganisms. Inocula consisted of PCB-dechlorinating microorganisms eluted from upper Hudson River sediments.

Materials and Methods

The procedure followed was to first weigh 2 g of sieved soil into each anaerobic culture tube. Depending on the treatment, sterile liquid medium or inoculum (10 mL) then was added while flushing with O_2-free N_2:CO_2 (80:20). Sterile (autoclaved) nonreduced media consisted of 1) minimal salts; 2) minimal salts plus 0.1 percent methanol; or 3) minimal salts plus 0.1 percent TSB. Sterile reduced media consisted of these same three media but purged of oxygen with nitrogen and amended with Na_2S (0.24 g/L). All media were buffered at pH 7. Inocula were prepared by eluting PCB-dechlorinating microorganisms from Hudson River sediments with each of these six media. After adding the proper inoculum to each tube, the tubes were sealed with Teflon-lined rubber stoppers and aluminum crimps. Controls were autoclaved for 1 hour at 121°C. Triplicate samples were analyzed every 4 weeks for 24 weeks. The entire contents of a culture tube were extracted for each observation, and a congener-specific PCB analysis was performed by capillary gas chromatography with electron capture detection.

To determine if the time required to achieve anaerobic conditions was related to the lag time before dechlorination or to the subsequent extent of dechlorination, monitoring the redox of the cultures was necessary. The redox indicator indigo disulfonate was added to parallel treatments for this purpose, reduced to a colorless form at an Eh of -125 mV. The concentration of the oxidized form was monitored photometrically during the first month of incubation.

Results

Dechlorination occurred only in inoculated treatments that received a carbon supplement (methanol or TSB) (Figure 1). The lag time was slightly less (8 weeks) in the TSB/Na_2S treatment than in the other dechlorinating treatments (12 weeks), possibly because reduced conditions were maintained more effectively (Figure 2). By the end of 24 weeks, about 0.69 and 0.62 *meta* plus *para* chlorines (m & p Cl) per biphenyl had been removed in the methanol and TSB treatments without reductant (Na_2S). The addition of Na_2S and methanol gave more extensive dechlorination (an average loss of 0.87 percent m & p Cl after 24 weeks) than methanol alone, but Na_2S did not stimulate further dechlorination with TSB. Thus, both inoculation and a carbon supplement were necessary to initiate PCB dechlorination in this soil. Apparently, indigenous microorganisms capable of PCB dechlorination were not abundant enough to express dechlorination activity within the 24 weeks that the experiment lasted.

The extent of dechlorination achieved in the inoculated treatments was not simply related to the rate at which reducing conditions were achieved, as indicated by the reduction of indigo disulfonate (Figure 2). Whether Na_2S was used, the inoculated methanol treatments took significantly longer to reduce all of the indigo disulfonate than the TSB treatments did. Without Na_2S, the same

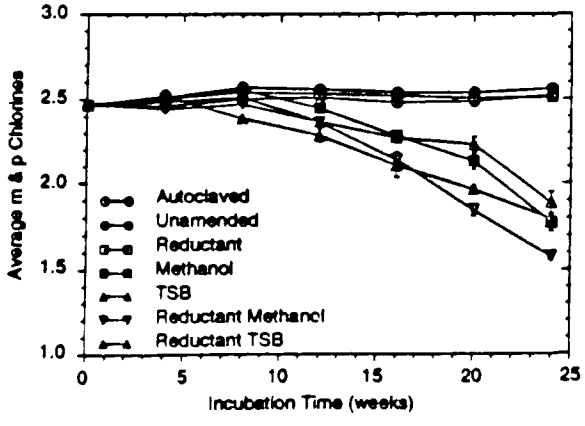

Figure 1. Dechlorination of PCBs in drag strip soil expressed as the decrease in the average number of *meta* and *para* chlorines over time.

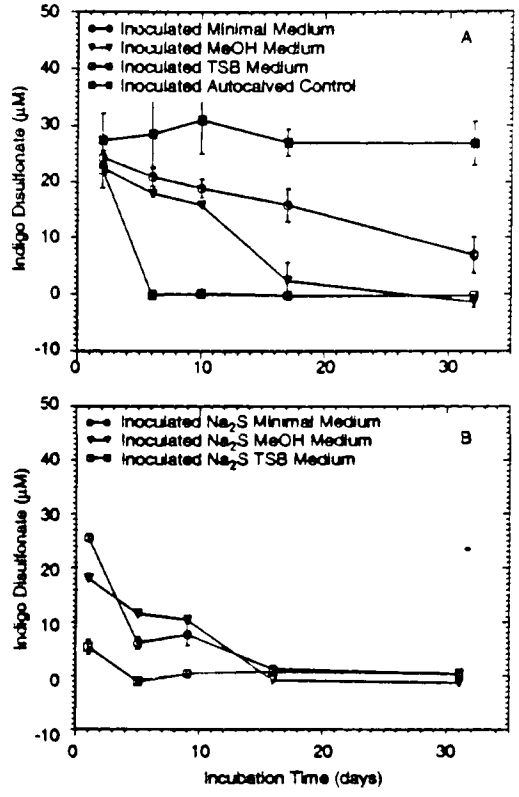

Figure 2. The redox indicator indigo disulfonate was used to follow changes in redox during the first month of incubation of the drag strip soil experiment. Reduced conditions are indicated when the concentration of the oxidized form (plotted) reaches zero. A = without chemical reductant (Na_2S), B = with chemical reductant.

extent of dechlorination was achieved with each carbon source, but with Na_2S greater dechlorination was achieved with methanol.

River Raisin Sediment Experiment

We are conducting a similar experiment to determine the minimal amount of manipulation necessary to dechlorinate PCBs in River Raisin sediments collected near Monroe, Michigan. In a previous research project, we found that little *in situ* dechlorination of PCBs had occurred in these sediments. PCB-dechlorinating microorganisms, however, exist in the sediments, the sediments support dechlorination in laboratory assays, and the PCBs are bioavailable because they were dechlorinated under 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 non-contaminated sediments. The treatability assay was conducted using air-dried River Raisin sediments. They were slurried with an equal weight of air-dried non-PCB-contaminated sediments and reduced anaerobic

mineral medium (RAMM). The slurry then was inoculated with microorganisms eluted from Hudson River sediment, and 2',3,4-trichlorobiphenyl (2-34-CB) in a small volume of acetone was added to a concentration of 500 µg/g sediment. The noncontaminated sediment was added to provide a source of undefined nutrients. The medium included essential minerals and the chemical reductant (Na_2S) to lower the initial redox potential. Inoculation ensured that PCB-dechlorinating microorganisms were present. The 2-34-CB was added because the addition of a single PCB (or polybrominated biphenyl) can somehow "prime" the dechlorination of PCBs already present in a contaminated sediment (6).

The question thus becomes: what aspects of our treatability assay are necessary to dechlorinate the PCBs present in the River Raisin sediments? We are conducting separate experiments with wet and air-dried River Raisin sediments to answer this question. With the air-dried sediments, the factors being considered are 1) addition of 2-34-CB; 2) addition of the mineral salts in RAMM; 3) addition of Na_2S; and 4) addition of the non-PCB-contaminated sediments. All treatments with the air-dried sediments were inoculated with microorganisms eluted from Hudson River sediments. These same four factors also are being addressed in the experiment with wet (i.e., never air-dried) River Raisin sediments. In this case, the necessity of inoculating with Hudson River microorganisms also is being tested. These experiments are still in progress, and data are not yet available.

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Potential Surfactant Effects on the Microbial Degradation of Organic Contaminants

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The biodegradation of poorly water soluble compounds in soil or sediment systems is believed to be limited by low bioavailability because of strong sorption of the compounds to natural organic matter (1-4). The use of surfactants to increase aqueous concentrations of these types of compounds, and therefore their bioavailability, often has been suggested as a way of overcoming this problem (5,6). Significant solubilization of the target compounds, however, usually occurs only above the critical micelle concentration (CMC) of the surfactant, a concentration often toxic or inhibitory to bacteria (7).

Petroleum sulfonate oil (PSO) surfactants are different from conventional surfactants in that they form stable microemulsions in water rather than micelles, thereby enhancing solubilization at low concentrations without apparent toxic effects to bacteria (5,8). We recently reported a 60-fold decrease in the apparent soil sorption coefficient (K^*) of 2,2',4,4',5,5'-hexachlorobiphenyl at a PSO aqueous concentration of only 30 ppm, and a 200-fold decrease in K^* at a 170 ppm PSO (4). We, therefore, propose to investigate the use of this class of surfactants in enhancing the anaerobic microbial dechlorination of polychlorinated biphenyls (PCBs).

Although conventional surfactants are ineffective at enhancing HCH solubility at concentrations below the CMC, evidence exists for stimulatory effects on biodegradation of aromatic hydrocarbons in soils even when surfactant-induced disassociation from soil was not significant, i.e., at concentrations below the CMC (9). For example, mineralization of phenanthrene was substantially enhanced in a muck soil in the presence of 10 µg of nonionic surfactant per gram of soil (10 ppm). Similar effects on biphenyl mineralization were not observed, and surfactant concentrations of 100 ppm were either less stimulatory or inhibited mineralization.

A few reports indicate that sub-CMC concentrations of surfactants may enhance anaerobic dechlorination of aromatic compounds. Dechlorination of pentachlorobenzene in sediment slurries was stimulated by Tween 80 concentrations of 0.06 µg/mL to 100 µg/mL and SDS concentrations of 0.3 µg/mL to 40 µg/mL (10), while Tween 80 at concentrations below the CMC slightly enhanced the dechlorination of hexachlorobenzene (11). Triton X-705 at 600 ppm decreased the lag time before PCB dechlorination took place in Hudson River sediment slurries but did not affect the subsequent rate (12). Concentrations of other surfactants tested (sodium dodecyl benzene sulfonate, Triton X-100, and X-045) were all at or above their CMCs and inhibited dechlorination. Because these secondary stimulatory effects can occur at surfactant concentrations below the CMC, they do not appear to be related to contaminant solubility enhancement. We are attempting to establish the stimulatory effects on PCB dechlorination of surfactant concentrations below the CMC for major types of nonionic, anionic, and PSO surfactants (Table 1) and to attribute these effects to either solubility enhancement or secondary mechanisms. The physiological or physical nature of such secondary mechanisms is being investigated.

Results

The surfactants used in this study are listed in Table 1. These include several nonionic surfactants that were selected to provide a range of CMC values, and because previous studies have shown that they provide beneficial effects on biodegradation as described above. We also have included a twin-head anionic surfactant to minimize surfactant sorption to soils.

One of the major objectives of this research is to evaluate the effectiveness of sub-CMC concentrations of

Table 1. Surfactants Proposed for This Study

Surfactant	CMC (mg/L)
Triton X-100	130 (7)
Triton X-405	620 (7)
Triton X-705	625
Tween 80	13 (13)
Alfonic 810-60	275 (9)
C_{16}DPDS (Dowfax 8390) (14)	4,000
Petroleum sulfonate oil	NA

NA = not applicable. These products form stable microemulsions in water and do not exhibit a CMC. They consist of petroleum sulfonate (61 to 63 percent) and mineral oil (33 percent).

surfactants in increasing the rate and extent of PCB dechlorination. To determine what the exact aqueous phase concentration in soil- or sediment-water slurries is and whether this concentration is above or below the CMC, we need to measure surfactant sorption (i.e., obtain sorption isotherms) by the soils and sediments. To accomplish this, we will use a batch equilibration technique, where the amount sorbed is determined from the difference between the initial (added) and final (after sorption) aqueous phase surfactant concentrations. The following three methods for measuring aqueous phase surfactant concentrations have been evaluated: 1) tensiometer; 2) UV-absorption; and 3) total organic carbon. Sorption isotherms developed using Method 1 indicated higher surfactant uptake by sediment then those obtained using Methods 2 and 3. We suspect that the presence of dissolved or suspended organic matter from the sediment may be influencing the surface tension measurement, and hence we have elected not to use this method. Methods 2 and 3 resulted in essentially identical sorption isotherms for Triton X-100 by Hudson River sediments. Method 3 is universally applicable to all the surfactants listed in Table 1, whereas Method 2 is only applicable to surfactants with the appropriate UV absorption properties. Hence, Method 3 is currently being used to obtain sorption isotherms for all the surfactants listed in Table 1. This information will quantitate the aqueous phase surfactant concentrations in our sediment slurries and determine whether these are above or below the CMC.

To separate solubility enhancement effects of surfactants (which could increase bioavailability and hence biodegradation rates) from the secondary effects of surfactants on biodegradation rates, we are evaluating the sorption of PCBs in sediment-water-surfactant systems above and below the CMC. We have now observed the effect of Triton X-100 on the sorption of 2,2',4,4',5,5'-PCB by soil by measuring the apparent sorption coefficient K^* at different aqueous surfactant concentrations (C_{tx}). At C_{tx} values below 200 ppm (approximately the CMC of Triton X-100), K^* values *increased* from ~ 500 to 1,200 with increasing surfactant concentration. In this concentration range, the added surfactant is strongly sorbed by soil, and the soil-bound surfactant in turn enhances PCB sorption. At higher C_{tx}s (above the CMC), K^* decreases rapidly and substantially because of the formation of surfactant micelles in solution that effectively dissolve PCBs and raise the apparent aqueous phase PCB concentration. These preliminary results strongly suggest that the enhanced contaminant biodegradation rates observed previously at low (below the CMC) surfactant concentrations are not due to increased bioavailability associated with solubility enhancement effects. Thus, other indirect or secondary effects may be responsible for the stimulating biodegradation rates at surfactant levels below the CMC. These mechanisms will be investigated in the future.

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Enhanced Dechlorination of PCBs in Contaminated Sediments by Addition of Single Congeners of Chloro- and Bromobiphenyls

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Bioremediation has been suggested as a technology that may be useful in decreasing the level of pollutants at contaminated sites. For polychlorinated biphenyl (PCB) contaminated sediments, reductive dechlorination reactions (anaerobic) preferentially transform the more highly chlorinated PCB congeners to less chlorinated derivatives, which are more amenable to aerobic degradation. In this instance, the anaerobic and subsequent aerobic processes are complementary and result in a reduction of toxic (higher chlorinated, coplanar) PCB congeners and possibly the biological destruction of PCBs through subsequent aerobic oxidations. Before using this method at a remediation site, the ability of indigenous microorganisms from the site to transform the pollutants must be assessed, to understand factors that control the dechlorination reactions, and to develop techniques to enhance microbial activities.

PCB transformation in anaerobic environments, such as sediments of lakes and rivers, could be inferred in the mid-1980s from the studies of Brown and coworkers (1). These investigators noted that historically contaminated sediments from the Hudson River exhibited an altered PCB congener profile compared with the congener profile of the original contaminating Aroclor. The alterations were characterized by a reduction in the concentration of the more highly chlorinated PCB congeners, with selective or preferential removal of *meta* and *para* chlorines, and an increase in the concentration of the more lightly chlorinated and *ortho*-substituted congeners. Thus, dechlorination of the more highly chlorinated PCB congeners was proposed to be catalyzed by anaerobic microorganisms residing in the contaminated sediment. The biologically mediated reductive dechlorination of PCBs from contaminated sediments was subsequently demonstrated in several laboratory investigations (2-4). In some studies, the microbial inoculum was obtained by "washing" PCB-contaminated sediments with anaerobic medium and collecting the supernatant (4,5). Recently, reductive dechlorination of PCBs was suggested to be enhanced when PCB-contaminated sediments are amended with PCB mixtures (Aroclors) or specific PCB/polybrominated biphenyl (PBB) congeners (6).

To date, only a limited number of studies have attempted to understand the factors that affect the reductive dechlorination of PCBs in historically contaminated sediments. Abramowicz et al. (7) reported that addition of inorganic nutrients enhanced reductive dechlorination of endogenous PCBs in laboratory incubations of Hudson River sediments. In a recent study using methanogenic sediment slurries contaminated with Aroclor 1260, Bedard and Van Dort (2) reported that addition of bromobiphenyl congeners stimulated the reductive dechlorination of endogenous (historical) PCBs. In an earlier study, Bedard and coworkers (8) reported that amendment of Woods Pond sediment with a high concentration (approximately 1 mM) of either 2,3',4',5-CB or 2,3,4,5,6-CB stimulated reductive dechlorination of endogenous PCBs and that transformation of congeners with *para* chlorines was especially evident.

The primary objectives of this study were to determine the reductive dechlorination potential of PCB-contaminated sediments from the Sheboygan and Ashtabula Rivers and to further test the hypothesis that addition of PCB and PBB congeners enhances the reductive dechlorination of endogenous (historical) PCBs by indigenous microbial populations.

Materials and Methods

PCB-contaminated sediments were collected from the Sheboygan River, near Sheboygan Falls, Wisconsin,

and from the Ashtabula River, near Ashtabula, Ohio. Grab samples of sediments were collected from all sites. Initial gas chromatography data indicated that a significant shift in the PCB congener profile had occurred since the time of PCB deposition, suggesting previous reductive dechlorination activity.

Biotransformation experiments were prepared by combining one volume of PCB-contaminated sediment with one volume of anoxic (N_2 sparged) site water and the mixture was stirred for approximately 5 min. Aliquots of the sediment slurry (equivalent to 5 g dry sediment) were dispensed into amber serum vials, and the sediment slurry was amended with various chlorobiphenyl or bromobiphenyl congeners dissolved in acetone. Initial experiments were conducted with PCB-contaminated Sheboygan River sediment (approximately 180 ppm total PCBs) and Ashtabula River sediment (approximately 100 ppm total PCBs) and were amended with penta-, hexa-, hepta-, or octa-chlorobiphenyl congeners. Additional experiments were performed with more heavily contaminated Sheboygan River sediment (approximately 1,000 ppm total PCBs) and were amended with either a di- or tetra-chlorobiphenyl congener or the corresponding di- or tetra-bromobiphenyl congener (final concentration of 1 mM). Autoclaved controls and nonautoclaved, unamended controls also were included in the study. Triplicate samples were analyzed at 4- to 8-week intervals for congener-specific PCBs using capillary gas chromatography and electron capture detection.

Results

Enhanced Dechlorination Using Specific PCB Congeners

Initial dechlorination experiments were conducted with PCB-contaminated (~180 ppm) Sheboygan River sediments amended with 20 ppm to 80 ppm of 2,2',3,3',4,5,5,6'-octachlorobiphenyl (octa-CB). The most prominent PCB homologues detected in the contaminated Sheboygan River sediments were trichlorobiphenyls and tetrachlorobiphenyls. The percentages of octa-CB remaining in the samples after anaerobic incubation for 8 months were 35 percent, 20 percent, and 10 percent, respectively, for sediments amended with 20 µg/g, 40 µg/g, and 80 µg/g. In all sediment experiments amended with octa-CB, there was a decrease in the concentration of hepta-, hexa-, penta-, tetra- and tri-CB congeners occurred as well as an increase in the concentration of di- and mono-CB congeners. The mole percentage of mono-CBs was less than 1 percent at the onset of the experiment (Figure 1A, Week 1); after

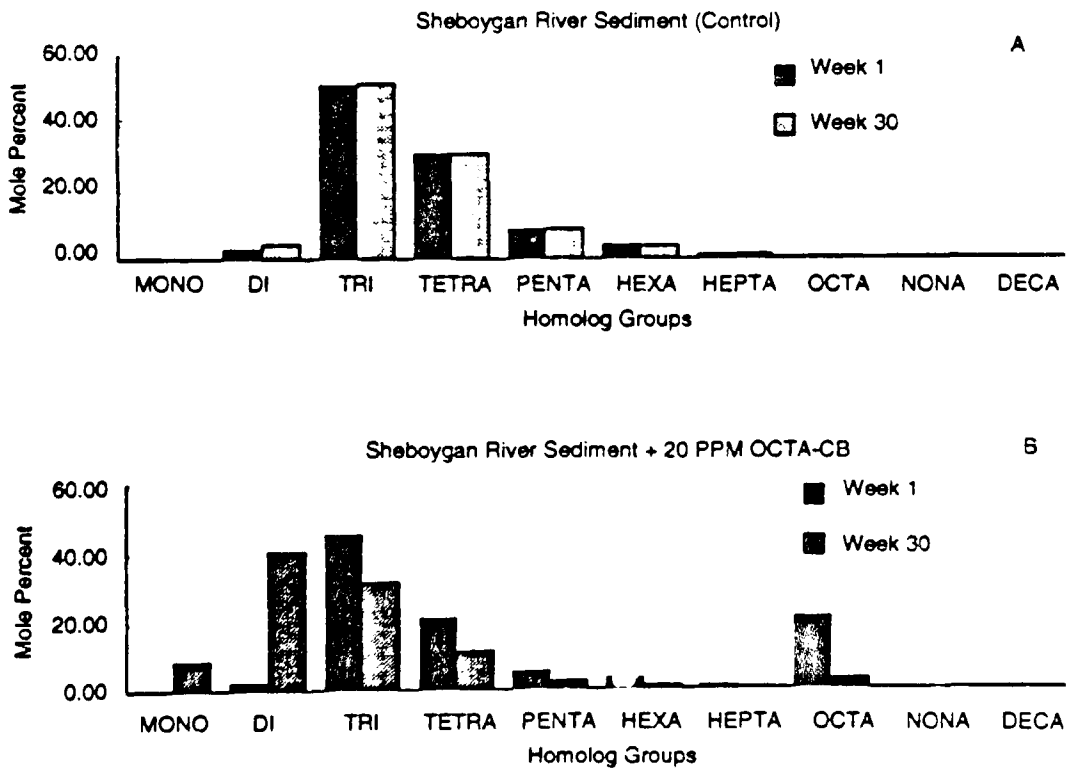

Figure 1. Profile of amended and endogenous PCB biotransformation in (A) unamended control sediments and (B) 20 ppm 2,2',3,3',4,5,6,6'-octachlorobiphenyl (octa-CB) amended sediments.

anaerobic incubation for 30 weeks, this homologue group accounted for approximately 8 percent of the total PCB congeners in sediments amended with 20 mg/g of octa-CB (Figure 1B). The major products of reductive dechlorination were di-CB congeners; this homologue group increased from 2.5 to 40 mole percent after 30 weeks of incubation. The most prominent di-CB peak detected in octa-CB amended sediments consisted of two *ortho*-substituted congeners (2,2'-CB and 2,6-CB). Two additional homologue groups, tri- and tetra-CBs, initially accounted for approximately 80 percent of the total PCB homologues in the contaminated Sheboygan sediments but were reduced to less than 50 percent of the total following 30 weeks' incubation in octa-CB amended experiments. The average number of chlorines per biphenyl (total of endogenous plus amended PCBs) decreased from 4.2 to 2.8 (±0.1), 2.5 (±0.3), and 2.2 (±0.3), respectively, in experiments amended with 20 µg/g, 40 µg/g, and 80 µg/g of octa-CB.

PCB-contaminated Ashtabula River sediments were spiked with 2,3,3',4,4'-pentachlorobiphenyl (penta-CB), 2,3,3',4,4',5-hexachlorobiphenyl (hexa-CB), or 2,2',3,4,5,6,6'-heptachlorobiphenyl (hepta-CB) or combinations thereof and incubated anaerobically. Dechlorination of the added congeners was observed after lag periods of 5, 4, and 3 months for experiments amended with either the penta-CB, hepta-CB, or hexa-CB, respectively. Addition of the chlorobiphenyl congeners singly or as mixtures resulted in enhanced reductive dechlorination of endogenous PCB congeners in a manner similar to that observed for Sheboygan River sediment amended with octa-CB. Appreciable decreases in the mole percentages of endogenous PCB homologue groups (tetra-CB and penta-CB) were coupled with increases in the mole percentages of mono-, di-, and tri-CB congeners. The average number of chlorines per biphenyl decreased from approximately 5.2 to 2.7 in Ashtabula River sediments amended with any of the three congeners tested. No significant changes in the distribution of the PCB homologue groups were noted in control experiments.

Dechlorination in the Presence of PBB/PCB Congeners

Recently, experiments have been initiated to test the hypothesis that amendment of PBB congeners enhances the dechlorination of PCBs in contaminated sediments. Highly contaminated (1,100 ppm PCBs) sediments from the Sheboygan River were amended with dibromo- or dichlorobiphenyl congeners, or with tetrachloro- or tetrabromobiphenyl congeners, and dehalogenation was followed over the course of 6 months incubation. After 6 months of incubation, no enhancement of dechlorination of endogenous PCBs has been detected in sediments amended with 2,2',4,5'-tetrabromobiphenyl or 2,2',4,5'-tetrachlorobiphenyl compared with controls. Both *meta* and *para* debromination of the added 2,2',4,5'-PBB congener, however, was evident after 1 month of incubation, with 2,2'-dibromobiphenyl observed as the major product. Approximately 25 percent of the parent 2,2',4,5'-PBB remained after 6 months' incubation. Dehalogenation of the amended 2,2',4,5'-PCB congener was more rapid than debromination of the corresponding PBB congener; more than 70 percent of the 2,2',4,5'-PCB was transformed to 2,2',4-PCB after 1 month's incubation. As with the added PBB congener, however, enhanced dehalogenation of the endogenous PCBs was not evident.

In a separate set of experiments, 2,4-, 2,5-, or 2,6-dibromobiphenyl or dichlorobiphenyl congeners were added to PCB-contaminated Sheboygan River sediments. Greater than 85 percent of the amended 2,4- and 2,5-dibromobiphenyl were debrominated at the *para* and *meta* positions, respectively, within the initial 3 months of incubation. No evidence of debromination of the amended 2,6-dibromobiphenyl was noted. Further, addition of the dibromobiphenyl congeners has not yet had an effect on the extent of dechlorination of the endogenous PCBs compared with controls. Of the dichlorobiphenyls examined, significant loss (40 percent) of only 2,5-dichlorobiphenyl has been observed. Dechlorination at the *meta* chlorine was accompanied by an increase in 2-chlorobiphenyl. Although results are only preliminary, a moderate reduction in the average number of *meta* plus *para* chlorines for endogenous PCBs appears to be in this data set.

The results from the present study demonstrate the dechlorination capacity of PCB-contaminated Sheboygan River and Ashtabula River sediments. No appreciable dechlorination of endogenous PCBs was observed in unamended sediment slurries. Several explanations are proposed for the stimulation of reductive dechlorination of endogenous PCBs in sediments by addition of specific PCB congeners: 1) the bioavailability of PCBs was enhanced, thus providing an available electron acceptor for oxidation reactions; 2) the growth of indigenous PCB dechlorinating microorganisms was stimulated; or 3) amended PCB congeners induced dechlorinating activity of indigenous microbial populations. Additional strategies should be considered for PCB bioremediation and may include increasing the physical-chemical availability of PCBs bound to sediments (for example, the addition of surfactants) or cycling between anaerobic and aerobic conditions.

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Effect of Heavy Metal Availability and Toxicity on Anaerobic Transformations of Aromatic Hydrocarbons

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The existence of co-contaminants (e.g., heavy metals and toxic organics) in impacted sediments has created concern over the potential for biodegradation to assist in remediating these sites. Heavy metals can be inhibitory to microorganisms and microbial processes, including the decomposition of organic matter and other biogeochemical processes (1). The characteristics of this inhibition for biodegradation of toxic organics are poorly understood because of the large number of variables involved. This study was initiated to determine the effect of heavy metals on reductive dechlorination of chlorinated aromatic organics. Experiments are being conducted with two metals, cadmium (a single valence [+2] transition metal) and chromium (a multivalence [+6 and +3] transition metal), and two chlorinated aromatics, hexachlorobenzene (HCB) and 2,3,4-trichloroaniline (2,3,4-TCA). The reductive dechlorination of these compounds has been demonstrated, and the degradation pathways are generally understood (2,3).

The interactions between metals and organic-degrading microbes or consortia are complex because the observed effects are largely a function of the bioavailability of both the metals and the organic compound. Studies have been conducted on aerobic biodegradation processes (4,5), but inhibition of anaerobic biodegradation is not understood. At present, the best information indicates that the soluble fraction of the co-contaminants is the "available" fraction to the microorganisms (6). Under anaerobic conditions, metals may be precipitated as sulfides or present as reduced forms of lower toxicity. Solubility and speciation of metals is strongly dependent on the redox potential and pH of the sediment. An excellent example is the solubility of chromium, which exists in two valence states with large differences in solubility—Cr(VI) and Cr(III)—depending on the redox potential of the sediment (7). Other metals with single valence states (e.g., Cd^{2+}, Zn^{2+}) adsorb onto redox-sensitive surfaces (e.g., iron and manganese oxides) and form various complexes under different redox conditions.

Results and Discussion

Experiments are being conducted to determine the effect of cadmium on reductive dechlorination of 2,3,4-TCA in previously uncontaminated anaerobic freshwater sediment environments, including a rice paddy soil, a cypress swamp soil, a bottomland hardwood soil, and a freshwater marsh soil. These soils differ widely in sediment properties, including the organic matter concentration, which ranges from 2.9 percent in the rice paddy soil to 74 percent in the freshwater marsh. 2,3,4-TCA is a particularly useful model compound because chlorine substituents are present at *ortho*, *meta*, and *para* chlorine positions. Representative results from several soils are discussed here. Microcosms, with continuous monitoring of the Eh and pH, were constructed using sediment slurries under anaerobic conditions. Sediments were amended with 2,3,4-TCA (200 mg/kg soil) and varying concentrations of Cd^{2+} (control, 10 mg/kg soil, 100 mg/kg soil, and 1,000 mg/kg soil). Periodically, subsamples of microcosms were removed for quantification of metals and 2,3,4-TCA. Gas chromatography/mass spectrometry was used to identify lower chlorinated aniline metabolites.

Degradation of 2,3,4-TCA in rice paddy soil is presented in Figure 1. Data are from representative replicates. When no Cd was added, dechlorination proceeded rapidly by removal of the *ortho* chlorine to form 3,4-dichloroaniline (3,4-DCA). 3,4-DCA appeared only transiently and was rapidly dechlorinated to 3-chloroaniline (3-CA). No further dechlorination was observed. When 10 mg/kg Cd was added, dechlorination also proceeded rapidly but by the removal of the *para* chlorine to form 2,3-DCA. Two monochloroanilines (2-CA and 3-CA) were subsequently formed in nearly equal amounts. When cadmium was added at higher concentrations (100 mg/kg and 1,000 mg/kg), no dechlorination was observed. Daily mass balance of chloroanilines for the microcosms in Figure 1 averaged 103 percent ± 33 percent.

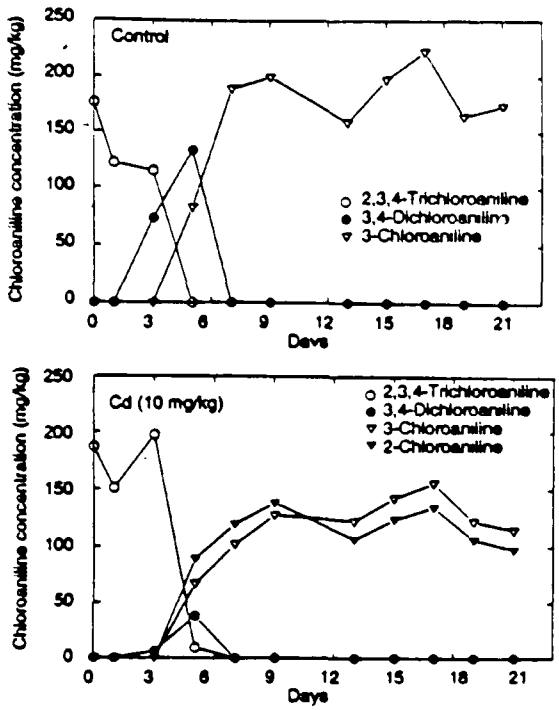

Figure 1. Dechlorination of 2,3,4-trichloroaniline in a control (no cadmium added) and cadmium amended (10 mg/kg soil) microcosm constructed from a rice paddy soil (Crowley silt loam). Soluble cadmium was < 20 µg/L for the control and 0.19 mg/L for the cadmium-amended microcosm. Soil Eh ranged from -200 to -250 mV.

This general trend also has been observed in the cypress swamp soil and freshwater marsh soil, despite wide differences in the degree of sorption of metals and organics in these soils. Studies are ongoing in the fourth soil (bottomland hardwood soil). The observed pattern is *ortho* dechlorination when no cadmium is added, *para* dechlorination when a critical level of cadmium is reached, and complete inhibition at another critical level of cadmium. The trend is poorly predicted by the *total* concentration of cadmium but appears to be well predicted by "soluble" cadmium (measured as porewater cadmium passing through a 0.45-mm filter). Of the three soils examined, *ortho* dechlorination occurred when soluble cadmium concentrations ranged from less than 20 mg/L to 32 mg/L. *Para* dechlorination occurred when soluble cadmium concentrations ranged from 0.15 mg/L to 0.2 mg/L. Complete inhibition occurred when soluble cadmium concentrations ranged from 0.2 mg/L to 7.4 mg/L. Further experimental replication may refine these ranges more accurately. These results are surprising in light of differences in pore water chemical composition between these flooded soils. MINTEQ, a geochemical speciation model, is being used to estimate concentrations of cadmium complexes, which may shed further light on these results.

Preliminary batch studies also have been performed to determine the effect of Cr(VI) on 2,3,4-TCA dechlorination in the bottomland hardwood soil. Results indicate that Cr(VI) additions affect the dechlorination of 2,3,4-TCA by increasing the lag time necessary for degradation to occur (Figure 2). Addition of Cr(VI) at 20 M, 50 M, 75 M, and 175 M all increased the lag time for dechlorination from approximately 2 to 10 weeks. Following the lag time, apparent rates of dechlorination of 2,3,4-TCA were unaffected by the initial chromium addition.

Biogeochemistry of chromium in the bottomland hardwood soil has been previously investigated (7). Addition of Cr(VI) under low Eh conditions is followed by rapid (1 min) reduction to Cr(III), followed by precipitation/sorption of Cr(III) from the soil solution. A critical Eh for the reduction process has been identified, +300 mV, below which the reaction proceeds rapidly. In the batch study (Eh = -200 mV), Cr(VI) was undetectable in solution (detection limit 5 ppb) immediately following addition, and only low concentrations of Cr(III) (50 ppb) were detected. Methanogenesis, as indicated by the accumulation of CH_4 in the vial headspace, was unaffected by additions of Cr(VI). The mechanism by which chromium inhibits dechlorination is unclear, although results suggest an initial toxic effect on the degrading population that requires time to overcome (lengthening lag time). This effect could be direct (mortality of some microbial population) or indirect (oxidation of some key reductant crucial to dechlorination).

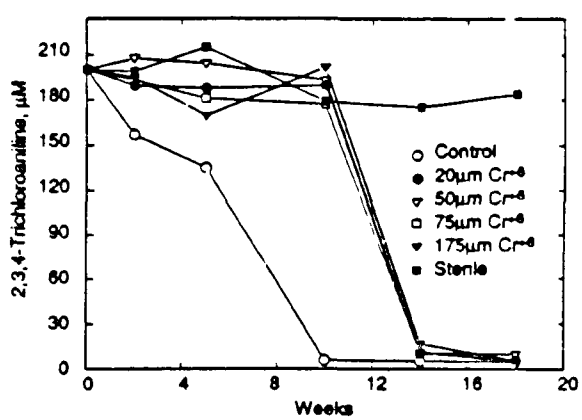

Figure 2. Effect of Cr(VI) on dechlorination of 2,3,4-trichloroaniline in flooded bottomland hardwood soil. Points are means of triplicate determinations. Coefficient of variation are < 20 percent with the exception of measurements at 2 weeks and 5 weeks for control samples (37 percent and 97 percent, respectively).

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Biodegradation of Petroleum Hydrocarbons in Wetlands Microcosms

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In the aftermath of several major environmental oil spills, it became apparent that spill preparedness did not include an up-to-date inventory of bioremediation strategies or adequate methods for assessing the efficacy of bioremediation under field conditions. Thus, field trials of bioremediation (1) preceded rigorous laboratory- and pilot-scale experimentation. A cooperative effort to develop protocols for evaluating bioremediation strategies has led to the adoption of a system of tiered assays for determining efficacy and environmental toxicity of products that might be applied to spilled oil. Protocols include 1) analytical methods for determining the extent of biodegradation; 2) toxicity assays for aquatic and sediment organisms; 3) flask experiments to determine potential for biodegradation; 4) and laboratory-scale microcosms for assessing the potential for degradation in prototype environments, including open water, beaches, and wetlands.

Development and Testing of Microcosm Protocols

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

Results

Sediment microcosms were constructed from homogenized marsh sediments from Sapelo Island, Georgia, and were flushed on a tidal basis with seawater adjusted to the salinity of the collection site (20‰). The tidal cycle was continued until a clear boundary distinguished the aerobic and anaerobic layers (3 mm to 5 mm) of the microcosm. Then, oil (521 fraction of Alaska North Slope crude, 0.5 mm depth/3.93 mL) was applied to the sediment surface. The numbers of hydrocarbon-degrading bacteria in the sediment prior to construction of the microcosms was in the range 10^3 cells/g to 10^4 cells/g, which is consistent with nonpristine coastal areas (2). Products to be tested were applied 1 day after the application of oil. The types of products submitted for testing in protocol development included microbial cultures, nutrients, surfactants, sorbents, and combinations thereof.

Figure 1 shows the composition of the oil, as determined by gas chromatography/mass spectrometry, after a 6-week incubation. The alkane constituents of the oil (Figure 1A) were appreciably degraded in all treatments relative to the original composition of the oil. The degradation in the nutrient treatment (Product D) was slightly greater than in the nonfertilized control. The addition of nutrients plus microbial inoculum (Product J) resulted in significant degradation of the full range of alkanes (C13 to C35); that degradation was primarily biological is indicated by the reduced ratios of C17:pristane and C18:phytane. Nevertheless, pristane and phytane were reduced in concentration, indicating that they also are subject to biodegradation, although at a slower rate than the normal alkanes. Thus, oil constituents that are more resistant to biological degradation than are pristane and phytane are more suitable for use as internal indices in longer incubation experiments; both hopanes (3) and C2-chrysenes have been proposed for this application.

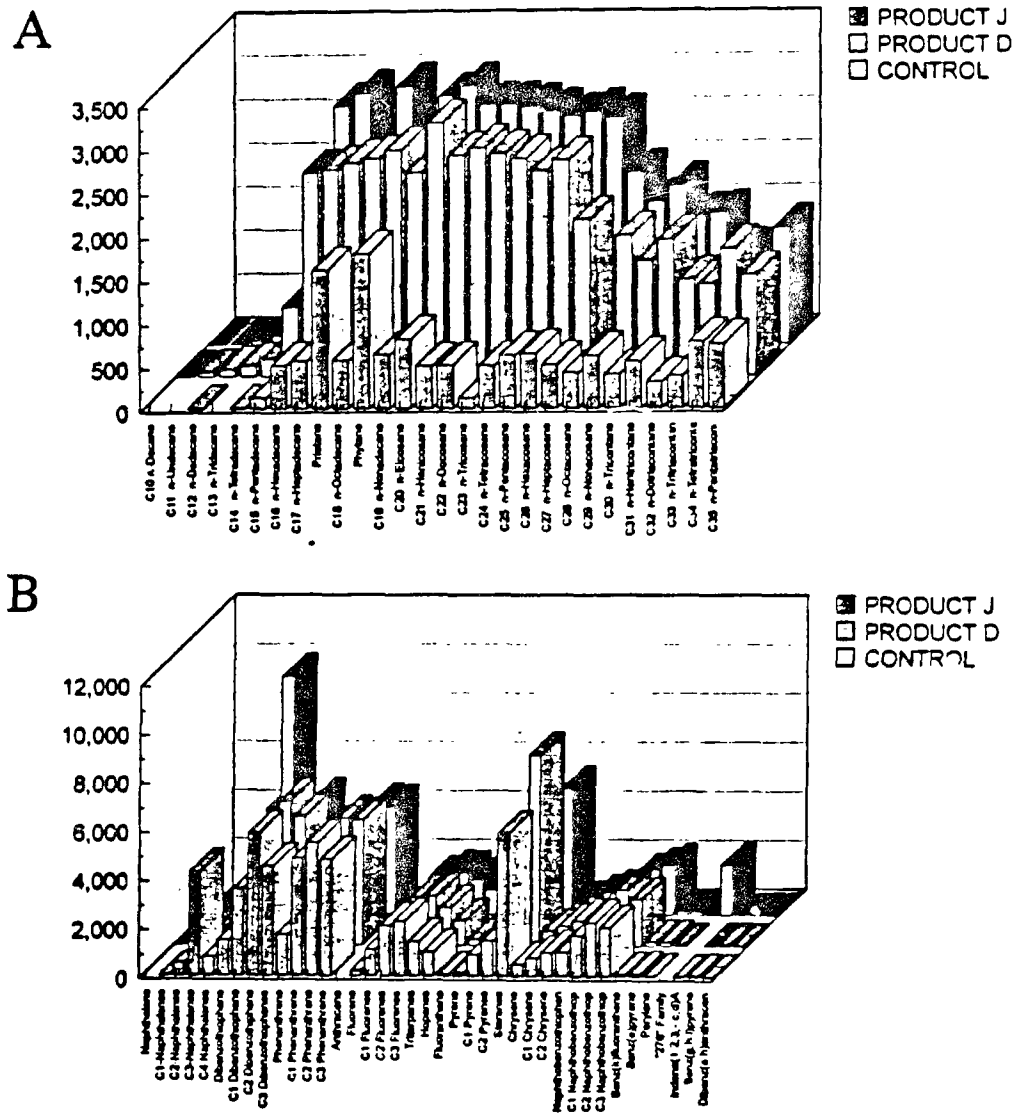

Figure 1. Abundance of selected aliphatic (A) and aromatic (B) constituents of Alaska North Slope crude oil after treatment for 30 days with a nutrient (Product D) and a microbial (Product J) bioremediation product in wetlands microcosms. Peak areas for individual hydrocarbons are referenced to hopane, an internal marker.

Degradation of the aromatic constituents of oil was negligible; only the naphthalene series differed in concentration between treated microcosms and controls after 6 weeks' incubation. The lack of degradation of aromatics in the continued presence of alkane constituents suggests that degradation of the two classes of compounds may be sequential, although Foght et al. (4) concluded that degradation of aliphatics and aromatics could occur concurrently if adapted organisms are present. To test whether alkane degradation goes to completion before the onset of degradation of aromatics, the length of the microcosm incubation period in subsequent experiments was increased from 6 weeks to 3 months.

Factors Influencing the Persistence of PAHs in Sediments

In light of the relative degradability of the alkane constituents of petroleum and the toxicity and carcinogenicity associated with the more recalcitrant polycyclic aromatic hydrocarbons (PAHs), the effectiveness of a remediation effort in reducing ecological risk depends largely on the degree to which the latter are degraded. Moreover, PAHs of industrial origin are of environmental concern as soil and sediment contaminants in their own right. Thus, the persistence of PAHs in the microcosms can be considered a shortcoming of bioremediation measures.

Several explanations have been proposed to explain the persistence of PAHs in the environment. Intrinsic controls on the rate of degradation include low solubility, toxicity, and interactions between PAH compound classes; extrinsic controls include environmental factors such as salinity, temperature, nutrient concentrations, and interactions between PAHs and other classes of compounds, including natural organic matter. Interactions between PAHs and other compounds may include co-metabolism, the competitive utilization of alternative substrates, or the absence of required inducer compounds.

Bauer and Capone (5) noted that preexposure of marine sediments to single PAHs enhanced subsequent degradation of those compounds and that cross acclimation occurred between select PAHs. Similarly, Kelley and Cerniglia (6) reported an interaction between fluoranthene and pyrene and concluded that the catabolism of fluoranthene, pyrene, and phenanthrene was catalyzed by a common enzyme system. Other researchers (7) observed that a mixed microbial community was required for the complete utilization of some PAHs.

Results

We tested the interactions between PAHs of different size classes to determine if interactions between PAHs were responsible for the persistence of those compounds in sediments. The presence of other PAHs, either grouped by size classes or as a mixture of 16 compounds, did not affect the mineralization of pyrene by an acclimated microbial culture introduced into sediment slurries with inorganic nutrients (Figure 2A). The same culture degraded pyrene more slowly when four-, five- and six-ring PAHs were present in mineral medium enriched with sediment organic extract (Figure 2B), and did not degrade pyrene at all when five- and six-ring PAHs were present in mineral medium (Figure 2C). We concluded that large PAHs are inhibitory to the activity of organisms capable of degrading pyrene, but that the inhibition is removed when the high molecular-weight compounds are sorbed to sediments or complexed with organic matter. Toxicity because of large PAHs, therefore, probably did not explain the persistence of PAHs in the microcosm trials.

Sediments that were inoculated with a culture that had not been recently exposed to PAHs adapted to degrade pyrene after a lag of 1 day, unless protein synthesis was inhibited with chloramphenicol (Figure 3). When the culture was preexposed to pyrene, the addition of chloramphenicol did not appreciably inhibit degradation upon subsequent exposure. Similarly, the antibiotic did not inhibit degradation of pyrene by a culture preexposed to phenanthrene, although protein synthesis was necessary for pyrene degradation by cultures preexposed to naphthalene. Therefore, we concluded that the cells

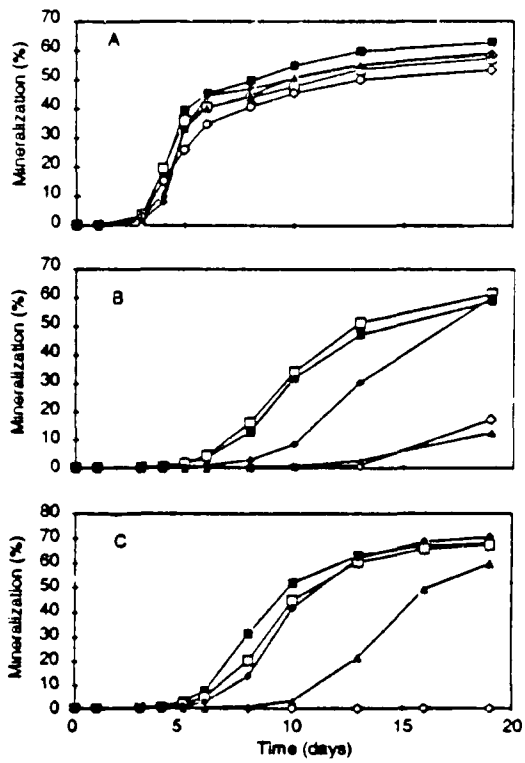

Figure 2. Mineralization of pyrene (6 µg/mL) by an enrichment culture in the absence of other PAHs (■) and in the presence of two- and three-ring PAHs (□), four-ring PAHs (◆), five- and six-ring PAHs (◇), and a mixture of 16 PAHs (▲) in sediment slurries amended with organic nutrients (A), minimal medium containing organic sediment extract (B), and minimal medium (C). The enrichment was previously acclimated in sediment slurries to a mixture of 16 PAHs. No mineralization occurred in sterile controls.

shared a common enzyme system for phenanthrene and pyrene, and another for naphthalene.

Ongoing Research

Current research includes the isolation and characterization of a *Mycobacterium* sp. capable of degrading pyrene as a sole carbon source. The isolate will be introduced into the mixed microbial community of the sediment microcosm to assess survival and impact on the degradation of PAHs. The microbial diversity in impacted and nonimpacted sediments will be assessed by whole genome hybridization, and specific probes will be used to compare the activities of oil degraders and lignocellulose degraders under various nutrient and surfactant treatments.

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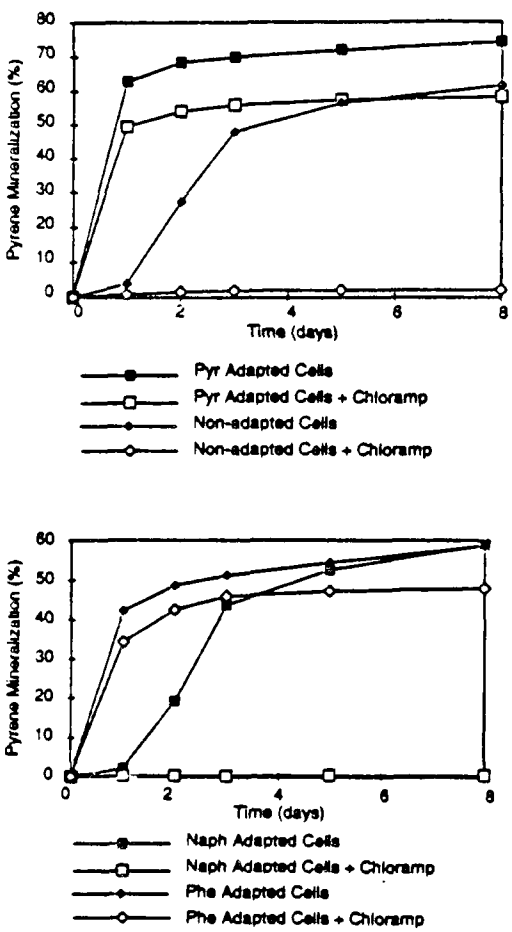

Figure 3. Mineralization of pyrene (5 µg/mL) in sediment slurries (10 percent w/v) by an enrichment culture acclimated by preexposure for 9 days to 50 µg/mL of the indicated PAHs. During the 9 days, the added PAHs were completely degraded.

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Biodegradation of Petroleum Hydrocarbons in Wetlands: Constraints on Natural and Engineered Remediation

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Sensitive wetland ecosystems are susceptible to impact from spilled and discharged oils. Major oil recovery and processing operations are located in wetland ecosystems, including Louisiana, where 40 percent of the U.S. coastal wetlands and 15 percent of U.S. crude oil production are located. Understanding the responses of these wetland ecosystems to oil-related impacts is critical for the design of remediation strategies. Bioremediation is particularly attractive because mechanical cleaning or washing operations are usually impossible because of the sensitivity of these systems. At present, however, little information is available on the constraints of bioremediating spilled oils in wetland ecosystems.

Coastal marshes are wetland ecosystems in the Gulf Coast region where oil production and transshipment are concentrated. Marsh soils differ from typical bottom sediments in fundamental ways that will affect bioremediation in these systems: 1) highly organic marsh soils store large amounts of nutrients but very little in readily available forms; 2) marshes are heavily vegetated with macrophytes that can serve as conduits for O_2 diffusion, dramatically increasing aerobic surface area in the rhizosphere of marsh soils; and 3) marshes are characterized by periods of flooding and drying, which expose a larger volume of porous soil to the atmosphere. Because these features of marshes and other wetland types are unique, this study was recently initiated to determine the constraints on natural and engineered oil biodegradation in wetlands. The project is a cooperative agreement with the EPA Environmental Research Laboratory in Athens, Georgia.

Background

Biodegradation of oil components in wetlands has been demonstrated (1) but rates of degradation are strongly dependent on environmental conditions. These conditions include temperature, salinity, Eh, pH, sorption, and the oxygen and nutrient status of the environment. Studies have documented changes in microbial populations in wetlands in response to spilled oils (2,3). These responses were generally increases in total microbial populations and increases in the ratio of oil degraders to total heterotrophs.

In general, wetlands are dominated by anaerobic processes: methanogenesis in freshwater wetlands and sulfate reduction in brackish and saline wetlands. Several novel microbial processes have been identified that degrade oil components under anaerobic conditions (4). Aerobic processes, however, are recognized to act on a broader spectrum of compounds and are more rapid and complete (e.g., mineralization to CO_2 and H_2O). In marshes, aerobic heterotrophic activity is concentrated at the sediment-water interface in a small (several millimeters) aerobic layer and around the rhizosphere of rooted marsh macrophytes. High sediment oxygen demand, created by a sequence of events leading from organic matter diagenesis, prevents further O_2 penetration.

The maintenance of this aerobic layer is critical to microbial degradation of petroleum hydrocarbons. In oil-impacted wetlands, petroleum components provide an additional overwhelming carbon source and potentially serve as a physical barrier for O_2 diffusion. Some of this limitation may be overcome by passive diffusion of O_2 through marsh plants, although the relative supply and demand of this process has not been calculated. Flooding/drying cycles, either tidal or seasonal, also will control O_2 supply to marsh soils. In addition to oxygen limitation, essential nutrients such as nitrogen may become limiting because of disruption of natural biogeochemical cycles and competition from highly productive macrophytes. Availability of nutrients such as nitrogen depends on microbial mineralization processes that convert nutrients to usable forms, which are rapidly assimilated by plants and microorganisms. This "tight" internal cycling is characteristic of marshes, where externally supplied nutrients are only a fraction of those required for observed plant (and microbial) growth.

Fertilization may be required to maximize a microbial response to oil.

Preliminary Results

Study sites that have been selected in the Barataria Basin, Louisiana, include a freshwater marsh and a salt marsh located along a salinity gradient extending toward the coast. Seasonal samples are being taken from these sites, and numerous nutrient, microbial, and geochemical analyses are being conducted relating to bioremediation potential. For example, samples taken during January/February 1994 were evaluated for aerobic biodegradation potential of two oil components, phenanthrene and hexadecane, using radiorespirometry. Surface marsh samples were removed from the marsh using thin-walled aluminum cores, homogenized, and dispensed in center-well respirometry vials. Slurries were amended with the labeled hydrocarbons in an oil matrix (~1 percent to 2 percent South Louisiana "sweet" crude, v/v), and $^{14}CO_2$ was quantified using liquid scintillation. Treatments included controls, killed controls, and fertilization (with nitrogen, phosphorus, and iron). Results indicate that fertilization can increase the extent of mineralization of hexadecane and phenanthrene. Fertilization approximately doubled the extent of hexadecane mineralization in both the salt and fresh marshes (Figure 1). Fertilization effects on phenanthrene were significant in the salt marsh but within the experimental error in the fresh marsh (Figure 2). Nutrient availability in the winter months are generally highest because of the lack of competition from growing plants; therefore, fertilization may have more dramatic effects in other seasons. Most probable numbers of oil-degrading microorganisms in the fresh marsh (10^3) were several orders of magnitude higher than in the salt marsh (10^1), which may explain observed higher rates of phenanthrene mineralization. Results will be contrasted with seasonal data taken over the next year.

Current work also is being conducted on other aspects of oil degradation in wetlands. The application of stable isotope techniques is being investigated as a method of measuring oil biodegradation in marshes. Marsh soils have characteristic $\delta^{13}C$ signatures because of the presence of nearly monospecific stands of plants that use either the C-3 or C-4 pathway (Table 1). Respired CO_2 reflects the carbon signature of the marsh soil. Crude oils also have stable, characteristic $\delta^{13}C$ signatures (6) that have been used to detect biodegradation in the subsurface (5). Measuring the $\delta^{13}C$ signature of CO_2 emitted from oiled marshes is being investigated as an indicator of the extent of oil mineralization in these wetlands. This measure may serve as a noninvasive technique for determining oil biodegradation in spill situations. Additional studies are being conducted on oil degradation using core and controlled Eh-pH microcosms. Variables being investigated include tidal and

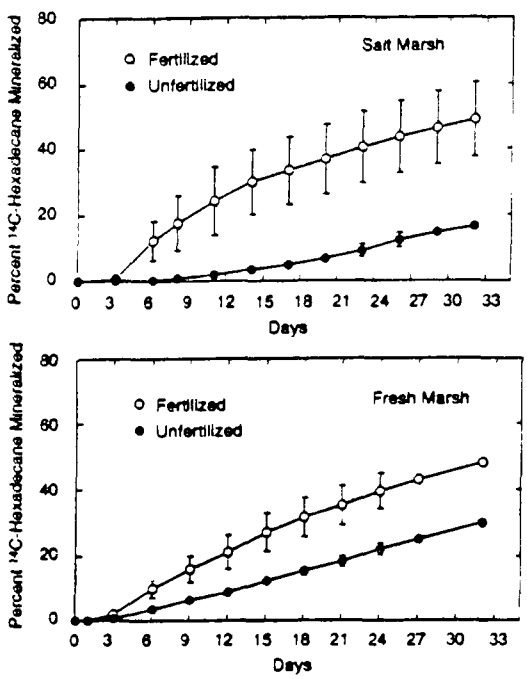

Figure 1. Mineralization of ^{14}C-hexadecane (in an oil matrix) in fertilized and unfertilized salt marsh and fresh marsh soils in coastal Louisiana (soil samples taken in February 1994).

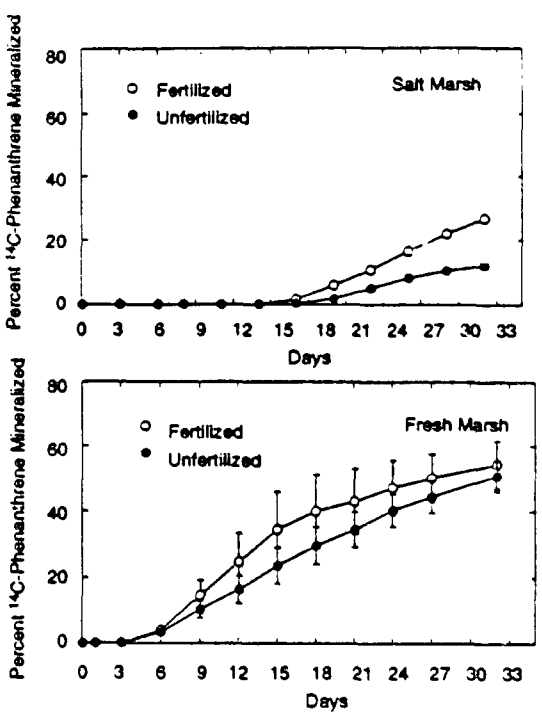

Figure 2. Mineralization of ^{14}C-phenanthrene (in an oil matrix) in fertilized and unfertilized salt marsh and fresh marsh soils in coastal Louisiana (soil samples taken in February 1994).

Table 1. $\delta^{13}C$ (‰) of Marsh Soils of Louisiana Coastal Region and of Petroleum Products (6,7)

Source	$\delta^{13}C$ (‰)
Fresh Marsh *(Panicum hemitomon)*	-27.9
Intermediate Marsh *(Sagittaria falcata)*	-26.6
Brackish Marsh *(Spartina patens)*	-14.9
Salt Marsh *(Spartina alterniflora)*	-16.5
Crude Oil	-30.6

flooding regime, fertilization, vegetation density, and soil oxygen demand. Gas chromatography/mass spectrometry analysis of crudes is being used to quantify 50 to 60 oil components, including alkanes, polycyclic aromatic hydrocarbons, naphthenes, and isoprenoids.

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Anaerobic Biotransformation of Munitions Wastes

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An environmental problem associated with U.S. military facilities is the presence of soil, sediment, surface water, and ground water contaminated with toxic explosive compounds. With the current emphasis on demilitarization and returning land to the private sector, the remediation of the contaminants from these sites has become important. Several types of remediation procedures are under investigation for the removal of munitions from soils and water. Incineration has been demonstrated to be an effective process for the remediation of soils from these sites. The physical process of wet air oxidation of munitions contaminants is under investigation, as well as several biological remediation procedures. Kaplan (1) reviews the literature concerning the biological degradation of munitions compounds and shows that under aerobic conditions the compound 2,4,6-trinitrotoluene (TNT) is degraded by a reductive process and is not mineralized but merely transformed, producing dinitrotoluenes and azoxy compounds as the products of metabolism. This degradation suggests that a process that is reductive in nature (i.e., anaerobic) would be the best approach to the treatment of soils contaminated with TNT. Under anaerobic conditions, reductive processes would occur at a faster rate, so lower amounts of the hydroxylamino intermediates would be produced and thus lower amounts of the azoxy dimers and polymers.

Current studies show that an aerobic treatment might be a possibility, using *Phanerocheate chrysosporium* (2-4). Boopathy et al. (5, 6) have published findings concerning the anaerobic degradation of TNT by a sulfate reducing bacteria. Many investigations are currently under way concerning the biological degradation of TNT, but the procedure outlined below is the first pilot-scale application of a biological technology for munitions degradation that has been demonstrated.

Background

A procedure for the anaerobic remediation of munitions compounds including TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and 1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) from contaminated soil has been developed (7-10) and is being demonstrated at Weldon Springs, Missouri. The procedure, first developed and demonstrated for the removal of Dinoseb from soils, involves flooding the soil with water and adding a carbon source with a high oxygen requirement (such as starch) (11-13). Aerobic heterotrophs deplete the oxygen from the aqueous phase while utilizing the starch. The aqueous/soil mixture then will be anaerobic, allowing the degradation of TNT, RDX, and HMX to occur. The procedure requires that the pH be controlled to between 6.5 and 7 and that the temperature be in the mesophilic range (8).

The pathway for TNT reduction as seen under anaerobic conditions is initially a reductive one, where first 4-amino-2,6-dinitrotoluene (4A), then 2,4-diaminotoluene (24DA), and finally 2,4,6-triaminotoluene (TAT) are produced. TAT very rarely accumulates in the cultures but is rapidly converted to 2,4,6-trihydroxytoluene (methylphloroglucinol, MPG) by some unknown mechanism. This conversion is followed by dehydroxylation reactions, leading ultimately to *p*-cresol, which can undergo ring cleavage either anaerobically or aerobically (14,15). Although the latter compounds do not accumulate in the soil during regular treatment procedures, they have been detected in laboratory cultures degrading TNT when yeast extract was added as a nutrient supplement for cultures enriched from soil.

A proposed improvement to the anaerobic remediation strategy is to implement an aerobic stage after the reductive stage of the procedure is complete. This

improvement would ensure mineralization of the carbon to CO_2 rather than a fermentation to several short chain fatty acids. This improvement requires that the addition of starch at the beginning of the procedure be reexamined, as excess starch always occurs when the treatment is complete; thus, oxygenating the system is very hard (7). To do this, the use of external carbon sources that were more defined and thus easier to control than starch were investigated. The use of a commercial soluble starch, glucose, and acetate was compared with the insoluble starch supplied by J.R. Simplot Co. (Boise, Idaho).

Laboratory experiments were conducted to determine the soil loading rates for the treatment of a soil from Umatilla, Oregon, contaminated with 12,000 mg TNT/kg soil, 3,000 mg RDX/kg soil, and 300 mg HMX/kg soil. These rates led to experiments designed to determine the effect of the reduced intermediates on the reduction of TNT and on the metabolism of the intermediates.

All experiments were performed using a 1 percent (w/v) addition of a soil that had been contaminated with Dinoseb and treated using the anaerobic procedure as an inoculum. Experiments to determine the effects of carbon source additions were performed using 4 percent (w/v) Umatilla soil in phosphate buffer. Experiments to determine the effects of 4A on metabolism were performed in cultures spiked with TNT and 4A at the levels indicated in Figure 3. Analyses were performed using narrow-bore high performance liquid chromatography, as described by Ahmad (16).

Results

The results of the experiments with various carbon sources led us to glucose as the carbon source of choice (Figure 1). Acetate was not used as a carbon source for oxygen depletion in these cultures. The reason is unknown, but the contaminants in the soil possibly either inhibited some reaction in the TCA cycle or the glyoxylate shunt, the two main pathways for the utilization of acetate. Commercially available soluble starch did not serve as a carbon source either, probably because of the absence of starch-degrading organisms in the soil inoculum. The insoluble starch was used as a carbon source for oxygen depletion in these experiments, as had been demonstrated previously (8,11). This starch contains its own microbial component (11), thus the presence of starch-degrading organisms in the soil was unnecessary. Cultures fed glucose reduced the redox potential to the lowest values and showed the fastest initial degradation of TNT.

When the amount of soil used in the treatment procedure was increased from 1 percent (w/v) to 4 percent (w/v), the first intermediate (4A) accumulated to an extent that had not been seen before (50 mg/L) (Figure 2). This accumulation was accompanied by a reduction in

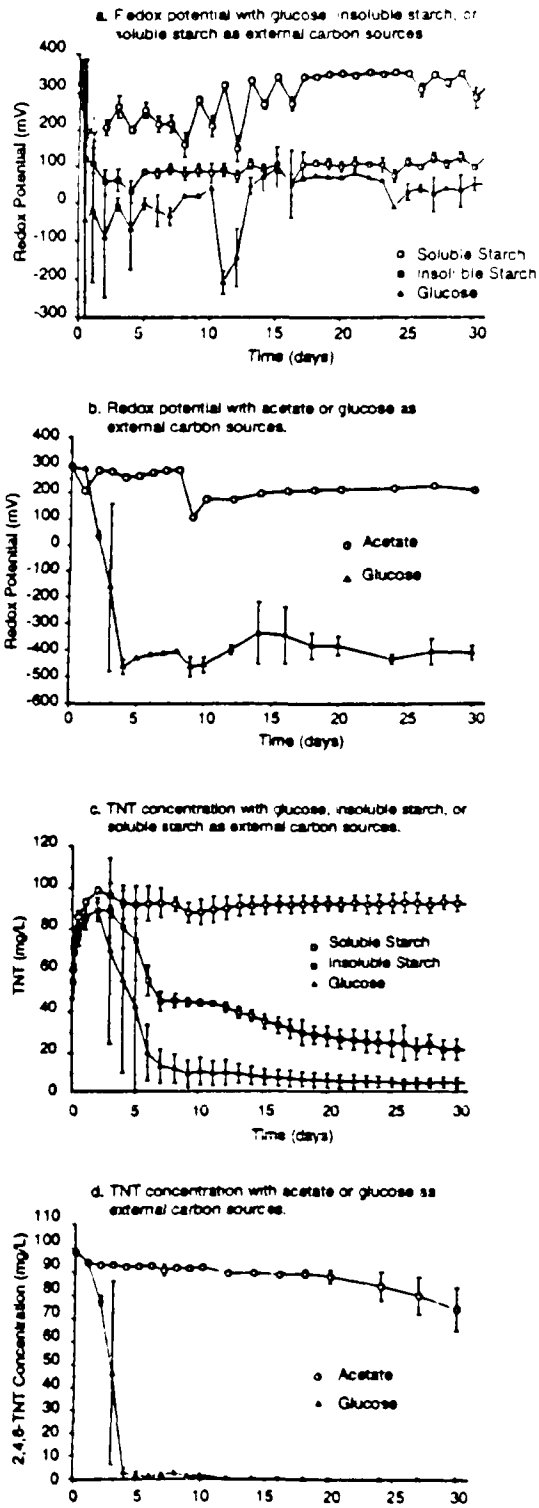

Figure 1. The effect of external carbon sources on redox potential and TNT degradation in cultures containing 5 percent Umatilla soil/phosphate buffer and inoculated with treated soil.

the rate and extent of reduction of TNT. To further examine this observation, experiments were conducted to determine the effects of 2A on the reduction of TNT and on the degradation of 2A. The results show that when 2A was spiked into the media containing TNT, a reduction in the rate and extent of degradation of TNT and 2A occurred (Figure 3).

Summary and Conclusions

Glucose was used successfully as an external carbon source, allowing an accurate calculation of the oxygen demand and a determination of the amount to add that would allow consumption of the oxygen present initially and maintenance of anaerobic conditions for a specified time. Calculations show that 28.8 mg/L of glucose must be supplied to remove all initial dissolved oxygen (DO) and keep the aqueous phase free of DO, assuming an initial DO of 9.08 mg/L, a reaeration rate of 0.908 mg/L, and an incubation time of 24 days. The calculation assumed that all glucose was used for oxygen consumption, and no fermentation of the glucose occurred. To correct for this, a figure of 100 mg/L glucose could be used as a conservative starting point. Future experiments at the University of Houston will determine whether this figure is sufficient to allow the creation of and to sustain anaerobic conditions for the required period, and whether the institution of an aerobic stage is beneficial to the procedure.

The process must be engineered towards rapid removal of intermediates rather than only rapid removal of TNT. This rapid removal will ensure that buildup of toxic intermediates will not occur and that the process may be performed reliably in the field. The development of more efficient inocula that will ensure efficient removal of intermediates produced during TNT degradation currently is under investigation at the University of Idaho and the University of Houston. The effects of the intermediates on the growth and metabolic activities of the organisms involved also is being investigated at the University of Houston.

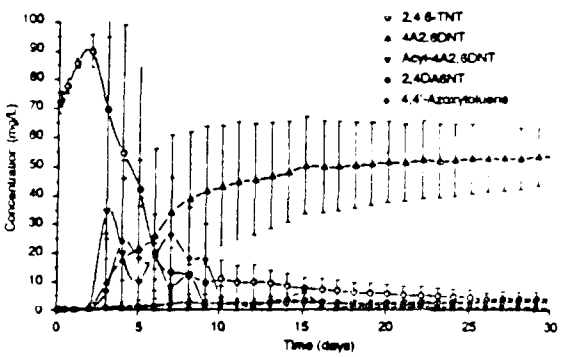

Figure 2. Concentrations of TNT and its metabolic intermediates during the anaerobic remediation of Umatilla oil in cultures inoculated with treated soil.

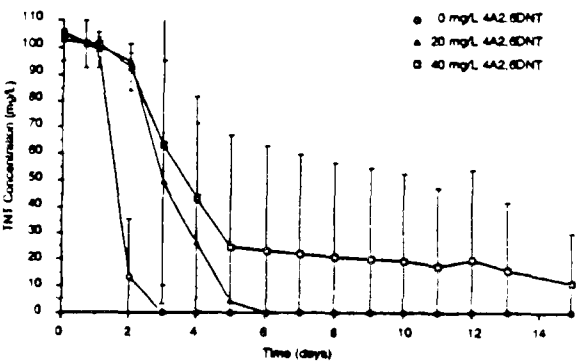

Figure 3. Concentrations of TNT in aqueous cultures inoculated with treated soil degrading 100 mg/L TNT in the presence of 4-amino-2,6-dinitrotoluene.

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Covalent Binding of Aromatic Amines to Natural Organic Matter: Study of Reaction Mechanisms and Development of Remediation Schemes

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Aromatic amines comprise an important class of environmental contaminants. Concern over their environmental fate arises from the toxic effects that certain aromatic amines exhibit toward microbial populations and reports that they can be toxic or carcinogenic to animals. Aromatic amines can enter the environment from the degradation of textile dyes, munitions, and numerous herbicides. Because of their importance as synthetic building blocks for many industrial chemicals, the loss of aromatic amines to the environment also may result from production processes or improper treatment of industrial waste streams. The high probability of contamination of soils, sediments, and ground-water aquifers with aromatic amines necessitates the development of innovative, cost-effective *in situ* remediation techniques for their treatment.

Numerous studies have demonstrated that aromatic amines become covalently bound to the organic fraction of soils and sediments through oxidative coupling or nucleophilic addition reactions (1-4). It is generally accepted that once bound, the bound residue is less bioavailable and less mobile than the parent compound. Thus, procedures for enhancing the irreversible binding of aromatic amines to soil constituents could potentially serve as remediation technologies.

Model studies suggest that oxidative enzymes derived from soil microorganisms play a significant role in catalyzing the formation of bound residues (5,6). Stimulation of these naturally occurring enzymes could provide an effective *in situ* method for the treatment of soils, sediments, and ground-water aquifers contaminated with aromatic amines (7). For example, Berry and Boyd (8) were able to enhance the covalent binding of the potent carcinogen 3,3'-dichlorobenzidine (DCB) in a soil by the addition of highly reactive substrates (i.e., ferulic acid and hydrogen peroxide). They concluded that by providing the indigenous peroxidase enzymes with highly reactive substrates, the overall level of oxidative coupling in the soil was increased, which lead to enhanced incorporation of DCB.

To gain a more in-depth understanding of the enzyme-mediated binding of organic amines to soils and sediments, we have studied the effects of enzyme amendments to sediments treated with aromatic amines such as aniline, reduction products of TNT and atrazine, and metabolic reaction products of atrazine.

Results and Discussion

Initially, experiments were conducted to determine the limiting factors controlling the binding of aniline to amended sediments. Figure 1 illustrates the effect of the addition of various combinations of horseradish peroxidase, H_2O_2, and ferulic acid to Beaver Dam sediment-water systems treated with aniline at an initial aqueous concentration of 5×10^{-5} M. In each case, the amendments were added 24 hours after the addition of aniline.

The data in Figure 1 show that the binding capacity of the sediment for aniline was limited prior to the addition of the amendments. Only 10 percent of the initial concentration of aniline was irreversibly bound to the untreated natural sediment. All amendments tested greatly enhanced the removal of aniline from the aqueous phase of the Beaver Dam sediment-water systems, as the concentration of aniline in the aqueous phase was below detectable limits in a matter of hours. The observation that the addition of H_2O_2 alone catalyzed the removal of aniline suggested that the sediment was not limited in peroxidase activity or oxidizable substrates.

To determine the effect of H_2O_2 on the binding of aniline in a sediment with no peroxidase, we monitored the aqueous concentration of aniline in both a nonsterile and

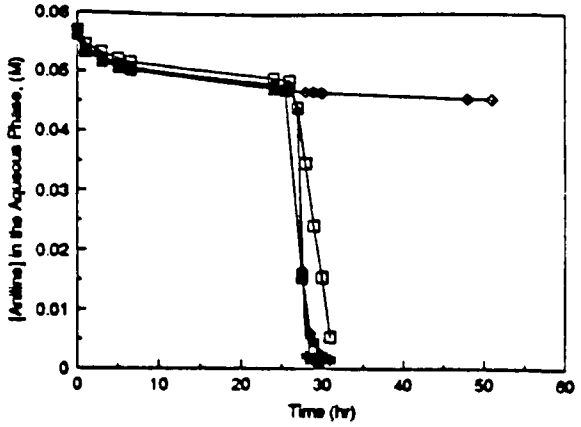

Figure 1. Effect of amendments on the aqueous phase concentration of aniline in Beaver Dam sediment-water system: (♦) control, no treatment; (■) ferulic acid, peroxidase, and H_2O_2; (★) ferulic acid and H_2O_2; and (□) H_2O_2.

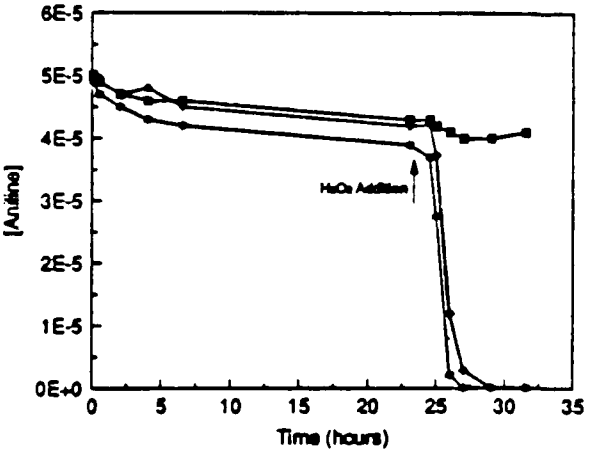

Figure 2. Effect of hydrogen peroxide treatment on the aqueous concentration of aniline in a Beaver Dam sediment-water system: (■) nonsterile control, no H_2O_2 treatment; (♦) nonsterile sediment treated with H_2O_2 at t=24 hr; and (★) heat-sterilized sediment treated with H_2O_2 at t=24 hr.

a heat-sterilized Beaver Dam sediment with and without the addition of H_2O_2 (Figure 2). The aqueous concentration of aniline was measured for 24 hr prior to the addition of H_2O_2. As before, the control study (no addition of H_2O_2) demonstrated the limited binding capacity of the sediment for aniline. Surprisingly, the addition of H_2O_2 24 hours after the initial addition of aniline had a significant effect on the aqueous concentration of aniline in both the sterile and nonsterile sediment-water systems.

Because our initial assumption was that heat sterilization would destroy peroxidase activity, the observation that treatment of the heat-sterilized Beaver Dam sediment-water system greatly enhanced the removal of aniline suggested that a mechanism other than peroxidase activation may exist. The high iron content of the sediment may have resulted in the iron-mediated reduction of H_2O_2 to form hydroxyl radicals (Fenton's reaction), which could subsequently react with aniline via hydrogen abstraction and ring addition. Recently, the chemical oxidation of chlorinated organics by addition of H_2O_2 to sand containing iron has been demonstrated by Ravikumar and Gurol (9).

In an attempt to determine if the iron-mediated reaction was occurring, two Beaver Dam sediment-water systems were treated with H_2O_2 24 hours prior to the addition of aniline. We hypothesized that if Fenton-type reactions were occurring, the extremely reactive hydroxyl radicals would react quickly with the organic matter and subsequently would not be available to react directly with aniline upon its addition 24 hours later. Surprisingly, at both concentrations of H_2O_2 studied, the binding capacity of the Beaver Dam sediment for aniline was increased by treatment with H_2O_2 24 hours prior to the addition of aniline. These findings suggest that hydoxyl radicals, like activated peroxidase, may react with organic matter to produce binding sites for compounds such as aromatic amines (Figure 3).

In summary, we feel that hydrogen peroxide treatment of soils and sediments contaminated with aromatic amines and other classes of reactive chemicals shows promise as a remediation method. We currently are extending this remediation technology to other aromatic amines of interest, such as TNT reduction products and atrazine and its metabolites, whose contamination of soils and sediments has been reported. Experiments are also in progress to further our understanding of the mechanisms by which H_2O_2 enhances the covalent binding of aromatic amines.

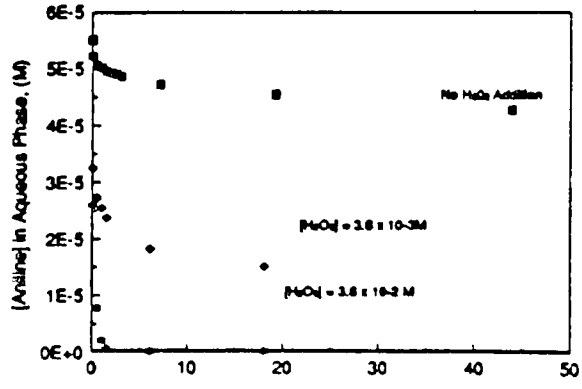

Figure 3. Effect of H_2O_2 treatment of a Beaver Dam sediment-water system 24 hours prior to the addition of aniline: Initial [aniline] = 5.5 x 10^{-5} M.

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Kinetics of Anaerobic Biodegradation of Munitions Wastes

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2,4-Dinitrotoluene (2,4-DNT) is formed during the manufacture of propellant and is commonly found in munitions wastewater. It has been found to be mutagenic in bacterial and mammalian assays and carcinogenic in animal studies (1). Because of its toxic nature and large-scale use, 2,4-DNT is listed as a priority pollutant by EPA. Early studies on the biodegradation of 2,4-DNT suggested that 2,4-DNT was resistant to biological treatment in aerobic processes such as activated sludge systems (2). Recently, some investigators reported complete degradation of 2,4-DNT by a pure aerobic culture (3,4). Industrial application of the aerobic biodegradation of 2,4-DNT, however, reveals that it is very difficult to achieve compliance with EPA discharge limits. Under anaerobic conditions, 2,4-DNT can be completely transformed to 2,4-diaminotoluene (2,4-DAT) with ethanol serving as the primary substrate (5). Subsequently, 2,4-DAT can be easily mineralized aerobically (5).

In this study, the anaerobic biotransformation of 2,4-DNT with ethanol serving as the primary substrate was investigated. The culture was acclimated in a chemostat with 2,4-DNT and ethanol as substrates. The pH and the temperature in the chemostat were kept at 7.2 and 35°C, respectively. The hydraulic retention time in the chemostat was 40 days. Biochemical methane potential (BMP) tests with 2,4-DNT and ethanol as substrates were conducted using an anaerobic respirometer with the culture from the chemostat serving as an inoculum. Sodium sulfide and L-cysteine hydrochloride were used to maintain a reducing environment for the BMP tests. The impact of the reducing agent on the biotransformation of 2,4-DNT and ethanol was studied. The effect of 2,4-DNT, the biotransformation intermediates, and 2,4-DAT on the bioconversion of ethanol also was investigated.

Results and Discussion

After steady-state operation was established in the chemostat (i.e., the effluent composition, the volumetric gas production rate and composition, and the biomass concentration in the chemostat had been constant for over 120 days), mixed culture from the chemostat was used as an inoculum for the BMP tests. The culture was transferred into the BMP reactors in an oxygen-free anaerobic chamber at 35°C. The pH and the temperature in the BMP reactors were kept the same as those in the chemostat. Different initial 2,4-DNT concentrations were used in the BMP tests, while the initial concentration of ethanol was the same in all of the reactors. Figure 1 illustrates the biotransformation process of 2,4-DNT, in the presence of ethanol and 50 mg/L sodium sulfide hydrate and 100 mg/L L-cysteine hydrochloride as the reducing agents. 2,4-DNT was completely transformed to 2,4-DAT, with 2-amino-4-nitrotoluene (2-A-4-NT) and 4-amino-2-nitrotoluene (4-A-2-NT) appearing as intermediates. The initial transformation rate decreased with increasing initial 2,4-DNT concentrations (Figure 1a). Note that at a low initial concentration of 2,4-DNT, a greater buildup of 4-A-2-NT occurred compared with 2-A-4-NT (Figures 1c and 1d). As the initial 2,4-DNT concentration increased, more 2,4-DNT was transformed via 2-A-4-NT (Figures 1c to 1f). A higher concentration of 2-A-4-NT than 4-A-2-NT was formed at the high initial 2,4-DNT concentration (Figure 1f). The results suggest two pathways leading to the complete biotransformation of 2,4-DNT to 2,4-DAT (Figure 2), with pathway (a) occurring faster at high initial 2,4-DNT concentrations and pathway (b) occurring faster at low initial 2,4-DNT concentrations.

Another BMP test was conducted under similar conditions except, in this instance, the reducing agent was 200 mg/L Na_2S $9H_2O$. The rate of biotransformation of 2,4-DNT was much higher, and 2,4-DNT exhibited much less inhibition to its biotransformation as a result of the presence of a higher concentration of sulfide. The presence of the higher concentration of sulfide provided a more reducing environment, which was favorable to the

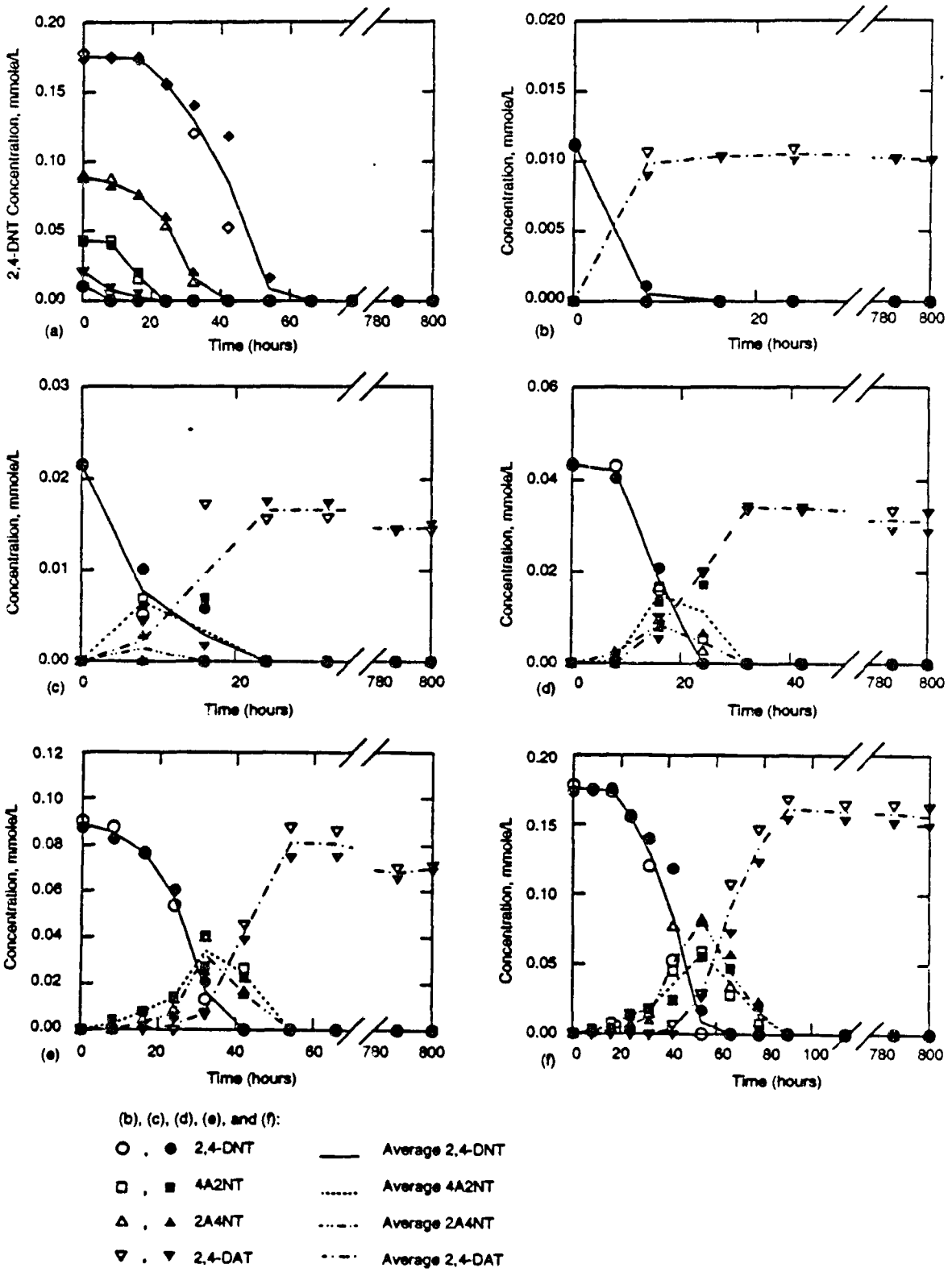

Figure 1. Anaerobic biotransformation of 2,4-DNT with ethanol as primary substrate.

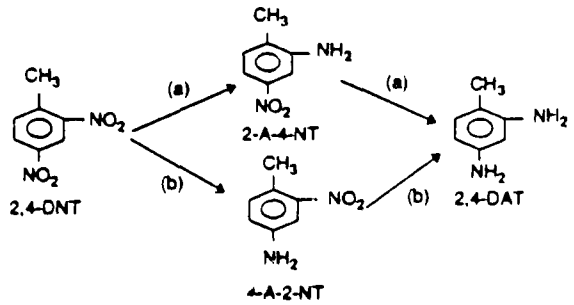

Figure 2. Pathway of anaerobic biotransformation of 2,4-DNT.

biotransformation of 2,4-DNT. An abiotic test was conducted to evaluate the potential for chemical reduction of 2,4-DNT. Results suggest that 2,4-DNT is chemically reduced to 2,4-DAT via 2-A-4-NT or 4-A-2-NT in the presence of high concentrations of sulfide and minerals.

The bioconversion of ethanol was also affected by the reducing agent used in the BMP test. L-cysteine hydrochloride is widely used as a reducing agent in anaerobic experiments. When L-cysteine (100 mg/L) and Na_2S (50 mg/L) were used as reducing agents in the co-metabolic biodegradation of 2,4-DNT, propionate was formed during the bioconversion of the primary substrate ethanol when the initial concentration of 2,4-DNT was lower than 6 mg/L (6). No such propionate production, however, was observed when sulfide (200 mg/L) was the sole reducing agent. L-cysteine hydrochloride may contribute to the formation of propionate during the fermentation of ethanol in the presence of 2,4-DNT.

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Biodegradation of Chlorinated Solvents

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Haloorganics comprise the largest single group of chemicals on the EPA list of priority pollutants (1) because many of these industrially important compounds have been demonstrated to be mutagenic and carcinogenic in mammals. Successful application of chlorinated solvent bioremediation requires extensive knowledge of underlying molecular mechanisms of biodegradation. Such knowledge will allow a rationale for selection of organisms and treatment schemes, and prevent slow, costly empirical approaches to bioremediate every different site.

Microbial action on chlorinated solvents often involves co-metabolism or cases of fortuitous metabolism, which provide no net benefit to the organism involved. An example of this is the bacterial degradation of trichloroethylene (TCE), a widespread ground-water pollutant. Gratuitous metabolism of TCE has been observed to be catalyzed by a number of different oxygenases: toluene dioxygenase (2,3), toluene-4-monooxygenase (4), ammonia monooxygenase (5), soluble methane monooxygenase (sMMO) (6), propane monooxygenase (7), toluene-2-monooxygenase (8), phenol hydroxylase (9), and isoprene oxygenase (10). Currently methanotrophs expressing sMMO oxidize TCE most rapidly in small-scale laboratory studies. In practice, the use of methanotrophs suffers from 1) inactivation of sMMO resulting from alkylation by acyl chlorides derived from TCE oxidation; 2) formation of toxic chloral hydrate as a TCE byproduct; 3) cooxidation of co-contaminants to more toxic materials (i.e., chlorobenzene to chlorophenols); 4) inhibition with methane; and 5) inability to maintain sMMO under field conditions.

In light of the above, other TCE-degrading organisms might outperform methanotrophs, or toluene dioxygenase-expressing strains, over sustained periods and under field conditions. One of our experimental models is the strain of *Pseudomonas cepacia* G4 (8,11), whose TCE-degrading ability is based on co-metabolic action of the toluene-2-monooxygenase system. The performance and safe application of TCE-biodegraders necessitates a greater understanding of the mechanisms of oxygen addition to TCE and rigorous determination of the final recoverable products. Purification of TMO activity from *P. cepacia* G4 will facilitate determination of the complete product stoichiometry of TCE oxidation. These questions are important in the context of understanding the physiological basis by which *P. cepacia* (toluene-2-monooxygenase, TMO) is less influenced by toxic effects resulting from TCE oxidation than are *Pseudomonas putida* F1 (toluene dioxygenase, TDO) and other organisms.

Understanding the biochemical basis of advantages of TMO over other chloroethene-degraders may open new, direct approaches for search of more effective strains and enzymes.

Physiology and Biochemistry of TCE Oxidation by *P. cepacia* G4

In Vivo Studies with P. cepacia G4

Generally, *in vivo* studies have focused on measuring the disappearance of chlorinated compounds. Supplementing this information, however, with a deeper knowledge of the products obtained from chlorinated solvent oxidation is crucial. TCE oxidation has been investigated most extensively, but only substoichiometric accounting of products has been accomplished. The present study addresses possible formation of epoxides from chloroethenes and of products arising from chloride migration during oxygen addition.

Identification of TCE Biodegradation Products

In experiments with TCE, 200 μM was essentially quantitatively degraded by *P. cepacia* G4. At that time, culture filtrates were extracted and analyzed by gas chromatography (GC) for the presence of the possible chloride rearrangement products 2,2,2-trichloroacetaldehyde and 2,2,2-trichloroethanol. Neither compound was detected above the level of 0.25 percent of the total TCE

transformed (less than 0.5 μM). Analysis of culture filtrates obtained in experiments with [^{14}C]-TCE and washed cell suspensions of *P. cepacia* G4 was performed by high performance liquid chromatography (Bio-Rad Aminex organic acid column). The major detectable metabolite, in all cases, comigrated with authentic glyoxylate and accounted for 2.5 percent, 29 percent, and 19 percent of the added TCE at 0 min, 30 min, and 60 min of incubation, respectively. (Zero time control contained live induced cells centrifuged with TCE, so several minutes elapsed before the cells were actually removed from the culture supernatant fluid.) In subsequent experiments with 10 mM glyoxylate added as cold trap, more than 60 percent of the products were accounted for as glyoxylate. The data indicate that glyoxylate is a likely major product and is further metabolized by *P. cepacia* G4. Two minor products also were observed transiently; one of them may be formate, the identity of other is unknown. These analyses provided no evidence for the formation of trichloroacetate, dichloroacetate, oxalate, and glycolate by *P. cepacia* G4 from [^{14}C]-TCE.

Evidence of Epoxide Formation from Chloroethenes by P. cepacia *G4*

Production of glyoxylate infers the formation of TCE-epoxide as precursor. While TCE-epoxide is unstable in water ($t_{1/2}$ < 1 min), *trans*-1,2-dichloroethylene epoxide undergoes hydrolysis and isomerization relatively slowly. *trans*-1,2-Dichloroethylene (trans-1,2-DCE) was used as a model compound to obtain evidence for epoxide formation. *trans*-1,2-DCE was readily oxidized by *P. cepacia* G4 induced with toluene vapor; at a starting concentration of 200 μM, 85 percent of *trans*-1,2-DCE was transformed after 60 min. Only 3 percent of the transformed *trans*-1,2-DCE was recovered, however, as its colored epoxide adduct with 4-(*p*-nitrobenzyl)-pyridine (12). Noninduced *P. cepacia* G4 showed no significant production of material forming the colored 4-(*p*-nitrobenzyl)-pyridine adduct.

GC/mass spectrometry (MS) and GC/Fourier transfer infrared (FTIR) was used to analyze pentane extracts of cell supernatants after incubation of *P. cepacia* G4 with *trans*-1,2-DCE. A compound was found with the same R_t, mass and infrared spectra as synthetic *trans*-1,2-DCE epoxide. Synthetic 2,2-dichloroacetaldehyde showed different R_t on the GC column used, and its MS fragmentation and infrared spectrum differed from that of the epoxide. These data indicate that *trans*-1,2-DCE epoxide is the major pentane extractable product formed.

Purification of Toluene-2-Monooxygenase (TMO)

Conditions have been established to obtain active crude extracts and to achieve partial purification of the components for this multicomponent enzyme system. Active crude extracts were obtained in a buffer consisting of 25 mM MOPs, pH 7.5, 200 μM $Fe(NH_4)_2(SO_4)_2$, and 5 mM cysteine. Partial purification of an NAD(P)H oxidoreductase, with an apparent molecular weight of 38,000 daltons on SDS-PAGE, has been accomplished through the use of ion exchange chromatography at different pHs. The reductase is capable of reducing cytochrome *c* and supports reconstituted toluene monooxygenase activity. In addition, the reductase from phenol hydroxylase of *Pseudomonas* sp. SF600 is capable of complementing the toluene-*ortho*-monooxygenase system, indicating a possible similarity between these enzyme systems.

Oxidation of Structural Analogues of Perchloroethylene (PCE) and TCE

Presently known aerobic biodegradation processes for chlorinated ethenes are based on the co-metabolic action of oxygenase enzymes involved in catabolic pathways providing effective utilization of compounds showing little or no structural relationship to TCE or PCE (e.g., toluene, isopropylbenzene, methane, camphor, isoprene). Analogues of chlorinated ethenes with one or more chlorine atoms replaced by methyl groups can be used for studies of biochemical mechanisms involved in oxidation of TCE, and of factors limiting activities of oxygenases on PCE. It is more important, however, that they can serve as potential structural surrogates of PCE and TCE that may support growth of bacterial strains and be useful carbon and energy sources for enrichment cultures to search for organisms and enzymes, effectively metabolizing PCE and TCE themselves. This approach requires prior synthetic work to obtain such surrogate substrates because the most promising compounds, such as 1,1-dichloro-2-methyl-1-propene or 1,1,2-trichloro-1-propene, are not available commercially. Neither TDO- or TMO-expressing strains are capable of oxidizing these compounds. The feasibility of use of methylated analogs of PCE and TCE as enrichment substrates will be analyzed.

Summary and Conclusions

The production of glyoxylate as a major TCE oxidation product differs from previous observations of *in vivo* and *in vitro* TCE oxidation catalyzed by toluene dioxygenase and methane monooxygenase. In the previous studies with TDO and sMMO, glyoxylate formation is a minor pathway. With *P. cepacia* G4 and TMO, the pathway in Figure 1 appears to be more prominent because of either possible enzyme participation in C-Cl bond cleavage reactions or a different intracellular environment that promotes glyoxylate formation from chemical decomposition of TCE-epoxide, thereby avoiding formation of toxic or alkylating intermediates.

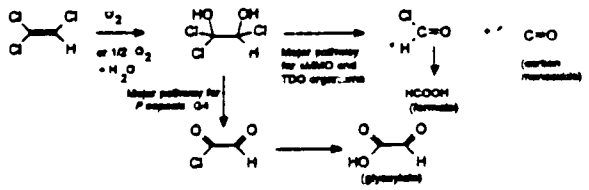

Figure 1.

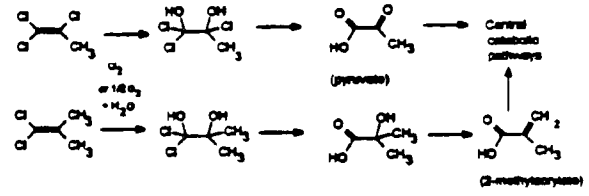

Figure 2.

Compared with TMO of *P. cepacia* G4, enzymes such as TDO or methane monooxygenase are inactivated *in vivo* by reactive intermediates generated during TCE oxidation; cells expressing these activities experience cytotoxicity from oxidizing TCE (13). Generally, most known TCE oxidation reactions are characterized by low reaction rates and formation of harmful metabolites. With respect to TCE (or PCE) co-metabolism, the bacteria cannot help themselves to select against or for such fortuitous reactions. These reactions provide no net benefit to cells as energy and carbon sources. Counter argument would point out that TCE and PCE are not natural products, and are found only recently in soil and water, so natural selection has not had time to select against this deleterious co-metabolism.

Using surrogate carbon and energy sources may offer a practical solution to finding microorganisms that 1) are capable of not forming toxic substrates; and 2) have higher reaction rates of TCE and PCE oxidation comparable with the conversion rates for growth (catabolic) substrates. Either direct dihydroxylation or a monooxygenation/hydration sequence would produce intermediates (Figure 2) capable of serving as carbon and energy sources. Therefore, the enrichment culture approach may provide a selection tool for finding new biological mechanisms capable of attacking the hindered double bond of PCE and TCE in an appropriate electrophilic environment.

Neither TMO or TDO can oxidize such hindered compounds as 1,1-dichloro-2-methyl-1-propene or 1,1,2-trichloro-1-propene. The less hindered compound, 1,1-difluoro-2,2-dichloroethylene, however, is oxidized by TDO and sMMO. This fact indicates that strong steric hindrance rather than the electrophilic environment of the double bond appears to be a limiting factor determining the success of oxidative reactions on PCE and TCE.

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Characterization of Bacteria in a TCE Degrading Biofilter

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A trichloroethylene- (TCE-) degrading vapor phase biofilter was investigated to determine the microbial population(s) mediating degradation. Initial observations suggested that ammonia-oxidizing bacteria could be responsible for TCE degradation. The biofilter being studied had been maintained in the presence of a gas stream containing methylene chloride, benzene, ethylbenzene, toluene, and TCE. During operation, a microbial community was established that could oxidize TCE when all other substrates were removed from the gas stream. Twenty to thirty percent removal of TCE at an inlet concentration of 21 ppmv (0.113 mg/L) and a gas residence time of 1 minute was experimentally observed. TCE degradation capability remained intact for more than 12 months. The standard OECD mineral salts solution with excess ammonia was trickled over the biofilter. The fact that ammonia was present in the nutrient solution provided circumstantial evidence that it could serve as a co-metabolite for nitrifying bacteria mediating TCE degradation. The ammonia monooxygenase (AMO) system, responsible for the conversion of ammonia to hydroxylamine, has been shown to carry out a co-metabolic oxidation of TCE (1,2). Characterization of the biofilter community was undertaken to establish if ammonia-oxidizing bacteria were responsible for TCE oxidation.

Background

Studies on the aerobic metabolism of TCE have shown that a diverse group of organisms can oxidize this compound in a co-metabolic fashion (3). The initial observation by Wilson and Wilson (4) demonstrating co-metabolism of TCE by methanotrophs was followed by reports of TCE degradation by toluene oxidizers (5), propane oxidizers (6), and ammonia oxidizers (1). Strategies for the treatment of TCE containing wastes often focus on the optimization of degradation using the addition of a co-metabolite to the appropriate group of organisms.

Experimental System and Results

The presence of nitrifying bacteria was monitored by most probable number (MPN) methodology and by gene probing with an AMO gene probe. The data generated showed that levels of ammonia oxidizers were low, generally below the level of detection of the AMO probe and 10^2 to 10^4 per gram of biofilter biomass. Following gene probing and MPN analysis, TCE degradation experiments were begun.

A series of TCE degradation experiments were conducted with biofilter biomass in a batch degradation assay using ^{14}C-TCE as a tracer and trapped $^{14}CO_2$ as the ultimate product of oxidation. The radiolabel experiments were conducted in 40.0-mL screw cap vials. The vials were capped with Teflon-lined septa, allowing injection into the vial. An inner vial containing 0.4 N NaOH was placed inside the larger to serve as a CO_2 trap. The trap was assayed by scintillation counting. The vials, inoculated with biomass, contained 2.0 mL of media and 38.0 mL of head space. After the appropriate incubation period, vials were acidified with 0.2 mL of 2 N H_2SO_4 to drive off CO_2. The sterile control values were subtracted from experimental values when determining conversion to CO_2. All data reported represent the mean value of three vials. Mass balance calculation on sterile controls were conducted by assaying the discharge per minute (dpm) in the NaOH trap, the aqueous phase, and a 2.0 mL hexane extract. Greater than 85 percent of added TCE could be accounted for at the end of the

experimental incubation time. Counts in the sterile control were always less than 2 percent of the total dpm added.

The initial phase of this study was designed to test the hypothesis that autotrophic ammonia-oxidizing bacteria were responsible for TCE degradation. Figure 1 shows the effect of nitrapyrin, an inhibitor of autotrophic ammonia oxidation, on TCE degradation (7,8). A number of batch treatments on the biomass were carried out as part of this study. The effects of ammonia, nitrate, phenol, and glucose, in both the presence and the absence of nitrapyrin, were examined. None of the treatments tested, including those to which nitrapyrin was added, greatly affect TCE mineralization. These results suggested that ammonia oxidizers were not responsible for TCE mineralization.

A time course experiment was conducted over a range of TCE concentrations in both the presence and the absence of ammonia. In this experiment, the oxidation of ammonia was assayed by a colorimetric method to detect both nitrite and nitrate. For this experiment, three TCE concentrations (0.021, 0.149, and 0.372 mg/L) and three time points (0, 20, and 44 hr) were chosen. Ammonia supplemented (+ ammonia) and nitrate (- ammonia) batch tests were inoculated with 0.003 mg of biofilter biomass. Data from this experiment are shown in Table 1. After 1 hour, no conversion of TCE to CO_2 was observed at any TCE concentration, either with or without ammonia. After 20 hours, TCE mineralization

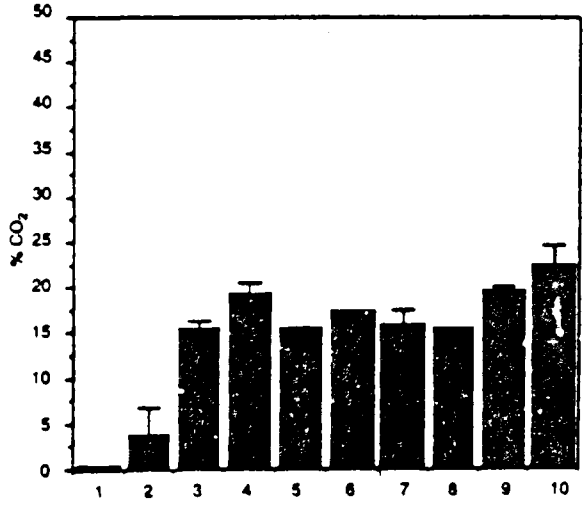

Figure 1. Nitrapyrin inhibition experiment. Biofilter biomass (0.01 mg biomass/vial) was used to test the effect of an inhibitor on TCE oxidation in the presence of various inducer compounds. Cultures were incubated in the presence of TCE (0.4 µg/vial) for 5 days prior to acidification. Results are reported as percent of added radiolabel recovered as CO_2: 1) heat-killed control; 2) time 0; 3) ammonia treated; 4) ammonia plus nitrapyrin; 5) nitrate; 6) nitrate plus nitrapyrin; 7) phenol treated; 8) phenol plus nitrapyrin; 9) glucose treated; and 10) glucose plus nitrapyrin.

occurred at lower TCE concentrations. No mineralization occurred at the highest TCE concentration at 20 hours or at 44 hours. Conversion to CO_2 in the vials at the lowest TCE concentration appeared to level off in 20 hours, showing little increase after 44 hours. The 0.149 mg/L TCE concentration continued to demonstrate increased TCE conversion at 44 hours. The effect of ammonia does not appear to be great at any concentration. A slight enhancement of mineralization in the ammonia-treated sample occurred after 20 hours, and a slight decrease in the ammonia-treated sample occurred after 44 hours. Vials from the 44-hours time point were assayed for nitrite and nitrate by colorimetric assay. No nitrite or nitrate was detected in any vials, suggesting that little ammonia oxidation was occurring. The nitrogen source had no effect on TCE mineralization. At this point, the biomass was examined to determine which organisms were mineralizing TCE without co-metabolite addition.

The persistence of aromatic hydrocarbon oxidizers in the biofilter suggests that they may be responsible for TCE oxidation. Enrichment cultures using biofilter biomass were incubated in 50.0 mL flasks in 10 mL of a mineral salts medium. These flasks were placed in 5-gal desiccators and exposed to 0.5 mL of either toluene or benzene. These flasks grew to turbidity and produced a yellow metabolite indicative of aromatic ring cleavage. The yellow metabolite was observed at the greatest dilutions tested (10^{-4}). These enrichment cultures were tested for mineralization in mineral salts in the absence of toluene or benzene, and showed high levels of TCE mineralization. The predominant culture appearing on vapor phase plates appears to be unique relative to previously described organisms and is being characterized. In contrast, TCE mineralization assays of positive MPN cultures did not mineralize TCE.

Conclusions

- Ammonia oxidizers are present in the biofilter, but at low levels.
- Removal of ammonia from the medium did not effect TCE mineralization by the biomass.
- Addition of the inhibitor nitrapyrin did not effect TCE mineralization by the biomass.
- Nitrifier enrichment cultures from the biofilter did not mineralize TCE.
- A high level of toluene/benzene oxidizers is present in the biofilter, and enrichment cultures can mineralize TCE without addition of an organic co-metabolite. These cultures are robust in the biofilter environment and have persisted in the biofilter for more than 1 year.

Table 1. TCE Mineralization by Biofilter Biomass with and without Ammonia Addition

NH_4	TCE	TCE Mineralization					
		1 hr		20 hr		44 hr	
	µg	%	µg	%	µg	%	µg
+	0.4	0.0	0.0	15.0	0.06	16.2	0.06
+	2.9	0.0	0.0	4.3	0.12	12.3	0.36
+	7.25	0.0	0.0	0.0	0.0	0.0	0.0
–*	0.4	0.0	0.0	11.6	0.05	15.3	0.06
–	2.9	0.0	0.0	3.0	0.09	13.1	0.38
–	7.25	0.0	0.0	0.0	0.0	0.0	0.0

*Nitrate substituted for ammonia
% = Percent of added radiolabel recovered as CO_2

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Bioremediation of TCE: Risk Analysis for Inoculation Strategies

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The introduction of non-native species has a colorful past for metazoan organisms. Controlled introductions of non-native or genetically engineered bacteria to date have not been documented to cause undesirable effects. The ubiquity of microorganisms has been largely assumed, providing a rationale for the safe release of "non-native" bacteria. The ubiquitous distribution argument assumes that all microorganisms have equal opportunity to occur in all environments, and that selective pressures determining distribution and abundance will eliminate introduced microorganisms that do not already occur in the target environment. The most successful introductions have resulted from isolating an organism from the targeted environment, modifying it, and returning it to its previous niche, e.g., *Rhizobium* spp. (1). An alternate strategy that has proved effective is to modify the environment to provide a niche for the phenotype of interest and to allow natural selective processes to occur (2). The success of these strategies supports the ubiquity argument. The history of virulent pathogen distribution, however, provides a model to warn us that the microbial world is not entirely homogeneous, and that some environments may be subject to invasion by non-native microorganisms. With the development of bacteria with potentially novel genetic combinations, we have a responsibility to determine if released organisms will be constrained by the selective pressures of the target environment.

Bacterial populations in nature are under constant selective pressures from physical and chemical conditions, substrate availability for growth, competition between species, and predatory/viral interactions. The balance of these forces determines both bacterial species composition and individual species' abundance. The relative significance of the biological factors (growth, competition, and predation) is determined by physical and chemical factors, as the limits of individual species' tolerance are reached within trophic or contaminant gradients. The addition of bacteria to environmental microbial communities may locally and temporarily change the balance of selective pressures, but these cells would ultimately face the selective forces of the target environment.

We have begun to address the abiotic and biological parameters for survival of *Pseudomonas cepacia* G4 PR-1 in laboratory microcosms utilizing ground water and sediment from the aquifer beneath the Borden Canadian Armed Forces Base in Ontario, Canada. This site is proposed for a bioremediation test using a funnel-and-gate technique (3) to control ground-water flow and force a trichloroethylene- (TCE-) contaminated plume through biocassettes colonized with PR-1. This bacterium constitutively expresses a toluene orthomonooxygenase that mineralizes TCE (4). The Borden aquifer is oligotrophic (3.5 to 6 mg DOC L^{-1}), with a ground-water flow of approximately 10 cm/day^{-1} through a well-sorted fine sand sediment (5). Determining the transport of bacterial cells from a treatment zone as well as their survival necessitates the development of field tracking methods for the organism and the plasmid that confers the ability to mineralize TCE.

Approach and Preliminary Results

Results obtained from analysis of the behavior of PR-1 in aquifer material in laboratory tests will be compared with the response at field scale during the release. This combination is hoped to highlight basic biological characteristics of bacteria that can be assessed in the laboratory; in this manner, future genetically engineered microorganism releases can be evaluated without expensive testing of the organism in mesoscale or semi-contained systems prior to release.

Characterization of Native Organisms

This initial phase is targeted toward identifying potential competitors, predators, and viruses in the target

environment. Selective plating and gene probing are employed to identify G4-like organisms that may be displaced by the addition of PR-1 or that may contribute to the loss of PR-1. Phenol-utilizing bacteria in the relatively pristine Borden aquifer represent about 3 percent of the colony-forming units (CFUs) obtained on the ground-water medium R2-A. In contrast, aquifer material from a TCE-contaminated site in Wichita, Kansas, had 62.6 percent of the R2-A CFUs appearing on phenol plates. Whether these differences will affect PR-1 survival remains to be determined.

We have enumerated protozoan predators of PR-1 in most probable number (MPN) growth assays using PR-1 cells as the growth substrate. Both flagellates (391 gdw^{-1}), naked amoebae (298 gdw^{-1}), and testaceans (52 gdw^{-1}) have been recovered that respond quickly and grow very well on PR-1 cells. The species diversity and numbers of protozoans recovered by this method are higher when sterile-filtered site ground water is used as a diluent rather than a phosphate buffer (6) or sodium pyrophosphate as a mild surfactant.

Both viruses and competitive interactions between PR-1 and native bacteria isolated on plates will be assayed using overlay plates with PR-1 cells and scoring for clearing zones. Native viruses have not been reported from aquifer environments as yet, but their widespread distribution in terrestrial and aquatic environments almost ensures their occurrence. Whether active viruses against PR-1 cells exist in the target environment remains to be determined.

PR-1 Tracking

A monoclonal antibody has been prepared against the o-side chain of PR-1 LPS (7). We have tested this monoclonal against a wide variety of bacteria, including other *P. cepacia* strains and isolates from the Borden aquifer, without evidence of cross reactivity. We have also tested the use of the monoclonal by tracking survival of PR-1 in laboratory microcosms by direct immunofluorescence and immunoblots of colonies from plates.

We are developing a polymerase chain reaction (PCR) detection assay for PR-1 utilizing the unique junction sites of Tn-5 from the insertion mutagenesis in both the plasmid and the genome. A set of three primers has been used to target an IS50 on the plasmid: two flanking primers and one asymmetrically situated in the interior sequence. This primer set yields a two band "fingerprint" when the PCR product is run out on gels.

PR-1 Survival

Tests for survival of PR-1 in ground water, sediment slurries in shake flasks, and flow through sediment columns are being conducted with the site material. Preliminary results suggest that the abiotic conditions of the aquifer are not limiting to PR-1 survival. When we introduced 1×10^7 PR-1 mL^{-1} into sterilized ground water, no loss of PR-1 cells was observed by immunofluorescent counts over 30 days, and plate counts dropped approximately an order of magnitude and then stabilized for 25 days. Seven months later, both direct counts and plate counts had dropped an additional order of magnitude each. In nonsterile ground water, however, PR-1 was eliminated within 10 days, despite a stable population of total bacteria determined by direct counts with the fluorochrome DAPI. In shaken sediment slurries, 2×10^7 PR-1 was eliminated within 4 days, and numbers of protozoa increased concomitant with the decrease in PR-1, suggesting that predation may be an important mechanism for loss of the bacterium from the system. Shifts in the bacterial community structure were apparent in the slurries based on colony morphologies on the heterotrophic medium R2A.

Presterilized and nonsterile sediment columns were set up using 50 cm long × 2 cm diameter tubes with 10 sampling ports sealed with silicone stoppers. A continuous culture of PR-1 set to a generation time of approximately 100 hours and a cell yield of 6×10^7 cells mL^{-1} was used as a source to feed to the top of the columns, with excess flow shunted off to a waste container. Flow through the column was controlled by a pump at the column outflow and set to 10 cm/day^{-1} as found in the aquifer. PR-1 cells were detected in the effluents with fluorescent antibodies after one void volume passed through the column (4.5 days). After two void volume replacements, the inflow of cells was stopped and switched to basal salts in an attempt to elute PR-1 from the columns. As in the ground water and sediment slurries, PR-1 persisted at higher levels in the sterile versus the nonsterile column, and we detected high numbers of bacterivorous flagellates in the nonsterile system. Unlike the ground water and sediment slurries, PR-1 persisted through 22 days of elution in the presence of predators. Extraction of the sediments with 0.1 percent sodium pyrophosphate at the termination of the experiment indicated that more of the PR-1 cells in the nonsterile system were particle associated than free in the pore water compared with the presterile system.

Conclusions

The preliminary results from our laboratory tests indicate that the abiotic conditions of the aquifer will not affect the persistence of PR-1, but losses to biological vectors will be a major factor. Cells free in the pore water will be quickly eliminated, but PR-1 may find refuge from predation in association with sediment particles that will allow long-term persistence of the organism in the target environment.

Acknowledgments

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Studies on the Aerobic/Anaerobic Degradation of Recalcitrant Volatile Chlorinated Chemicals in a Hydrogel Encapsulated Biomass Biofilter

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Trichloroethylene (TCE) and tetrachlorethylene (PCE) are organic solvents most frequently detected as ground-water contaminants. Both TCE and PCE undergo reductive dechlorination in anaerobic environments. PCE is aerobically recalcitrant.

In an ongoing biofilter study, experimental work is being conducted to evaluate the potential of gel-entrapped biomass for treating volatile chlorinated solvents, such as TCE and PCE, in the gas phase. Entrapped biomass offers the possibility of aerobic/anaerobic environments in the gel bead interior while aerobic conditions are maintained outside the bead. The reduced environment allows contaminants such as TCE and PCE to be degraded in a biofilter column packed with gel beads containing entrapped biomass.

Background

TCE degrades under anaerobic conditions, forming intermediates such as vinyl chloride, dichloroethylenes, and ethylene (1). TCE also degrades under aerobic conditions usually as a co-metabolite in the presence of a primary substrate. A number of compounds serve as primary substrates for TCE degradation, including aromatics, such as toluene and phenol (2,3); alkanes, such as methane and propane (4,5); and 2,4 dichlorophenoxyacetic acid (1). These microorganisms degrade TCE because of the enzymes expressed in response to the primary substrate; for example, toluene monooxgenase, which enables microorganisms to degrade toluene and other aromatics, allows degradation of TCE. The primary metabolite-to-TCE ratio has been found to be 2 g/g to 40 g/g in a recent study (6). Studies of TCE degradation (6) were conducted in a gas-lift loop reactor. TCE concentrations of between 300 µg/L (60 ppmv) and 3,000 µg/L (600 ppmv) were degraded with 95 percent or better efficiency. Results of another TCE study indicate that certain bacteria may be able to express the above enzyme even in the absence of toluene or phenol (7). Recently, biofiltration studies with a 25 ppmv gas-phase inlet concentration of TCE in a celite-pellet packed bed have shown that TCE can be successfully degraded with phenol present in the trickling nutrients (8).

Materials and Methods

Activated sludge biomass in an aqueous bioreactor was acclimated to toluene and TCE by exposing the sludge to air contaminated with toluene and TCE for a period of 30 days. The reactor was supplied with mineral nutrients, and the inlet and exit gas phase concentrations were periodically analyzed. After acclimation was achieved, complete toluene conversion and about 30 percent TCE conversion were observed in the reactor. The biomass was then removed from the reactor, mixed with k-Carragenan at 50°C, and extruded into 0.5 cm × 1.5 cm cylindrical beads. The beads, once extruded, were quenched in a mineral medium and then packed in a biofilter. The experimental biofilter consists of a 1-in. diameter, 5-in. height bed packed with k-Carragenan beads, with biomass encapsulated in each bead.

Contaminated air stream was obtained by injecting the substrate into the air stream by means of a syringe pump (Harvard Apparatus, Model 11). The flow rate of air was controlled by an MKS thermal mass flow controller (Controller 1259, Control Module 247). Because both air flow rate and substrate injection rate were precisely controlled, uniformity of the substrate composition in the air stream was ensured. The contaminated air stream was introduced at the bottom of the biofilter to ensure uniform distribution. OECD nutrient solution was introduced at the top of the biofilter bed at a flow rate of 300 mL/day. TCE concentrations were analyzed on a

Hewlett-Packard 5710A gas chromatograph with a 20-ft long, 1/8-in. diameter column having the packing (PT 10-percent Alltech AT-100 on Chromosorb W-AW 80/100). Carrier gas was nitrogen, and the detector was flame ionization (FID). Chloride ion concentrations in the nutrient solution were measured by an Orion solid-state chloride ion combination electrode (#9617BN) on an Accumet 1003 pH/mV/ISE meter. The pH of nutrient solutions was measured by a combination pH electrode connected to the above meter. Ammonia-nitrogen concentration in nutrient solution was measured by an Orion gas sensing ammonia electrode (#9512BN). Nitrite ions in nutrient solutions were detected using a Hach NI-7 nitrite detection kit.

Results and Discussion

Separate studies were conducted with toluene at 300 ppmv inlet concentration at various gas phase residence times. Figure 1 shows the removal efficiency as a function of gas phase residence time for toluene. Toluene degrades aerobically in the biofilter, achieving 100-percent removal efficiency at less than 1 min residence time.

Studies also were conducted with 25 ppmv inlet concentration of TCE at various gas phase residence times. No toluene was present in the inlet gas stream. Complete mineralization of TCE was observed at a gas residence time exceeding 4 min, suggesting a nonaerobic pathway. Corresponding increases in chloride ion were observed in the liquid nutrient phase, which demonstrated that TCE was mineralized to carbon dioxide and chloride ion. No partially chlorinated by-products were observed in the exit gas phase.

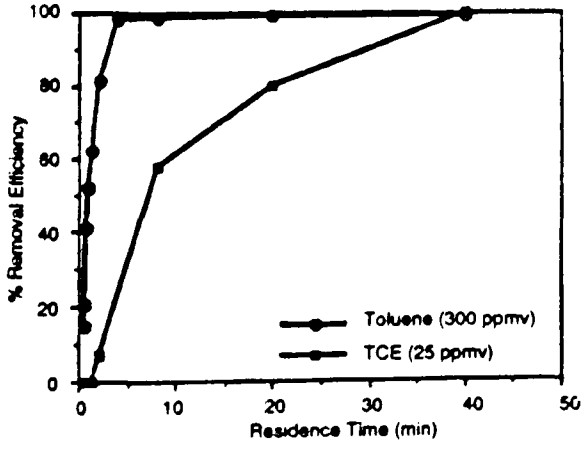

Figure 1. Plot of percent removal efficiency for toluene and TCE in the gel-bead biofilter with encapsulated biomass. Toluene and TCE studies were conducted separately.

Studies are currently being conducted to 1) measure the dissolved oxygen concentration as a function of depth in the hydrogel bead using a microsensor; 2) investigate the effect of bead-size on reactor removal efficiency for TCE (as the bead size decreases, the extent of the anaerobic zone is expected to decrease); 3) develop a mathematical model for the hydrogel bead biofilter and validate the model using the experimental data; and 4) extend the TCE study to other chlorinated solvents, such as PCE.

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Metabolites of Oil Biodegradation and Their Toxicity

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Development of strategies for the bioremediation of crude oil and refinery processed petroleum must build on a basic understanding of microbial degradation of oil and its many chemical constituents, as well as the limitations imposed on these processes by environmental factors. Numerous studies document microbial activities on bulk oil and its components (1,2), yet little is known of the formation, accumulation, and toxicity of compounds during oil biodegradation. Recent reports of petroleum-derived oxidation products in ground water (3) and in the tissues of mollusks (4) indicate the need to characterize products formed during crude oil biodegradation and to assess their environmental effects. This work addresses some of these questions.

Amounts of neutral and acidic materials recovered from different oil-degrading cultures (from both marine and terrestrial sources) were significantly greater than from sterile controls. Biologically generated neutral materials were toxic (100-percent mortality) to larvae of *Mysidopsis bahia* (5), to grass shrimp embryos (6), and to embryos of *Menidia beryllina* (7) at concentrations matching those at which they were formed in cultures. *Menidia* embryos exhibited developmental defects. Work is continuing to define the nature of the toxic components of these neutral fractions, their precursors in oil, and the microorganisms and processes that lead to their formation.

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TCE Remediation Using a Plasmid Specifying Constitutive TCE Degradation: Alteration of Bacterial Strain Designs Based on Field Evaluations

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An integrated study was undertaken to determine the potential for field application of altered strains of *Pseudomonas cepacia* G4 ($PR1_{23}$ and $PR1_{31}$) developed by us for the bioremediation of trichloroethylene (TCE). The investigation demonstrated the ability of $PR1_{23}$ to degrade TCE without inducer substrates via the constitutive expression of toluene *ortho*-monooxygenase (TOM). Two fundamental areas of research are detailed: 1) the effectiveness of the $PR1_{23}$ phenotype in a field bioreactor; and 2) laboratory transfer of the constitutive degradative phenotype to two new bacterial strains selected for their capacity to colonize bioreactor matrices. $PR1_{23}$ was field tested in a 100-L plugged flow reactor receiving contaminated water at 2 L/min and a daily batch input of cells (6 L) for a period of 2 weeks. Under these conditions, $PR1_{23}$ was able to effectively degrade TCE and *cis*-DCE in contaminated aquifer water at concentrations up to 700 µg/L (70- to 95-percent removal). The $PR1_{23}$ constitutive TOM phenotype, therefore, was desirable and effective. $PR1_{23}$, however, gave no indication of successful colonization of the reactor matrix, and biodegradation activity quickly fell following cessation of cell input. The TCE degradative genes and the genetic alteration responsible for their constitutive expression are present on a self-transmissible plasmid (pTOM). $PR1_{31}$ was used to allow transmission of the degradative plasmid ($pTOM_{31c}$) containing the constitutive TOM phenotype to two alternate *Pseudomonas* strains selected for superior colonization potential.

Both strains acted as competent recipients for $pTOM_{31c}$, constitutively expressing the encoded TOM and forming active biofilms in laboratory columns containing a diatomaceous earth matrix. This nonrecombinant transfer of constitutively expressed TCE degradative genes to bacteria prescreened for their stability in a particular environment represents a significant advantage over past strategies, which require that conditions be tailored to $PR1_{23}$ or $PR1_{31}$ survival. Such an approach may be extended to *in situ* treatment scenarios by transferring the constitutive phenotype to strains actually isolated from TCE-contaminated sites. The resulting organisms would have the advantage of being intrinsic to the particular site and of possessing an effective, nonrecombinant degradative activity.

Portions of this research were performed under a grant from the U.S. Air Force, Armstrong Laboratories, Environics Directorate, Tyndall Air Force Base, Florida.

Degradation of a Mixture of High Molecular-Weight Polycyclic Aromatic Hydrocarbons by a Mycobacterium Species

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A *Mycobacterium* sp., which was previously tested for its ability to mineralize several individual polycyclic aromatic hydrocarbons (PAHs), simultaneously degraded phenanthrene, anthracene, fluoranthene, pyrene, and benzo[a]pyrene in a six-component synthetic mixture. Chrysene, however, was not degraded to any significant extent. When provided with a primary carbon source, the *Mycobacterium* sp. degraded more than 74 percent of the total PAH mixture during 6 days of incubation. The *Mycobacterium* sp. appeared to degrade phenanthrene preferentially. No significant difference in degradation rates was observed between fluoranthene and pyrene. Anthracene degradation was slightly delayed, but, once initiated, degradation proceeded at approximately the same rate. Benzo[a]pyrene was degraded to a lesser extent. Additionally, degradation of a crude mixture of benzene-soluble PAH components from sediments resulted in a 47-percent reduction of the material in 6 days compared with autoclaved controls. Initial experiments using environmental microcosm test systems indicated that mineralization rates of individual [^{14}C] labeled compounds were significantly lower in the mixtures than in equivalent doses of these compounds alone. Mineralization of the complete mixture was estimated conservatively to be between 49.7 percent and 53.6 percent in 12 weeks. Mineralization was nearly 50 percent within 30 days of incubation when all compounds were radiolabeled. These results strengthen the argument for the potential application of this *Mycobacterium* sp. in bioaugmentation of PAH-contaminated wastes.

Bioavailability Factors Affecting the Aerobic Biodegradation of Hydrophobic Chemicals

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We are currently studying interactions between complex waste mixtures and microorganisms that are capable of transforming organic components of these mixtures. Our goal is to integrate methodologies used to study the abiotic behavior of hydrophobic organics in soil with the biological degradation of the organics. Sorption of hydrophobic compounds, such as polychlorinated biphenyls (PCBs), to soil represents a potential barrier to their degradation and detoxification in the environment, and influences the relative accessibility of these compounds to a number of physical, chemical, and biological processes. We find the concept of bioavailability a unique opportunity to couple interesting basic research to applied bioremediation problems. Our long-term objectives include 1) the study of the desorption of PCBs from historically contaminated soils and sediments; 2) the determination of the influence of co-contaminants, cosolvents, and surfactants on PCB desorption enhancement; and 3) the coupling of PCB desorption and biodegradation kinetics. The soil that we are studying is from a former racing drag strip in Glen's Falls, New York, contaminated with Aroclor 1242. Previous studies have shown that approximately half of the PCBs present in the soil are unavailable for aerobic biodegradation. This surface soil, classified as a sand (95 percent sand, 4.2 percent silt, and 0.8 percent clay), contains 1.9 percent organic carbon and 1.43 percent oil and grease. Mineralogical analyses show that the soil minerals consist of 40 percent quartz, 45 percent chlorite, and 15 percent Ca-albite (all low internal surface-area minerals). Heavy metal analysis suggests that only lead levels are somewhat high, averaging 190 ppm. Specific surface-area analysis indicates a low value of 0.1444 m^2/g. The total pore volume is 0.0016 cm^3/g, and the average pore diameter is 443.78 Å. We also are characterizing the following drag strip soil fractions individually: medium sand (2.00 mm to 0.425 mm), fine sand (0.425 mm to 0.08 mm), and silt/clay (< 0.08 mm). Studies on the biodegradation of PCBs found in each of the three fractions suggest that biodegradation of PCBs from the silt/clay fraction is less than biodegradation from the fine and medium sand fractions. Since the silt/clay fraction represents the major reservoir for organic carbon, oil and grease, heavy metals, and PCBs due to its high surface area, the release of PCBs from this fraction may be essential to enhancing PCB biodegradation. The biodegradation of the PCBs found in this fraction is currently the focus of our studies. We are using the traditional batch method to examine congener-specific desorption from the drag strip soil and the three

fractions. In addition, we will compare the miscible displacement technique with results from batch studies. The miscible displacement technique uses preparative high performance liquid chromatography (HPLC) glass columns packed with drag strip soil and high-precision HPLC pumps to provide a steady flow rate. Column effluent fractions are collected after passage through a flow-through variable-wavelength UV detector. Both the batch method and miscible displacement technique allow us to examine the influence of cosolvents and surfactants (biological and synthetic) on PCB desorption and mobility. Enhanced desorption and mobility may contribute to increased availability to biodegradation processes. In addition, we are examining the biodegradation of the oil and grease in the drag strip soil. Analysis of the oil and grease by column chromatography shows the distribution of organics to be 81.9 percent hydrocarbons, 16.9 percent polars, and 1.2 percent asphaltenes. This oil is very weathered and contains few readily biodegradable components. We are in the process of enriching for microorganisms capable of transforming this oil matrix and will test whether biodegradation of the oil results in nhanced availability and biodegradation of the PCBs present.

Section Six
Hazardous Substance Research Centers

The Hazardous Substance Research Centers (HSRC) conduct EPA research on bioremediation under the direction of ORD's Office of Exploratory Research (OER). Research is sponsored by the following centers: Northeast Hazardous Substance Research Center (Regions 1 and 2), Great Lakes and Mid-Atlantic Hazardous Substance Research Center (Regions 3 and 5), South/Southwest Hazardous Substance Research Center (Regions 4 and 6), 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).

The symposium's poster session included presentations on in situ attenuation of chlorinated aliphatics in glacial alluvial deposits; scaling up from a field experiment to a full-scale demonstration of in situ bioremediation of chlorinated solvent groundwater contamination; the bioavailability and transformation of highly chlorinated dibenzo-p-dioxins and dibenzofurans in anaerobic soils and sediments; localization of tetrachloromethane transformation activity in *Shewanella putrfaciens* MR-1; the formation and transformation of pesticide degradation products under various electron acceptor conditions; laboratory and field investigations of bioremediation of aromatic hydrocarbons at Seal Beach, California; and pneumatic fracturing to enhance in situ bioremediation.

In Situ Attenuation of Chlorinated Aliphatics in Glacial Alluvial Deposits

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The National Center for Integrated Bioremediation Research and Development (NCIBRD) has located operations at the recently decommissioned Wurtsmith Air Force Base (WAFB) in Oscoda, Michigan. NCIBRD is dedicated to the evaluation of decontamination technologies for hazardous wastes and remediation of spill and disposal sites. These activities are administered by the University of Michigan and oversight is provided by a science advisory board comprised of the directors of five Hazardous Substance Resource Centers, representatives of the EPA Biosystems Group, and nationally recognized engineers and scientists from government and private sectors.

WAFB is ideally suited for *in situ* bioremediation research activities. The 7-square-mile base is bordered by the Au Sable River to the south and west, and by Van Etten Lake to the east. The property sits on a 20-m bed of highly transmissive glacial sand underlain by a thick silty-clay aquitard. The ground water is found at about 6 m throughout the study area. The U.S. Air Force has been working with the U.S. Geological Survey (USGS) to characterize the extent of contamination at WAFB for the past 12 years, resulting in a large database and an array of approximately 600 permanent monitoring wells. An excess of 70 sites are tainted by a variety of sorbed, dissolved, and nonaqueous-phase petroleum hydrocarbon mixtures, chlorinated solvents, and heavy metals. Air Force remediation activities have been limited to the installation of three conventional air strippers for the containment of the largest plumes. These systems will provide the capture zone needed for the eventual controlled release of tracer chemicals, allowing an in-depth field study of the fate and transport of contaminants.

The USGS database provided information indicating that natural bioattenuation of aromatic and chlorinated aliphatic compounds was occurring at WAFB. A sampling program is currently being implemented to study the process at two of these sites: FT-02 (a heavily used fire training area) and OT-16 (a former jet engine test cell).

Fire training was conducted at FT-02 from 1952 to 1993. Typically, 8,000 L of jet fuel (and some incidental chlorinated solvents) was pumped over a simulated aircraft structure, ignited, and extinguished. Unfortunately, unburned fuel and solvents infiltrated into the aquifer. The USGS and Air Force installed 49 monitoring wells in 17 clusters to track the movement of the plume originating from this site. Preliminary well monitoring and solid borings have shown evidence of a large plume, with total volatile organic compounds exceeding 1,000 mg/L, that is undergoing natural biotransformation. Concentrations of these compounds in the aquifer solids reflect co-metabolic transformations; in other words, upgradient vadose zone levels of trichloroethylene (5 mg/kg), BTEX (600 mg/kg), and dissolved oxygen decrease and concentrations of cis-1,2-dichloroethylene increase to 5 mg/kg downgradient from the site. This site is located approximately 300 m from OT-16 and is hydraulically connected; plumes from these sites are believed to merge downgradient.

The jet engine test cell was used for a variety of test activities. Cleanup of this structure typically involved washing solvents off the floor into an oil-water separator, which eventually failed, allowing the solvents to enter the aquifer. The plume contains high concentrations of BTEX (4 mg/L) and moderate amounts of chlorinated solvents (70 mg/L). The Air Force installed 19 wells downgradient of this site, but little sampling has been done. NCIBRD has just begun site characterization efforts at this site.

Future work at these two sites will supplement existing physical-chemical information with location and geophysical surveys, meteorological monitoring, additional borings and monitoring well emplacements, soil gas surveys, permanently installed water level recorders, grain-size and hydraulic conductivity determinations, as well as chemical property measurements (e.g., mineralogy, carbonate, organic carbon, metal and metal oxide content, cation-exchange capacity, etc.). In addition, routine well sampling will document not only contami-

nant concentrations but also changes in metabolic levels in the aquifer. This effect will support experimental applications of *in situ* remediation technologies to be conducted by consulting, private industry, and academic professionals.

In Situ Bioremediation of Chlorinated Solvent Ground-Water Contamination: Scaling up from a Field Experiment to a Full-Scale Demonstration

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Studies conducted at an experimental field site at Moffett Naval Air Station have demonstrated that trichloroethylene (TCE) can be effectively biodegraded co-metabolically through the introduction into the subsurface of a primary substrate (such as phenol or toluene) and oxygen to support the growth and energy requirements of a native population of microorganisms. Additional preliminary experimental work at Moffett Field now has been conducted in preparation for a full-scale demonstration.

A full-scale demonstration at a real hazardous waste site is likely to encounter a plume with multiple contaminants. It was desirable, therefore, to determine how other contaminants which could potentially be present might affect the rate and extent of TCE degradation. In particular, previous laboratory studies at Stanford University have indicated that the degradation products of 1,1-dichloroethylene (DCE) are toxic to methane-oxidizing bacteria. Follow-on field work conducted at Moffett Field demonstrated that the presence of 1,1-DCE inhibited TCE degradation by phenol-oxidizing microorganisms. Thus, 1,1-DCE should not be present at the site selected for a full-scale demonstration of this technology.

An effective method to provide the indigenous microorganisms with sufficient oxygen to oxidize the primary substrate is needed for the field demonstration. In past studies at Moffett Field, molecular oxygen has been used as an oxygen source. Molecular oxygen, however, is difficult to transfer to solution. Hydrogen peroxide is an alternative oxygen source that has been used in bioremediation of petroleum hydrocarbons and is much easier to apply to the subsurface than molecular oxygen. Preliminary work at Moffett Field showed that hydrogen peroxide worked as effectively as molecular oxygen in degrading TCE.

Another question that needs to be answered prior to full-scale implementation of this technology is how best to mix a primary substrate, an oxygen source, and TCE and to deliver the mixture to the microorganisms. At Moffett Field, mixing of these three components was accomplished aboveground, with the mixture then introduced into the subsurface through an injection well. In a full-scale demonstration, the TCE will, of course, already be in the ground water. A major objective of this demonstration will be to investigate how a primary substrate and an oxygen source can be efficiently mixed and transported to indigenous microorganisms, to promote co-metabolic degradation of TCE. For the demonstration, a subsurface recirculation system similar to that described by Herrling (1) and McCarty and Semprini (2) is expected to be used. The remediation system will consist of a single well, screened at two depths. In operation, a submersible pump installed between the two screens would draw TCE contaminated water into the well at one screened interval. The primary substrate and oxygen will then be introduced into the water through feed lines, and the water, which now contains TCE, primary substrate, and oxygen, will be discharged into the aquifer from the second screened interval. In essence, an *in situ* treatment zone will be created in the aquifer around the discharge screen. Based on the Moffett Field results, this treatment zone is expected to cover an area within approximately 1 day's groundwater travel distance out from the well.

Ultimately, these studies, in which the laboratory and the Moffett Field site are being used to make predictions regarding processes and to help design systems at a "real-world" site, hopefully will help lead to a better understanding of how laboratory and field investigations can best be scaled up to make better real-world predictions.

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Bioavailability and Transformation of Highly Chlorinated Dibenzo-p-dioxins and Dibenzofurans in Anaerobic Soils and Sediments

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Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are introduced via several industrial and municipal channels into both aerobic and anaerobic environmental compartments. Because of their high toxicity and uncertain genotoxic potential, their determination and fate in environmental samples is of great interest. The fate of highly chlorinated PCDD/PCDF congeners was studied in both high and low organic carbon anaerobic microcosm incubations. The inocula were derived from historically contaminated anaerobic environments such as polychlorinated biphenyl-contaminated sediments and creosote-contaminated aquifer samples, and were amended with a mixture of aromatic and aliphatic acids for methanogenic growth. The samples were analyzed and quantified using high resolution gas chromatography coupled with an electron capture detector and a low resolution mass selective detector operated in selected ion monitoring (SIM) mode ([M^+], [M^++2], and [M^++4] ions). Recovery efficiencies after soxhlet extraction and sample cleanup were 40 to 70 percent, based on 1,2,3,4-tetrachlorodibenzo-*p*-dioxin as an internal standard. The long-term (2 years) removal patterns of sediment-sorbed PCDDs/PCDFs in both sediments could be explained by labile and resistant PCDD/PCDF desorption components, presumably because of intraparticle diffusion-controlled mass transfer limitations. Mass transfer limitations were based on incubation time-dependent decreased extraction efficiencies of PCDDs/PCDFs from inactive controls. The net first-order initial rate constants of disappearance ranged from 0.30 to 0.75 ($\times 10^{-3}$) d^{-1} for aquifer sediments and from 0.46 to 1.87 ($\times 10^{-3}$) d^{-1} for high organic carbon Hudson River sediments. Moreover, the overall decrease in PCDDs/PCDFs from the sediment particles in active microcosms sacrificed after 30 months was as much as 20 percent greater compared with the autoclaved controls. Lesser chlorinated congeners were found in all active microcosms analyzed. Isomer-specific analysis of the lesser chlorinated congeners indicated that the 1,4,6,9-chlorines were removed preferentially, thus enriching the medium in 2,3,7,8-substituted congeners and increasing the overall relative toxicity. These observations contribute to our knowledge regarding the fate of PCDDs/PCDFs in anaerobic soils and sediments, and indicate the importance of congener "fingerprinting" during environmental source/fate analysis.

Localization of Tetrachloromethane Transformation Activity in Shewanella Putrefaciens MR-1

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Investigations of pollutant transformation by pure cultures may enhance our understanding of *in situ* natural attenuation processes in these environments. *Shewanella putrefaciens* MR-1, an Fe(III)- and Mn(IV)-reducing facultative anaerobe, has been shown to dechlorinate tetrachloromethane (CT) to chloroform (24 percent), after growth under nitrate- or Fe(III)- respiring conditions. Mass balance for carbon included 56-percent incorporation in biomass, 4.1-percent formation of nonvolatile products, and 5.5-percent mineralization. Product distribution was independent of growth conditions. Amendment of MR-1 cell suspensions with lactate, formate, or hydrogen increased CT transformation activity, while methanol did not. The rate and extent of CT transformation increased for MR-1 cells grown with electron acceptors having more positive half-reduction potentials ($E^{o'}$). Nitrate did not inhibit CT transformation. In the presence of Fe(III), reductive dechlorination was enhanced and resulted in the production of dichloromethane (DCM), presumably by abiotic mechanisms involving Fe(II).

In MR-1 cell extracts, NADH was the most effective electron donor for CT transformation. Addition of FMN increased the activity 3- to 10-fold. Furthermore, CT transformation activity has been localized primarily to membrane fractions (89 percent).

The effects of respiratory inhibitors on CT transformation activity have been examined. Rotenone, an inhibitor of NADH dehydrogenase, reduced CT transformation activity in MR-1 whole-cell suspensions using lactate or NADH as an electron donor. Quinacrine, an inhibitor of flavins, enhanced this activity. No significant effect was seen in the presence of pCMPS, sodium azide, and sodium cyanide or in the presence of the cytochrome inhibitors HQNO and Antimycin A. These results suggest that transformation of CT may be mediated by a nonheme electron transfer agent.

Respiratory mutants of MR-1 have been screened for CT transformation activity. Rates of CT transformation for MR-1 mutants in Fe(III) reductase, Mn(IV) reductase, or fumarate reductase were equivalent or greater than those for the MR-1 wild-type strain. MR-1 mutants that did not synthesize menaquinones (MK) and so lost the ability to couple nitrate, Fe(III), or fumarate reduction for growth also lost 90 percent of CT transformation activity. When cell suspensions of MK-deficient mutants were complemented with an MK precursor, CT transformation rates returned to MR-1 wild-type levels. These results indicate that MK or another electron transfer mediator reduced by MK but not a terminal reductase may be responsible for CT transformation by MR-1.

Formation and Transformation of Pesticide Degradation Products Under Various Electron Acceptor Conditions

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Pesticide contamination of ground-water supplies is a serious and growing problem in the United States. More than 600 active chemicals exist that are used to protect crops from target pests (1). Pesticides can remain in the environment for a long time, entering the air or ground-water supply by partitioning to or diffusing through the soil column. Transformation of these chemicals to one or more principal metabolites often occurs with unknown and unmonitored results. To develop systems to destroy these contaminants and formulate intelligent policies to regulate or restrict their use, an understanding of the reactions that these compounds undergo in the environment is essential.

The herbicides alachlor and atrazine, the two most commonly used pesticides in the nation, together account for 25 percent by weight of total pesticide use (2). These herbicides are also the two most frequently detected pesticide contaminants in ground-water supplies in the Midwest (2). Many xenobiotics can undergo mineralization to carbon dioxide and water by biological means; alachlor and atrazine, however, undergo very little mineralization under typical environmental conditions. Mineralization has been observed by only a few researchers, generally at quantities of less than 5 percent of the initial herbicide concentration. As a single exception, a recently completed study revealed that atrazine, when serving as the sole nitrogen source for a microbial population, was mineralized at levels of greater than 80 percent of the initial concentration, with a half-life of 0.5 to 2.0 days using a microbial consortium that had undergone more than 5 months of subculturing and enrichment in the laboratory (3). With little natural mineralization occurring under typical environmental conditions, transformation intermediates of alachlor and atrazine may be formed and may be accumulating in the soil and ground water.

The specific objectives of this research project were to identify the transformation products of alachlor and atrazine under four common electron acceptor conditions (aerobic, denitrifying, sulfate-reducing, and methanogenic) and, to the extent possible, determine kinetic coefficients that describe the rate of formation and disappearance of these metabolites.

Experimental Design

Four 9-L, fill-and-draw reactors were established to maintain specific environmental conditions. Each reactor was fed a mineral nutrient solution typical of ground water under the redox condition of interest. Temperature was maintained at 20°C in the dark to mimic environmental conditions. Each of the reactors was fed acetate as the carbon and energy source, with some of the batch denitrifying experiments carried out with citrate as an electron donor as well. Alachlor and atrazine were fed at approximately 100 µg/L each, along with a phosphate buffer to maintain a neutral pH. In addition, the specific electron acceptor for each system was added in excess: O_2 for the aerobic reactor, KNO_3 for the denitrifying reactor, and $MgSO_4 \cdot 7H_2O$ (at a high sulfate-to-organic ratio) for the sulfate-reducing reactor. The bacteria in each system were acclimated to alachlor and atrazine prior to the start of the experiments.

Control experiments were set up to determine which physical and chemical means of alachlor and atrazine transformation were important. The potential role of the phosphate buffer in catalyzing chemical hydrolysis of alachlor and atrazine was studied with a phosphate control. Reactions with resazurin, a color indicator of redox potential used in the denitrifying reactors, also were studied using several control reactors with varying resazurin concentrations. A mercuric-chloride-killed biological control was used to investigate sorption to biomass, and to further assess the role of resazurin. Finally, a deionized water control was employed to identify mixing problems, the significance of alachlor and atrazine sorption to the reactor itself, and potential volatilization, chemical hydrolysis, or photolysis reactions.

All experiments were carried out in a batch format. An initial dose of alachlor and atrazine was added to the reactor and allowed to mix for approximately 45 min, then samples for pesticide analysis were taken from the reactor at various time intervals. The denitrifying and control experiments were carried out in 2-L Pyrex bottles built like the larger 9-L reactors, so several different conditions could be tested without affecting the stock enrichment culture. The experiments involving the methanogenic and sulfate-reducing systems were carried out in the 9-L reactors.

Results

Initially, alachlor and atrazine disappeared in batch reactors maintained under all terminal electron acceptor conditions except aerobic conditions. Further experiments involving the aerobic reactor were abandoned because of the absence of noticeable degradation of parent compounds. Resazurin was added only to the denitrifying reactors to indicate whether the proper conditions were maintained. This compound was found to be involved in the abiotic transformation of alachlor and atrazine. Second-order degradation constants for alachlor and atrazine transformation are given in Table 1; these constants are averaged values for four experiments for each of the different terminal electron acceptor conditions. Each of these rate constants has been corrected for the abiotic transformation of atrazine and alachlor in the denitrifying reactors due to resazurin, and the abiotic transformation of alachlor in the methanogenic and sulfate-reducing reactors due to the bisulfide ion. Therefore, the values given in Table 1 represent only the biological transformation of alachlor and atrazine.

The standard deviation of these rate constants is relatively high, for two reasons. First, in the denitrifying experiments duplicate reactors were used that contained different quantities of biomass and most likely slightly varying microbial populations as well. A slight change in the relative numbers of the different microorganisms present could result in the differences that were observed in alachlor and atrazine transformation rates among the different reactors. For the experiments involving the methanogenic and sulfate-reducing environments, one reactor was used for the four experiments. Upon complete degradation of alachlor, 1 to 2 weeks were allowed to pass with no pesticides added to the reactors while electron donor and acceptor levels were maintained. At this point, alachlor again was dosed to the reactors, and the next experiment was started. Over the course of the four experiments, the rate of alachlor transformation decreased considerably under both methanogenic and sulfate-reducing conditions. At the end of the fourth experiment, no acetate utilization was observed in either reactor, and no methane production occurred in the methanogenic reactor. At this point, 2 L of fresh ground-water media was added to each of the reactors and the normal fill-and-draw feeding was resumed, but no pesticides were added to either reactor. After 2 months, no recovery of either population was observed. This effect on the microbes was thought to have been a result of the buildup of nonmetabolizable and toxic alachlor or atrazine metabolites.

Several metabolites of alachlor were positively identified in these systems. Under denitrifying conditions with resazurin and organisms present, aniline, m-xylene, acetyl alachlor, and diethyl aniline were positively identified as products of alachlor degradation. Aniline, identified and quantified by gas chromatography/mass spectrometry (GC/MS), appeared between Days 12 and 17 of the 45-day experiment and had degraded below detection limits by the last day. At the maximum aniline concentration, 35 percent of the initial alachlor added had degraded to aniline. Aniline formation and degradation constants are listed in Table 2; these rate constants are based on the assumption that aniline is formed as a direct result of alachlor degradation and that biomass remains constant throughout the experiment. Aniline formation was assumed to have occurred to some maxima, at which point degradation began. Experiments are presently under way to study the degradation of aniline in reactors fed only this compound. The presence of aniline in ground water as a result of alachlor degradation is possible, but the high rate of aniline

Table 1. Second-Order Degradation Constants for Alachlor and Atrazine under Three Terminal Electron Acceptor Conditions

	Second-Order Degradation Constant	
Conditions	**Alachlor**	**Atrazine**
Denitrifying Reactor	7.9×10^{-5} ($\pm$ 4.1×10^{-5}) L/mg VSS day	6.7×10^{-5} (5.3×10^{-5}) L/mg VSS day
Methanogenic Reactor	2.9×10^{-3} ($\pm$ 1.6×10^{-3}) L/mg VSS day	8.4×10^{-5} L/mg VSS day
Sulfate-reducing Reactor	1.5×10^{-2} ($\pm$ 1.4×10^{-2}) L/mg VSS day	6.5×10^{-5} L/mg VSS day
Resazurin	5.0×10^{-2} ($\pm$ 5.4×10^{-2}) L/mg res day	4.2×10^{-2} (4.2×10^{-2}) L/mg res day
Bisulfide Ion (4)	1.5×10^{-3} L/mg VSS day	—

Table 2. Second-Order Formation and Degradation Constants for Aniline in the Reactor Containing Both Resazurin and Denitrifying Organisms

Second-Order Formation Constant	Second-Order Degradation Constant
8.4×10^{-5} L/mg VSS day	4.8×10^{-3} L/mg VSS day

removal by aerobic microorganisms makes the persistence of this substance for a period of longer than a few days unlikely. Under reducing conditions in an aquifer, however, aniline may persist for a few weeks or conjugate to form compounds such as diphenylamine.

In the denitrifying reactors containing resazurin and acetate-utilizing organisms, m-xylene, a suspected human carcinogen, appeared between Days 17 and 22 of the experiment and had disappeared by Day 31. On Day 22, the highest m-xylene concentration was present in the reactor sample and corresponded to approximately 9 percent of the initially fed alachlor.

m-Xylene also was detected in an abiotic reactor containing only resazurin, atrazine, and alachlor under denitrifying conditions. On Day 45, the highest observed m-xylene concentration was present in this reactor and accounted for 17 percent of the initial alachlor concentration. Because this compound also is readily biodegradable, it is unlikely that m-xylene would persist in ground water as a result of alachlor contamination and subsequent transformation. The role of resazurin was not clearly defined. Biomass growth was observed in the reactor containing only resazurin, alachlor, and atrazine, indicating that resazurin most likely served as an electron donor for organism growth. Therefore, it is unclear whether resazurin itself or the organisms that were capable of growth on only resazurin were responsible for the formation of m-xylene in this reactor.

One of the denitrifying reactors contained only biomass; in this reactor, neither aniline nor m-xylene was detected. Resazurin, or perhaps some compound that facilitates electron transfer, such as vitamin B_{12}, may be required for at least one step in the degradation pathway that leads to aniline and m-xylene production.

In the methanogenic and sulfate-reducing reactors, diethyl aniline and acetyl alachlor were detected. Because these conditions are highly reducing, acetyl alachlor is an expected product and is likely formed as a result of reductive dechlorination. Acetyl alachlor could not be quantified because the sample received from Monsanto had evaporated to a residue. Diethyl aniline is a product of further microbial attack of the ether and carbonyl groups of alachlor. At the highest observed concentration, diethyl aniline represented 9 percent and 20 percent of the initial alachlor added to the system in the methanogenic and sulfate-reducing reactors, respectively. Two unidentified metabolites, SM1 and SM2, accumulated in both reactors, perhaps causing the toxicity that eventually caused the organisms to stop their degradation of acetate, alachlor, and atrazine.

Using the gas chromatograph with both an electron capture detector (GC/ECD) and a nitrogen-phosphorous detector (GC/NPD), along with the GC/MS, many transformation products were observed in all of the reactors yet could not be positively identified. By preliminarily identifying these compounds using a spectra library from the National Bureau of Standards on the GC/MS, an idea of the identity of some of these products was gained. Some of the compounds were long, branched, saturated, and unsaturated hydrocarbon chains and were probably caused by the breakdown and microbial metabolism of acetate and citrate. Other compounds appeared to be caused by the conjugation or substitution of two or more substances. Transformation products appeared to be formed by many different mechanisms, such as dealkylation or reductive dechlorination, and had widely varying concentration profiles. Compounds like acetyl alachlor in the denitrifying reactor appeared and disappeared in a few days. Other compounds, such as diethyl aniline and the unknown metabolites SM1 and SM2 detected in the methanogenic and sulfate-reducing reactors, were long-lived, persisting in the reactor over a period of weeks.

No transformation products of atrazine were identified under any of the conditions investigated. Since atrazine disappearance was measured in the denitrifying, methanogenic, and sulfate-reducing systems, and complete mineralization to carbon dioxide and water was very unlikely, metabolites should have been formed in these reactors. The C-18 solid-phase extraction column used is reportedly not very effective at trapping polar substances. It is likely that polar transformation products such as hydroxyatrazine were produced; the polar products probably were lost during sample extraction because only those compounds that were extractable by the use of the C-18 column were analyzed. Their loss is a possible explanation for the lack of detected transformation products of atrazine. As new solid-phase extraction columns are developed for effective extraction of pesticides and their polar metabolites, more transformation products will be identified in these systems.

Summary and Conclusions

The speed and specific degradation steps followed in the transformation of alachlor and atrazine, and the various degradation products that are formed as a result of this transformation, are strong functions of

environmental conditions, namely, the terminal electron acceptor conditions present. In alachlor degradation, aniline and m-xylene were products detected only in the denitrifying reactors. On the other hand, acetyl alachlor was identified under denitrifying, methanogenic, and sulfate-reducing conditions. The product formation and transformation patterns during alachlor degradation were very different in each of these systems. Analytical limitations prevented the identification of likely pola. products of atrazine degradation. Further study is required to identify more of the metabolites that are formed and to try to formulate a degradation pathway for alachlor and atrazine. The electron acceptors present, and consequently the microbial population developed in these systems, affect the rate of herbicide transformation, the pathway that this degradation takes, and the products that are formed that may accumulate in the systems. The conditions under which herbicide degradation takes place also can result in the formation of compounds that are human health hazards and could be a threat to ground-water supplies.

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Bioremediation of Aromatic Hydrocarbons at Seal Beach, California: Laboratory and Field Investigations

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The objective of this study was to develop our understanding of processes that are important in the anaerobic biodegradation of aromatic hydrocarbons in contaminated ground-water aquifers. The focus of the investigation was a site at the Seal Beach Naval Weapons Station in Southern California, where a significant gasoline spill resulted in contamination of the groundwater aquifer. The project was divided into laboratory and field components, which were interrelated. The goals of the laboratory experiments were to determine the capability of the aquifer microbial community to transform aromatic hydrocarbon compounds under various anaerobic conditions and to understand the effect of environmental factors on the transformation processes. Field experiments were carried out on site at Seal Beach. The objectives of the field experiments were to evaluate potential *in situ* application of anaerobic bioremediation processes and to attempt to apply laboratory results to the field. The results from the field experiment will be used to design a remediation proposal for the aquifer at the Seal Beach site.

Approach and Results

Laboratory Study

In a laboratory microcosm experiment, we evaluated several factors that were hypothesized to influence field-scale bioremediation. Individual monoaromatic compounds (e.g., benzene, toluene, ethylbenzene, and m-, p-, and o-xylene) were the primary substrates. To test the influence of liquid-phase composition on the hydrocarbon degradation potential of Seal Beach aquifer sediment, the sediment was placed in native ground water, native ground water with nutrient amendments, and various other laboratory media formulations including denitrifying, sulfate-reducing, and methanogenic media. In replicate bottles during the first 52 days of the study, toluene and m+p-xylene (here, m-xylene and p-xylene were measured as a summed parameter) were biotransformed in the unamended ground-water samples under presumed sulfate-reducing conditions. Addition of nitrate to the ground water increased rates of toluene biotransformation coupled to nitrate reduction, stimulated biotransformation of ethylbenzene, and inhibited the complete loss of m+p-xylene that was observed when nitrate was not added and sulfate-reducing conditions prevailed. Addition of the nutrients ammonia and phosphate had no effect on either the rate of aromatics transformation or the distribution of aromatics transformed. In contrast to nitrate-amended ground water, ethylbenzene was always transformed first followed by toluene in the microcosms prepared with denitrifying media. In sulfate-reducing media, lag times were increased, but toluene and m-xylene were ultimately transformed just as in the microcosms with ground water alone. Although methane had been detected in the field, there appeared to be no transformation activity in the methanogenic microcosms during the period of the experiment.

Bioreactor Study

A pilot-scale facility consisting of 90-L reactors was constructed at the Seal Beach site. The facility was designed for the operation of three anaerobic *in situ* bioreactors. The reactors consisted of aquifer sediment filled stainless steel cylindrical vessels with the capability to control and monitor both hydrodynamic flow and supplements to the composition of the native groundwater influent. Initial operation of the three anoxic/anaerobic reactors focused on evaluating anaerobic bioremediation strategies for aromatic hydrocarbons under existing (presumed sulfate-reducing) and enhanced denitrifying conditions. Bioreactor results were consistent with the laboratory microcosm experiments. Toluene and m+p-xylene were degraded in both the unamended and nitrate-amended bioreactors. Degradation of ethylbenzene was stimulated by nitrate addition. Evidence indicated that benzene or o-xylene was not

transformed in either reactor. The final percentage removal efficiency appeared to be higher in the unamended bioreactor, where flow was slower.

Field Study

Field experiments have been conducted to assess anaerobic bioremediation of a test zone within the contaminated aquifer at the Seal Beach site. A network of eight observation wells and one extraction well was installed at the Seal Beach site. Hydrodynamic evaluation of the well field indicated that two of the wells were satisfactory for further experimentation. Experiments have been conducted using a slug test experiment design in which a single well was used for the injection of the "slug" or test pulse and the same well was used to extract the test pulse. The results of the experiments were inferred by differences measured in the samples collected during extraction. Since the native ground water contained a variety of electron acceptors and the water used for the injected pulses was water that had previously been extracted from the test zone, the ground water was treated to control the concentration of all electron acceptors during the injection of the test pulse. Before injection, the desired salts were added back to the deoxygenated injection stream and the stream metered into the injection well. Sodium bromide was added as a conservative tracer. Under this scenario, the different electron acceptors investigated (e.g., nitrate and sulfate) could be added as desired. During initial tracer studies, the injection water was organics free, and thus the source of the organics was desorption from the *in situ* aquifer solids. In subsequent and ongoing bioremediation studies, benzene, toluene, ethylbenzene, m-xylene, and o-xylene were added with the injection pulse at a concentration of approximately 200 µg/L each.

The initial bromide tracer data showed stable tracer concentrations and indicated no substantial encroachment of native ground water detected in the first 0.4 pore volumes. A very small hydraulic gradient existed at the site, hence recovery of the bromide mass from the test wells ranged from 93 to 99 percent with the extraction of three pore volumes over a 103-day period. During the tracer test, the equilibrium desorption concentrations for the aromatic hydrocarbons when the electron acceptors nitrate and sulfate were absent from the ground water were evaluated. Benzene, ethylbenzene, and o-xylene concentrations remained relatively stable and thus appeared to be at an equilibrium. The toluene and m+p-xylene concentrations had a downward trend relative to benzene once the native ground water encroached after approximately 0.4 pore volumes, suggesting that the nitrate and sulfate concentrations available in the native ground water supported some biological activity in the latter part of the experiment for toluene and m+p-xylene removal.

In a nitrate augmentation experiment, nitrate and aromatics were added to the injection pulse, resulting in complete consumption of toluene and ethylbenzene followed by m-xylene within the first 2 weeks. o-Xylene was degraded slowly, and its concentration approached zero by Day 60. No apparent loss of benzene occurred when compared with the inert tracer. The addition of nitrate to the test region appeared to enhance the natural anaerobic denitrifying population, confirming the presence of an already active nitrate-reducing population in the aquifer whose activity was enhanced by the addition of nitrate. With the exception of o-xylene transformation, these results were comparable with those from the nitrate-amended microcosm and bioreactor experiments, wherein toluene, ethylbenzene, and m-xylene were transformed under denitrifying conditions.

During the tracer study, methane was detected in the test wells. With the encroachment of the native ground water and associated increase in nitrate and sulfate concentrations, the methane concentration decreased to values close to zero, suggesting that nitrate and sulfate inhibit methanogenesis at this site.

Additional experiments are under way to determine more precisely some of the kinetic constants in the aquifer under denitrifying conditions and to evaluate rates and removal of aromatics under sulfate-reducing and methanogenic conditions.

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Pneumatic Fracturing To Enhance In Situ Bioremediation

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In situ bioremediation often is limited by the transport rate of nutrients and electron acceptors (e.g., oxygen, nitrate) to microorganisms, particularly in soil formations with moderate to low permeability. An investigation is under way to integrate the process of pneumatic fracturing with bioremediation to overcome these rate limitations. Pneumatic fracturing is an innovative technology that uses high pressure air to create artificial fractures in contaminated geologic formations, resulting in enhanced air flow and transport rates in the subsurface. The pneumatic fracturing system also can be used to inject nutrients and other biological supplements directly into the formation.

A project to investigate the coupling of these two technologies has been sponsored by EPA under the Superfund Innovative Technology Evaluation (SITE) Emerging Technologies Program and is scheduled for completion in the summer of 1994. Laboratory and field studies are being carried out simultaneously to degrade benzene, toluene, and xylenes (BTX) in gasoline. The laboratory studies are examining the physical and biological processes at and near the fracture interfaces, including diffusion, adsorption, and biodegradation. Both column and batch studies are being used to observe and quantify the individual and combined effects of these processes. For the field portion of the studies, a pilot demonstration is under way at an industrial site contaminated with gasoline that is underlain by fill and natural claylike soils. First, a full-size prototype of the integrated pneumatic fracturing/bioremediation system was developed. The site then was pneumatically fractured, and periodic injections of nutrients are continuing over a period of 10 months. Off-gases from the monitoring wells are being analyzed for BTX, oxygen, methane, and carbon dioxide to evaluate process effectiveness. Preliminary results from the laboratory studies and field demonstration available at the time of the conference will be presented.

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