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May 1993

HEBERT

Symposium on Bioremediation of Hazardous Wastes: Research, Development, and Field Evaluations

Abstracts

The Fairmont Hotel
Dallas, TX
May 4-6, 1993

U. S. ENVIRONMENTAL PROTECTION
AGENCY
1445 ROSS AVENUE
DALLAS, TEXAS 75202

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CONTENTS

Page

Bioremediation Field Initiative 1

Retrospective Performance Evaluation on In Situ Bioremediation:
Site Characterization 3
John T. Wilson and Don H. Kampbell

Retrospective Performance Evaluation on In Situ Bioremediation:
Modeling and Risk Assessment 10
Tissa H. Illangasekare, David C. Szlag, and John T. Wilson

Evaluation of Full-Scale In Situ and Ex Situ Bioremediation of
Creosote Wastes in Soils and Ground Water 14
Ronald C. Sims, John E. Matthews, Scott G. Huling,
Bert E. Bledsoe, Mary E. Randolph, and Daniel Pope

An Evaluation of Concurrent Bioventing of Jet Fuel and Several
Soil Warming Methods:
A Field Study at Eielson Air Force Base, Alaska 20
Gregory D. Sayles, Robert E. Hinchee, Catherine M. Vogel,
Richard C. Brenner, and Ross N. Miller

Documenting Bioventing of Jet Fuel to Great Depths:
A Field Study at Hill Air Force Base, Utah 27
Gregory D. Sayles, Robert E. Hinchee,
Richard C. Brenner, and Robert Elliott

Application of Wood-Degrading Fungi to Treat Contaminated Soils 34
John A. Glaser, Richard T. Lamar, Mark W. Davis, and Diane M. Dietrich

Performance Evaluation 39

Field Evaluation of Phenol for Cometabolism of Chlorinated Solvents 41
Gary D. Hopkins, Lewis Semprini, and Perry L. McCarty

Innovative Bioremediation Strategies for Creosote:
Characterization and Use of Inocula 47
James G. Mueller, Suzanne E. Lantz, Jian-Er Lin, and P. Hap Pritchard

Decontamination of PCB-Contaminated Sediments Through the Use of
Bioremediation Technologies 51
John F. Quensen, III, Stephen A. Boyd, James M. Tiedje, and John E. Rogers

CONTENTS (cont.)

Page

Natural Anaerobic Bioremediation of TCE at the St. Joseph, Michigan, Superfund Site 57
Peter K. Kitanidis, Lewis Semprini, Don H. Kampbell, and John T. Wilson

Comparison of Bioventing and Air Sparging for In Situ Bioremediation of Fuels 61
Don H. Kampbell, Christopher J. Griffin, and Frank A. Blaha

Determining the Genotoxicity of Bioremediation Products 68
Larry D. Claxton, S. Elizabeth George, Robert W. Chadwick, Virginia S. Houk, L.E. Rudd, J.J. Perry, and John T. Wilson

Field Research 71

Field Demonstration of a Constitutive TCE-Degrading Bacterium for the Bioremediation of TCE 73
Malcolm S. Shields, Michael Reagin, Robert Gerger, Rhonda Schaubhut, Robert Campbell, Charles Somerville, and P. Hap Pritchard

Bioremediation of TCE: Monitoring the Fate and Effects of a Microorganism Used in a Field Bioaugmentation Study 80
M.S. Shields, R. Snyder, M. Reagin, R. Gerger, R. Campbell, C. Somerville, and P.H. Pritchard

Factors Determining the Effectiveness of Microbial Inoculation in Soils and Sediments: Effectiveness of Encapsulation 86
Jian-Er Lin, James G. Mueller, and P. Hap Pritchard

Combining Treatability Studies and Site Characterization for Rational Design of In Situ Bioremediation Using Nitrate as an Electron Acceptor 90
S.R. Hutchins, D.H. Kampbell, M.L. Cook, F.M. Pfeffer, R.L. Cosby, J.T. Wilson, B. Newell, J.A. Johnson, V. Ravi, and J.K. Rumery

Rate and Extent of Natural Anaerobic Bioremediation of BTEX Compounds in Ground Water Plumes 100
Morton A. Barlaz, Michael B. Shafer, Robert C. Borden, and John T. Wilson

Pilot-Scale Research 107

Treatment of PCP-Contaminated Soils by Washing with Ethanol/Water Followed by Anaerobic Treatment 109
Amid P. Khodadoust, Julie A. Wagner, Makram T. Suidan, and Steven I. Safferman

CONTENTS (cont.)

Page

Preliminary Evaluation of Attachment Media for Gas Phase Biofilters 115
Francis L. Smith, George A. Sorial, Paul J. Smith, Makram T. Suidan, Pratim Biswas, and Richard C. Brenner

Process Research 121

Methanogenic Degradation Kinetics of Nitrogen and Sulfur Containing Heterocyclic Aromatic Compounds in Aquifer-Derived Microcosms 123
E. Michael Godsy, Donald F. Goerlitz, and Dunja Grbić-Galić

Anaerobic Degradation of Halogenated and Nonhalogenated Phenolic Compounds 129
M.M. Häggblom, M.D. Rivera, and L.Y. Young, and J.E. Rogers

Anaerobic Biodegradation of 5-Chlorovanillate as a Model Substrate for the Bioremediation of Paper-Milling Waste 131
Barbara R. Sharak Genthner

Bioremediation of Soils and Sediments Contaminated with Aromatic Amines 137
Eric J. Weber, David L. Spidle, and Kevin A. Thorn

Anaerobic Biotransformation of Munitions Wastes 139
Deborah J. Roberts, Stephen Funk, Don L. Crawford, and Ronald L. Crawford

Cometabolic Biodegradation of 2,4-Dinitrotoluene Using Ethanol as a Primary Substrate 145
Jiayang Cheng, Makram T. Suidan, and Albert D. Venosa

Effects of Metals on Anaerobic Treatment Processes 149
W. Jack Jones and In Chul Kong

Fate of Highly Chlorinated Dibenzo-*p*-Dioxins and Dibenzofurans in Anaerobic Soils and Sediments 155
Peter Adriaens, Dunja Grbić-Galić, and Gregory D. Sayles

Bioreactor Treatment of Nitrate Contamination in Ground Water: Studies on the Sulfur-Mediated Biological Denitrification Process 161
Michael S. Davidson and Thomas Cormack

Chemical Interactions and pH Profiles in Microbial Biofilms 166
Joseph R.V. Flora, Makram T. Suidan, Pratim Biswas, and Gregory D. Sayles

CONTENTS (cont.)

Page

Characterization of Biofilter Microbial Populations 170
Alec Breen, Alan Rope, John C. Loper, and P.R. Sferra

Fundamental Studies in the Development of the Gas Phase Biofilter 174
Rakesh Govind, Vivek Utgikar, Yonggui Shan, Wang Zhao, Madan Parvatiyar, Stephan Junginzer, and Dolloff F. Bishop

Sequential Anaerobic/Aerobic Treatment of Contaminated Soils and Sediments 184
Grace M. López, Gregory D. Sayles, Karen Bühler, Dolloff F. Bishop, In S. Kim, Guanrong You, Petra Klostermann, Margaret J. Kupferle, and Douglas S. Lipton

Poster Session 189

Approaches to the Development of Comparative Genotoxicity Risk Assessment Methods for Evaluating Hazardous Waste Control Technologies 191
Larry D. Claxton

Modulation Effects of Biotechnology Microbial Agents Following Pulmonary Exposure of Mice 193
S. Elizabeth George, Michael J. Kohan, John P. Creason, and Larry D. Claxton

Field Treatment of BTEX in Vadose Soils Using Hydrofracturing, Vacuum Extraction, and Biofilters 194
Dolloff F. Bishop, Wendy Davis-Hoover, and Rakesh Govind

PCB Biodegradation during Aerobic Treatment of Sludge from the French Limited NPL Site 197
J.W. Anderson, T. Smith, and J.T. Wilson

Development and Evaluation of Composting Techniques for Treatment of Soils Contaminated with Hazardous Waste 204
John A. Glaser, Carl L. Potter, Edward D. Kennedy, Jeffrey J. McCormack, Joseph B. Farrell, and Michael Najar

Engineering Optimization of Slurry Bioreactors for Treating Hazardous Waste in Soil and Sediments 206
John Glaser, Paul McCauley, Majid Dosani, Jennifer Platt, Edward Opatken, and Diana Roush

Biotreatability of a Vadose Zone Soil Contaminated with Dioctyl Phthalate 207
Don H. Kampbell, Dennis D. Fine, and Jerry W. Anderson

CONTENTS (cont.)

Page

Innovative Bioremediation Strategies for Creosote: Geographic Diversity of PAH Degradation Capabilities at Wood-Treating Sites 208
James G. Mueller, Suzanne E. Lantz, Richard Devereaux, Deborah L. Santavy, and P. Hap Pritchard

Design of an Expert System to Select an Appropriate Bioremediation Technique 214
Raymond C. Loehr and Greg E. Schmidt

A Data Visualization System for Bioremediation Analysis 217
Lewis A. Rossman, Kevin Savage, and John Franco

Poster Presentations Supported by EPA's Hazardous Substance Research Center Program 219

Development of a Knowledge-Based Bioremediation Adviser 221
Shu-Chi Chang, Peter Adriaens, Iris D. Tommelein, and Timothy M. Vogel

Use of Composting Technologies to Treat a TNT-Contaminated Soil 224
James H. Johnson, Jr., Mohammed Moshin, Lily Wan, and Abdul Shafagatti

Effect of Pore-Scale Hydrodynamics on Bulk Reaction Rates 225
Bruce B. Dykaar and Peter K. Kitanidis

In Situ Bioremediation Using a Recirculation Well 227
M.M. Lang, L. Semprini, and P.V. Roberts

Chlorinated Aliphatic Hydrocarbon Biodegradation by Methanotrophic Bacteria 229
Laurence H. Smith, Tomas Henrysson, and Perry L. McCarty

Anaerobic Biodegradation of BTEX Compounds at Seal Beach, California 231
Harold A. Ball, Martin Reinhard, and Eva O. Orwin

Biotransformation of Indole and Quinole under Denitrifying Conditions 232
J.N.P. Black, R.M. Kauffman, D. Denney, and D. Grbić-Galić

Cometabolism of TCE by Nitrifying Bacteria 233
Michael Hyman, Roger Ely, Sterling Russell, Ken Williamson, and Daniel Arp

Assessing the Effect of Environmental Conditions on Chlorophenol Reductive Dechlorination Pathways and Kinetics 235
Sandra Woods, Sheryl Stuart, David Nicholson, Teresa Lemmon, James Ingle, and John Westall

CONTENTS (cont.)

Page

Spatial Distribution of Nonaqueous Phase Liquid in Sand Cores Using X-ray Computed Tomography 243
John L. Holmes, R. Lee Peyton, and Tissa H. Illangasekare

Scale-Up Implications of Respirometrically Determined Microbial Kinetic Parameters . . 245
P.J. Sturman, R.R. Sharp, J.B. DeBar, P.S. Stewart, A.B. Cunningham, and J.H. Wolfram

Dissipation of Polycyclic Aromatic Hydrocarbons in the Rhizosphere 246
M. Katherine Banks and A. Paul Schwab

Effect of Irreversible Sorption on Bioavailability 247
Amy T. Kan, Gongmin Fu, Mason B. Tomson, and Calvin H. Ward

The Effect of Population and Substrate Interactions on SBR Design Optimization 249
B.C. Baltzis, G.A. Lewandowski, S. Dikshitulu, and K.W. Wang

BIOREMEDIATION FIELD INITIATIVE

RETROSPECTIVE PERFORMANCE EVALUATION ON IN SITU BIOREMEDIATION: SITE CHARACTERIZATION

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INTRODUCTION

Bioremediation is difficult to assess in heterogeneous geological material. Often, oily phase material is associated with fine textured material with low hydraulic conductivity. Remedial fluids tend to pass around the fine textured material. Because the flux of nutrients and electron acceptor through the fine textured material is small, there is little opportunity for bioremediation, and significant concentrations of contaminants can remain in subsurface material.

These relationships are illustrated in a case history from an industrial site in Denver, Colorado (1). A temporary holding tank under a garage leaked used crankcase oil, diesel fuel, gasoline, and other materials into a shallow water table aquifer. Figure 1 shows the relationship between the garage, the work pit containing the leaking holding tank, and the approximate area of the spill.

REMEDIAL EFFORT

Remediation involved removal of separate oily phases, in situ bioremediation with hydrogen peroxide and mineral nutrients, and bioventing. Ground water flow under ambient conditions was to the north or northeast. The flow of water during the remediation paralleled the natural gradient. Water was produced from a recovery well on the northeast side of the spill RW-1 in Figure 1. The flow from the well was split. Part of the flow was amended with hydrogen peroxide and nutrients and recharged to the aquifer in a nutrient recharge gallery on the south side of the spill. The remainder of the flow was delivered to a ground water recharge gallery located to the south of the nutrient recharge gallery. From 3 to 6 gpm was delivered to the nutrient recharge gallery, and 4 to 8 gpm was delivered to the ground water recharge gallery for a total flow of 9 to 11 gpm. The system was operated from October 1989 to March 1992. At a flow of 10 gpm, from 10 to 15 pore volumes would have been exchanged in the area between the nutrient recharge gallery and the recovery well.

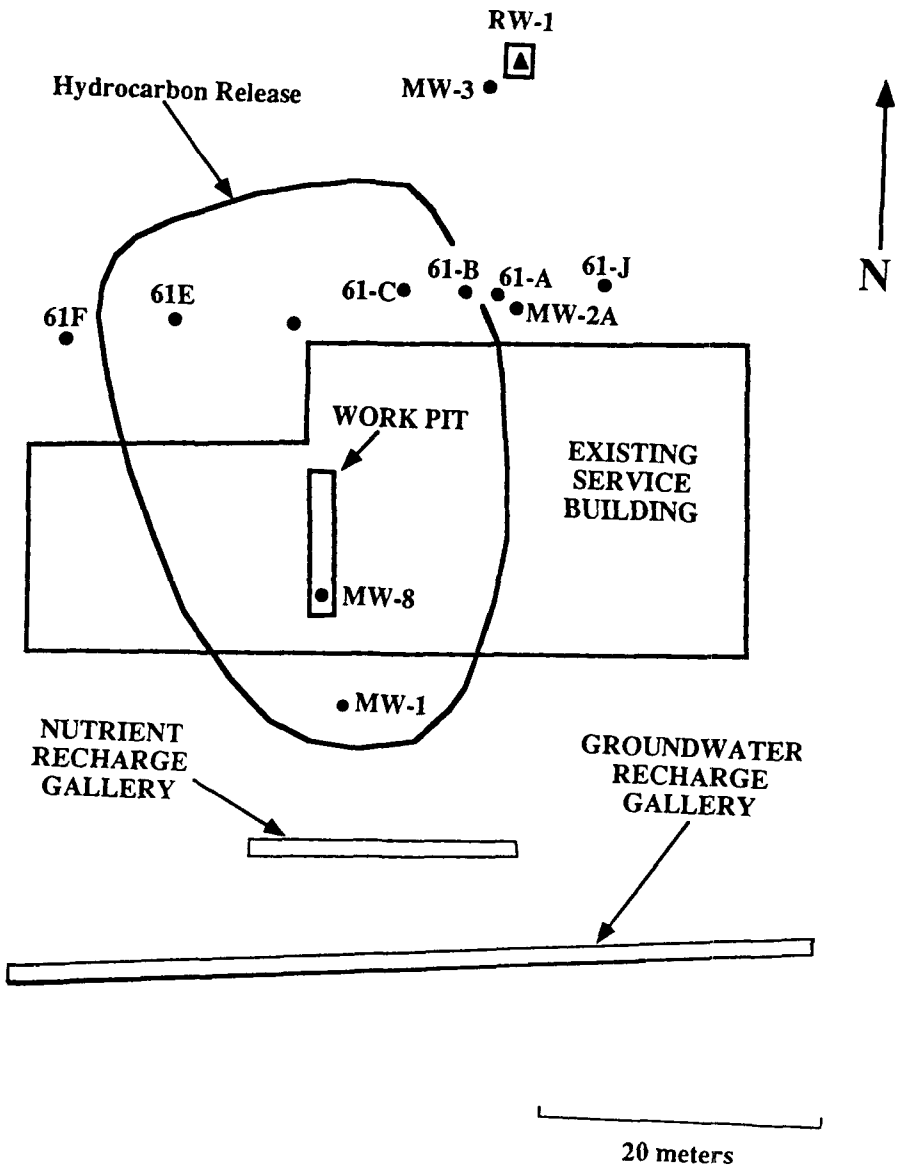

Figure 1. Infrastructure at an in situ bioremediation project in Denver, Colorado. A holding tank in a work pit under a garage leaked petroleum hydrocarbons to the water table aquifer. Ground water was pumped from a recovery well (RW-1) and filtered through activated carbon. The flow was split; part was amended with hydrogen peroxide and mineral nutrients, and recharged in a nutrient recharge gallery. The remainder was recharged in a ground water recharge gallery. The system was designed to sweep hydrogen peroxide and nutrients under the service building. MW-1, -2A, and -3 are monitoring wells. 61A through 61J are boreholes for cores.

GROUND WATER REMEDIATION

Table 1 compares the reduction in concentration of benzene and total benzene, toluene, ethylbenzene, and xylene (BTEX) compounds in ground water that was achieved by in situ bioremediation at the site in Denver. As shown in Figure 1, monitoring wells MW-1 and MW-8 are in areas with oily phase hydrocarbons. Well MW-2A is just outside the region with oily phase hydrocarbons. Well MW-3 is a significant distance from the region with oily phase hydrocarbons; it sampled the plume of contaminated ground water that moved away from the spill.

Prior to remediation, concentrations in wells MW-1 and MW-8 were equivalent. Well MW-1 was closest to the nutrient recharge gallery, and the aquifer surrounding MW-1 was completely remediated; BTEX compounds were undetectable in ground water. In well MW-8, immediately adjacent to the point of release, the concentration of benzene was reduced at least one order of magnitude, and the concentration of benzene and BTEX compounds in well MW-3 also were reduced an order of magnitude.

Water in the monitoring wells and the recirculation well contained low concentrations of contaminants by March 1992. Active remediation was terminated and the site entered a period of post-remediation monitoring.

REMEDIATION OF AQUIFER SOLIDS

In June 1992, core samples were taken from the aquifer to determine the extent of hydrocarbon contamination remaining, and to determine whether a plume of contamination could return once active remediation ceased. The site was cored along a transect downgradient of the release. The transect extended laterally from clean material, through part of the spill, into clean material on the other side (Figure 1). In each borehole, continuous cores extended vertically from clean material above the spill, and through the spill to clean material below. The cores were extracted and analyzed for total petroleum hydrocarbons (TPH) and for the concentration of individual BTEX compounds.

The relationship among the land surface, the water table, the region containing hydrocarbons, and the bedrock are presented in Figure 2. Significant amounts of hydrocarbons remain within a narrow interval approximately 2.0 feet thick, near the water table. The total saturated thickness of the aquifer was approximately 20 feet. At the time of sampling, the elevation of the water table was 5280.5 feet above mean sea level (AMSL) and all the hydrocarbons were below the water table.

The highest concentrations of hydrocarbons at the site in Denver were obtained in samples from the borehole (D), which was closest to the work pit. Table 2 presents the vertical distribution of BTEX compounds and TPH in borehole D. The material in the interior of the spill had higher proportions of BTEX compounds. Table 3 makes the same comparison at the most contaminated depth interval along the transect. Material closer to the spill had higher concentrations of TPH and greater relative proportions of BTEX compounds.

Apparently at the Denver site, a cortex of material that has been physically and biologically weathered surrounds a central core of material that has not been depleted of BTEX compounds. The concentration of an individual petroleum hydrocarbon in solution in ground water in contact with oily phase hydrocarbon can be predicted by Raoult's law. The solution concentration in water should be proportional to the mole fraction of the hydrocarbon in the oily phase.

Table 1. Reduction in Concentration of Hydrocarbon Contaminants in Ground Water Achieved by In Situ Bioremediation

Well	Benzene			Total BTEX		
	Before	During	After	Before	During	After
	----(μg/liter)---					
MW-1	220	<1	<1	2,030	164	<6
MW-8	180	130	16	1,800	331	34
MW-2A	?	11	0.8	?	1,200	13
MW-3	11	5	2	1,200	820	46
RW-1	<1	2	<1	<1	2	<1

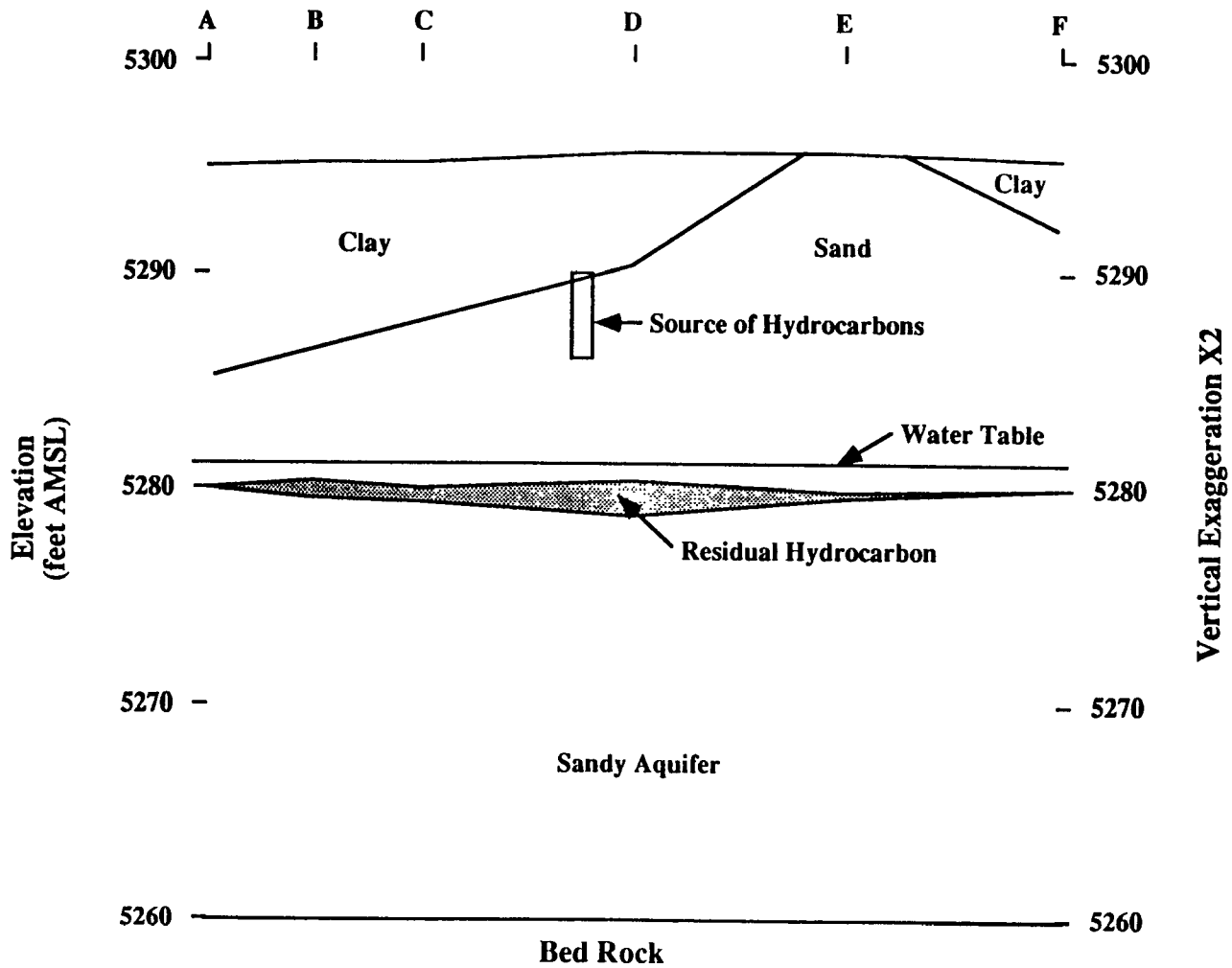

Figure 2. Cross section showing the vertical relationship of the land surface, water table, and residual hydrocarbon remaining after bioremediation, and the lower confining layer of the aquifer. The cross section runs through core boreholes depicted in Figure 1.

Table 2. Vertical Extent of Total BTEX Compounds and Total Petroleum Hydrocarbons at Borehole D, the Most Contaminated Borehole in the Transect (Figure 1)

Elevation	TPH	BTEX	Benzene	Color and Texture
(feet AMSL)	--(mg/kg)--			
281.14 to 5280.31	<44	<1	<0.2	Brown sand
5280.31 to 5279.97	227	5.1	<0.2	Brown sand
5279.97 to 5279.56	860	101	<0.2	Black sand
5279.56 to 5279.14	1176	206	4.3	Black sand
5279.14 to 5278.97	294	27	0.68	Black sand
5278.97 to 5278.64	273	7.4	0.26	Black sand
5278.64 to 5278.22	<34	<1	<0.2	Black sand
5278.22 to 5277.14	<24	<1	<0.2	Brown to yellow sand

Table 3. Lateral Distribution of Total BTEX Compounds and Total Petroleum Hydrocarbons along the Transect (Figure 1) at the Most Contaminated Depth Interval

Borehole	TPH	BTEX	Benzene
	--(mg/kg)--		
B	167	0.8	<0.2
C	156	3.5	<0.2
D	1,176	260	4.3
E	156	3.5	0.06

RETROSPECTIVE PERFORMANCE EVALUATION ON IN SITU BIOREMEDIATION: MODELING AND RISK ASSESSMENT

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INTRODUCTION

Conventional methods for determining the extent of cleanup at a bioremediation site can often be misleading. Monitoring wells may show very low or zero levels of contaminants after active bioremediation, but then the levels may increase over time. In most cases, regulatory authorities require a direct measure of the residual nonaqueous phase liquids (NAPLs) after bioremediation in addition to monitoring well data. Often the relative composition of the oily phase is assumed to remain constant during bioremediation. This is a conservative assumption and generally leads to target levels of total petroleum hydrocarbons (TPH) concentrations on the order of 10 to 100 mg/kg aquifer material. Many bioremediation schemes, however, may preferentially degrade the compounds of regulatory concern leaving relatively high TPH levels in the soil that pose a minimal risk. This modeling study focuses on developing a methodology to evaluate the possible risk, if any, associated with benzene, toluene, ethylbenze, and xylene (BTEX) sources left in soils after the implementation of a bioremediation scheme. This developed methodology will assist in providing answers to the following questions: 1) Will BTEX reappear in ground water? 2) How long will it take the plume to reappear? 3) What concentration level may be expected? The results of the case study will also assist in providing a technical basis for monitoring schedules, locating compliance wells, and constructing rational criteria for site closure.

SITE DESCRIPTION AND CHARACTERIZATION

The type and extent of contamination beneath a service building at an industrial site in Denver, Colorado, is documented in a companion paper by Wilson and Kampbell (1). In brief, used hydrocarbons (crankcase oil and gasoline) were released from an underground holding tank. These hydrocarbons exist as light nonaqueous phase liquids (LNAPLs) and have formed a smear zone approximately 1 to 2 ft thick near the water table. Soil was sampled at approximately 10-ft intervals transverse to the original NAPL plume in order to determine the remaining concentrations of TPH and BTEX. Test were conducted to determine the hydraulic conductivity in the vicinity of the contaminant source.

Hydraulic conductivity data were collected using a pump test, laboratory parameter tests, and bail tests. The pump test provides a conductivity value that is representative over the aquifer cross section but may not be representative of the aquifer material containing entrapped LNAPL. Laboratory parameter tests conducted on 28 soil core samples showed hydraulic conductivity varies two orders of magnitude across the site with some highly permeable channels evident. Relatively low permeabilities are found in the soils that are visibly stained with hydrocarbons and are closest to the water table. These data and observations of the samples during coring reveal the presence of layering in the aquifer.

MODEL SELECTION

The following observations at the site and in the laboratory indicated the need for three-dimensional simulation: 1) Visual inspection of aquifer material indicated the presence of coarse gravel lenses, clayey sands, and sands of varying gradation; 2) LNAPL plumes are inherently three-dimensional, forming thin pancakelike plumes in the capillary fringe and just beneath the water table; 3) LNAPL can become entrapped in coarse lenses, which act as preferential flow channels, well beneath the water table; 4) Solute plumes are not vertically homogeneous and biological activity will not be uniformly distributed vertically.

Bypassing of the LNAPL plume by nutrients and electron acceptor, attributable to a lowered hydraulic conductivity of the central part, resulted in high TPH and BTEX levels in some cores. Coupled with the clear need for a three-dimensional model are other criteria such as availability, ease of use, reliability, and cost. MODFLOW, a three-dimensional ground water flow model developed by the U.S. Geological Survey (USGS), was selected to simulate the ground water flow. Solute transport is simulated with a three-dimensional random walk called RAND3D.

MODELING APPROACH

The problem domain was modeled as a rectangular area 300 ft long and 200 ft wide. Two wells outside of the modeled domain were used as reference head locations for general head boundaries. The NAPL contaminant zone covers approximately 1,600 ft^2 area and a soil depth of approximately 1.7 ft. In order to accurately assess the mass flux from the LNAPL contaminant source, three layers were chosen in the model. The upper two layers are 1 ft thick and the bottom layer is 18 ft thick. The LNAPL organic contaminant is confined to the upper two layers.

Data for the period June 8, 1989, to April 1, 1990, were used for calibration. The goodness of fit between the model and measured well data was characterized by a mean residual and standard

deviation of the mean residual. The best fit model was obtained by assigning the pump test average hydraulic conductivity to the bottom layer, which carried the majority of water.

The main focus of this modeling effort is to determine how much contaminant mass will be transported from the remaining residual and if it will generate a plume of regulatory concern. A monitoring well screened only in the upper two layers would detect the highest contaminant concentrations. A pumping well screened over the entire aquifer thickness also is being considered. In this case, however, dilution will play a major role in reducing the maximum concentrations. Two significant assumptions are used in the solute transport modeling: 1) the concentration of BTEX in the source zone remains constant, and 2) water flowing from the contaminated cells is in equilibrium with the residual NAPL. Using the heads generated by MODFLOW, the ground water velocity through each source cell was calculated. The known NAPL BTEX concentration was then used to calculate the equilibrium concentration and, consequently, the mass flux. Estimated benzene mass fluxes were converted into particle inputs for each layer. The particle tracking model was used to simulate solute transport using the velocity field generated from MODFLOW.

RESULTS AND DISCUSSION

Any simulation of solute transport requires specification of the contaminant source. For the case of a NAPL spill, the source function will consist of a continuous mass flux of solute from the residual NAPL phase to the aqueous phase. Many researchers have shown that equilibrium is quickly reached in spill scenarios if the ground water velocity is low and the "residence time" of the water in contact with NAPL is "long enough." From a regulatory standpoint the assumption of equilibrium is conservative, as greater mass fluxes cannot be achieved. The water flux can vary significantly in the source zone often giving a misleading indication that the contaminant transport is rate limited. This primary problem has been a focus of our work. Preferential flow paths often develop within the source zone in areas with low BTEX and TPH concentrations, allowing water flow to bypass the more highly contaminated areas. Laboratory determination of the hydraulic conductivity in the samples containing high amounts of TPH confirms this observation.

A key result of the modeling study is that the solute plume emanating from a NAPL source is not homogeneous. In general, the solute plume will consist of subplumes over different depth intervals, of widely different concentrations, and moving at different velocities. A regulatory question posed earlier in this paper concerns how long the compliance wells should be monitored. The answer: when all the subplumes have reached steady-state. The plumes in the middle and bottom layer have reached or are close to equilibrium by 330 days. The plume in the top layer, which incidently has the highest benzene concentration, has not reached equilibrium at 420 days.

The design of the compliance wells will have tremendous impact on the actual sampled concentration. If the wells are bailed or pumped so that the well volume is completely mixed, significant dilution will occur. The existing monitoring wells at the site are screened over the top 5 ft of the aquifer. The maximum concentration achieved in the well screened over a 5-ft interval reaches a steady-state concentration of 26 ppb. If that well is screened over the entire saturated thickness, a concentration of 15 ppb is achieved. Even greater dilution will occur if the well is pumped.

Several operational considerations for risk assessment and compliance well monitoring can be made from the modeling study: 1) A benzene plume will reestablish itself at the site, but it will be three orders of magnitude lower than the federal maximum contaminant level (MCL). New

standards may be set and the risk from this plume may be deemed significant. 2) The local hydraulic conductivity plays a significant role in determining the contaminant mass flux and in creating subplumes of different concentration and velocity. 3) Compliance well monitoring will have to be continued past August 1993 so that solute plumes in all levels will reach steady-state. 4) Retardation coefficients and effective porosity data would significantly improve the time of arrival estimate of the solute plume. 5) Compliance well design should be carefully considered when sampling a three-dimensional plume, as the well design can lead to significant contaminant dilution.

CONCLUSIONS

A modeling methodology for the retrospective evaluation of bioremediated aquifers contaminated with organic chemicals was developed. The primary hypothesis on which the methodology was based is that during the spill, the NAPL contaminant becomes entrapped preferentially in coarse formations in the saturated zone and fine formations in the unsaturated zone. This hypothesis is supported by laboratory (2) and field data. Flow channels created by naturally occurring aquifer soil heterogeneities as well as macroscale entrapment of the NAPL also will produce preferential paths for the treating agents. The proposed methodology requires that these local heterogeneities in the contaminant zone of the spill be captured. The standard pump tests that provide the regional values for transmissivity will not have the adequate resolution to capture these spill-site-scale heterogeneities. Even though the hydraulic conductivity values determined in the laboratory on disturbed soil samples were used in this study, a more appropriate characterization method would be well-designed bail tests (or slug tests), which capture the local layered heterogeneities more accurately. These local hydraulic conductivity values allow us to obtain the velocity field in the contaminant zone and to subsequently determine the contaminant mass flux. Solute breakthrough curves determined by this method can then be used to conduct risk analysis and to provide a rational basis for post-remediation well monitoring.

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EVALUATION OF FULL-SCALE IN SITU AND EX SITU BIOREMEDIATION OF CREOSOTE WASTES IN SOILS AND GROUND WATER

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Ada, OK

Objectives of the bioremediation field initiative are to 1) document more fully the performance of full-scale bioremediation field applications in terms of treatment effectiveness, operational reliability, and cost; 2) provide technical assistance to EPA and state agency site managers that are overseeing or considering the use of bioremediation; and 3) develop a treatability data base that will be available through EPA's Alternative Treatment Technology Information Center (ATTIC). The performance evaluation project in Libby, Montana, mainly focuses on the first objective.

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. Two forms of wood preservative were used at the site: creosote, containing polycyclic aromatic hydrocarbons (PAHs) and pentachlorophenol (PCP). PAHs are currently the primary components of concern at the site. The performance evaluation project, now in the third year, is under the direction of Dr. Ronald Sims of Utah State University.

The bioremediation performance evaluation consists of three phases: 1) summarizing previous and current remediation activities; 2) identifying critical site characterization and treatment parameters necessary to evaluate bioremediation performance for each of the bioremediation treatment units; and 3) evaluating bioremediation performance based on the information obtained.

Three biological treatment processes are addressed in the bioremediation performance evaluation: 1) above-ground fixed film bioreactor for treatment of extracted ground water from the upper aquifer; 2) surface soil bioremediation in a prepared-bed, lined land treatment unit (LTU); and 3) in situ bioremediation of the upper aquifer at the site. Each biological treatment process will be evaluated with regard to design, performance, and monitoring activities. Figure 1 illustrates the relative locations of the three treatment processes at the site.

BIOLOGICAL TREATMENT PROCESSES

The upper aquifer above-ground treatment unit is provided for biological treatment of extracted ground water for removal of PAHs and PCP prior to reinjection via an infiltration trench. The subsequent biological treatment consists of two fixed-film reactors operated in series. The first reactor 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.

An evaluation of the system components for equalization and for biotreatment is being conducted. Equalization system components include four ground water extraction wells and an equalization tank consisting 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. Figure 2 illustrates the components of the above-ground treatment system for extracted ground water.

The LTUs have been used for bioremediation of contaminated soil 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. After being pretreated in the waste pit area, soil is further treated in one of two prepared-bed, lined LTUs. 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) $\leq$ 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 LTU cell 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. 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.

Contaminated soil is applied and treated in lifts (approximately 9 in. thick) in the designated LTU. Degradation rates, volume of soil to be treated, initial contaminant concentration, degradation period, and LTU size determine the time required for remediation of a given lift. Based on an estimated 45-day timeframe for remediation of each applied lift to acceptable contaminant levels, 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 pilot in situ bioremediation system for the upper aquifer area involves the addition of hydrogen peroxide and inorganic nutrients to stimulate the growth of contaminant-specific microbes. The hydrogen peroxide injection system was designed to maintain a concentration of approximately 100 mg/L of hydrogen peroxide. Injection flow rate is approximately 100 gpm into three injection

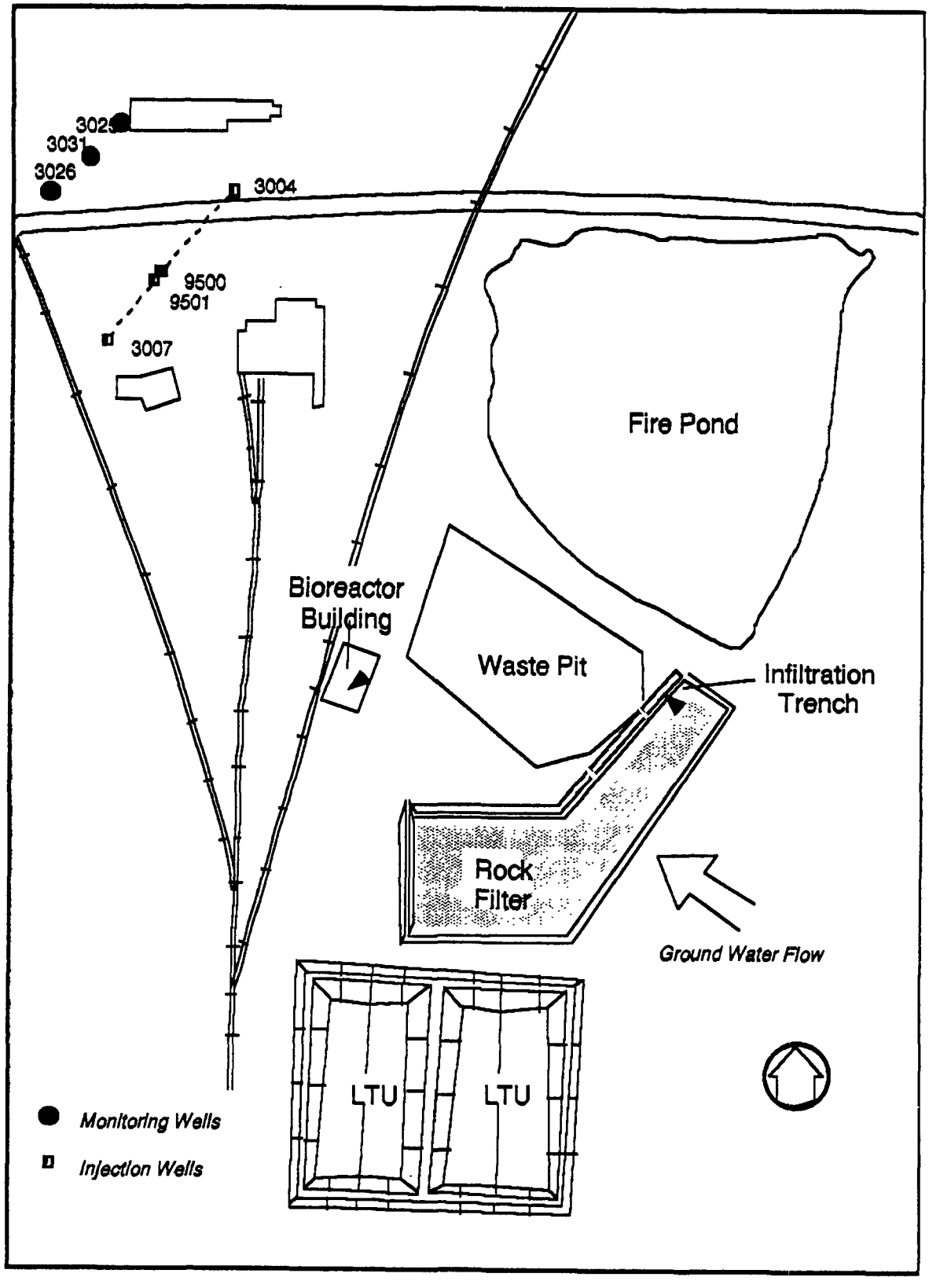

Figure 1. Relative locations of the three treatment processes at the site, including the bioreactor building for treatment of extracted ground water, LTUs, and monitoring and injection wells for in situ treatment.

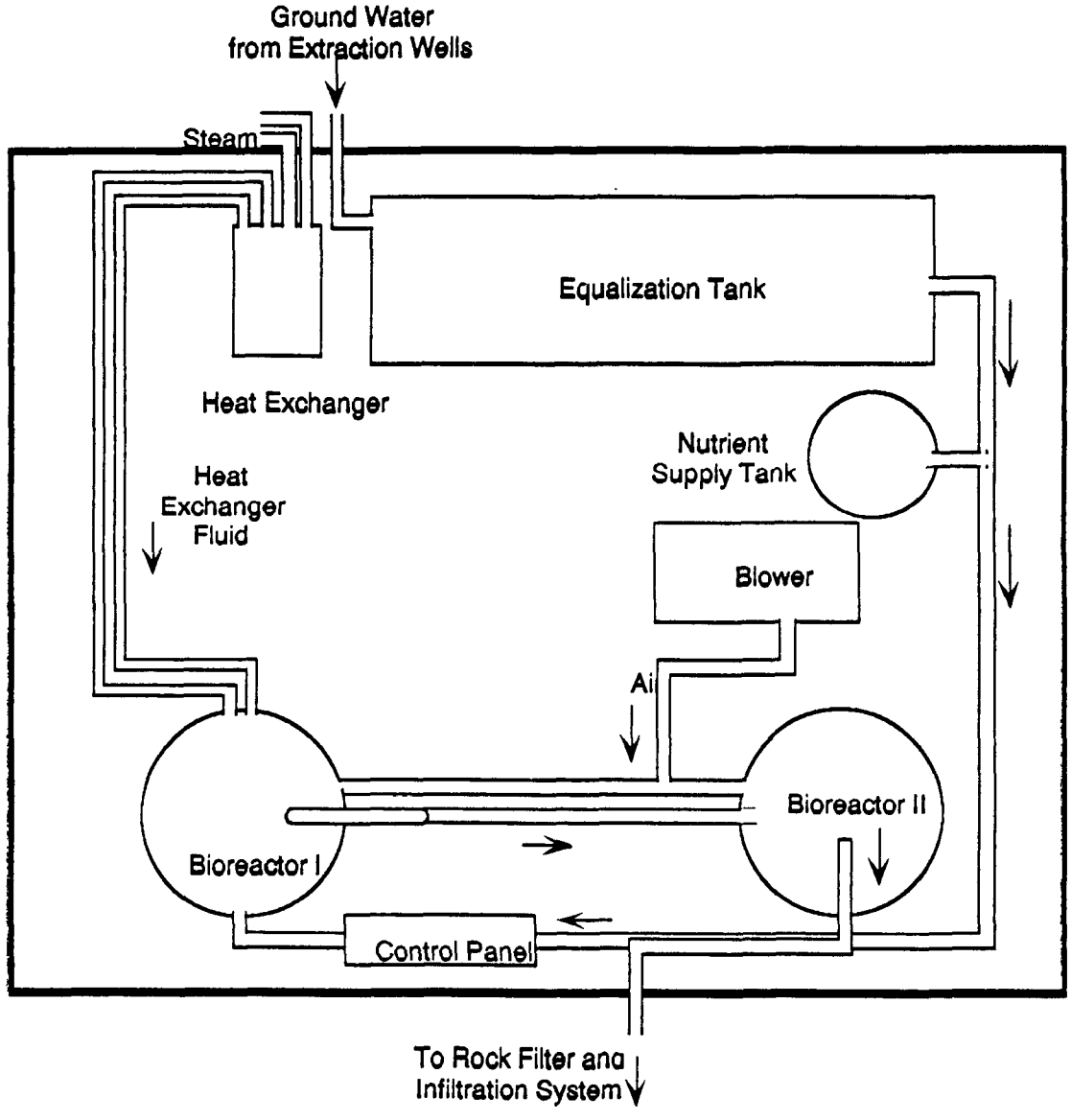

Figure 2. Components of the above-ground treatment system for bioremediation of extracted ground water.

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 and 1 mg/L of nitrogen and phosphorus, respectively.

The ROD calls for cleanup levels in the upper aquifer of 40 parts per trillion (ppt) for 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/or inorganic compounds.

PERFORMANCE EVALUATION ACTIVITIES

Performance evaluation of the upper aquifer above-ground treatment system involves evaluation of the bioreactor system. Treatment evaluation is focused on characterizing performance with regard to system capability to remove PAHs and PCP from the ground water, optimizing operation within the bioreactors, and investigating the fate of contaminants in the bioreactors under environmental operating conditions at the site. The above-ground treatment system was sampled during 1991 and 1992 for chemical, physical, and biological parameters. In addition, a hydraulically equivalent 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 will be combined with data provided by Champion International to provide an in-depth evaluation of performance.

Performance of the soil bioremediation system in the LTUs involves evaluation of the reduction in concentration of PAHs and PCP with time and with depth within the LTUs. The primary purpose of the LTU soil sampling program being carried out in this project is 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 biodegradation potential of similar types of waste 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 to more 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 = 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
V_s = the sample variance

In general, sampling efforts to minimize V_t will result in the best 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 will 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 provide representative information about the transformation of PAHs and PCP within the LTUs.

The LTUs were sampled in May, June, July, and September of 1991, and September of 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 in situ bioremediation system has 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 has included measurement 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 PAH and PCP concentrations in treated and background areas at the site. Laboratory mass-balance experiments using radiolabeled target compounds have been used in conjunction with field-scale measurements to provide additional information concerning biotic reactions (mineralization) and potential abiotic reactions (poisoned controls).

PERFORMANCE EVALUATION REPORTS

Separate reports are being prepared that will address each of the three biological treatment systems at the site: 1) above-ground system for extracted ground water; 2) soil bioremediation in prepared-bed LTUs; and 3) in situ treatment. Information generated from full-scale characterization and monitoring, from pilot-scale studies, and from laboratory treatability studies will be combined with information supplied by Champion International to provide an integrated evaluation of bioremediation performance at the Libby, Montana, site that can be used to evaluate and select rational approaches for characterization, implementation, and monitoring of bioremediation at other sites.

AN EVALUATION OF CONCURRENT BIOVENTING OF JET FUEL AND SEVERAL SOIL WARMING METHODS: A FIELD STUDY AT EIELSON AIR FORCE BASE, ALASKA

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INTRODUCTION

Bioventing is the process of supplying 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. Since the bioventing equipment (air injection/withdrawal wells, air blower, and soil gas monitoring wells) is relatively noninvasive, bioventing technology is especially valuable for treating contaminated soils in areas where structures and utilities cannot be disturbed, such as at the Hill Air Force Base (AFB) site.

Through the Bioremediation Field Initiative, a cooperative program between EPA's Office of Research and Development and Office of Solid Waste and Emergency Response, EPA's Risk Reduction Engineering Laboratory began a 2.5-year field study of in situ bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Eielson 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 Laboratories, Columbus, OH. With the pilot-scale experience gained in these studies and others, bioventing should be available in the near future as a reliable, inexpensive, and unobtrusive means of treating large quantities of organically contaminated soils.

METHODOLOGY

Site history, characterization, installation, and monitoring were summarized previously (2). Figure 1 shows a plan view of the project. Briefly, four test plots have been established, all receiving

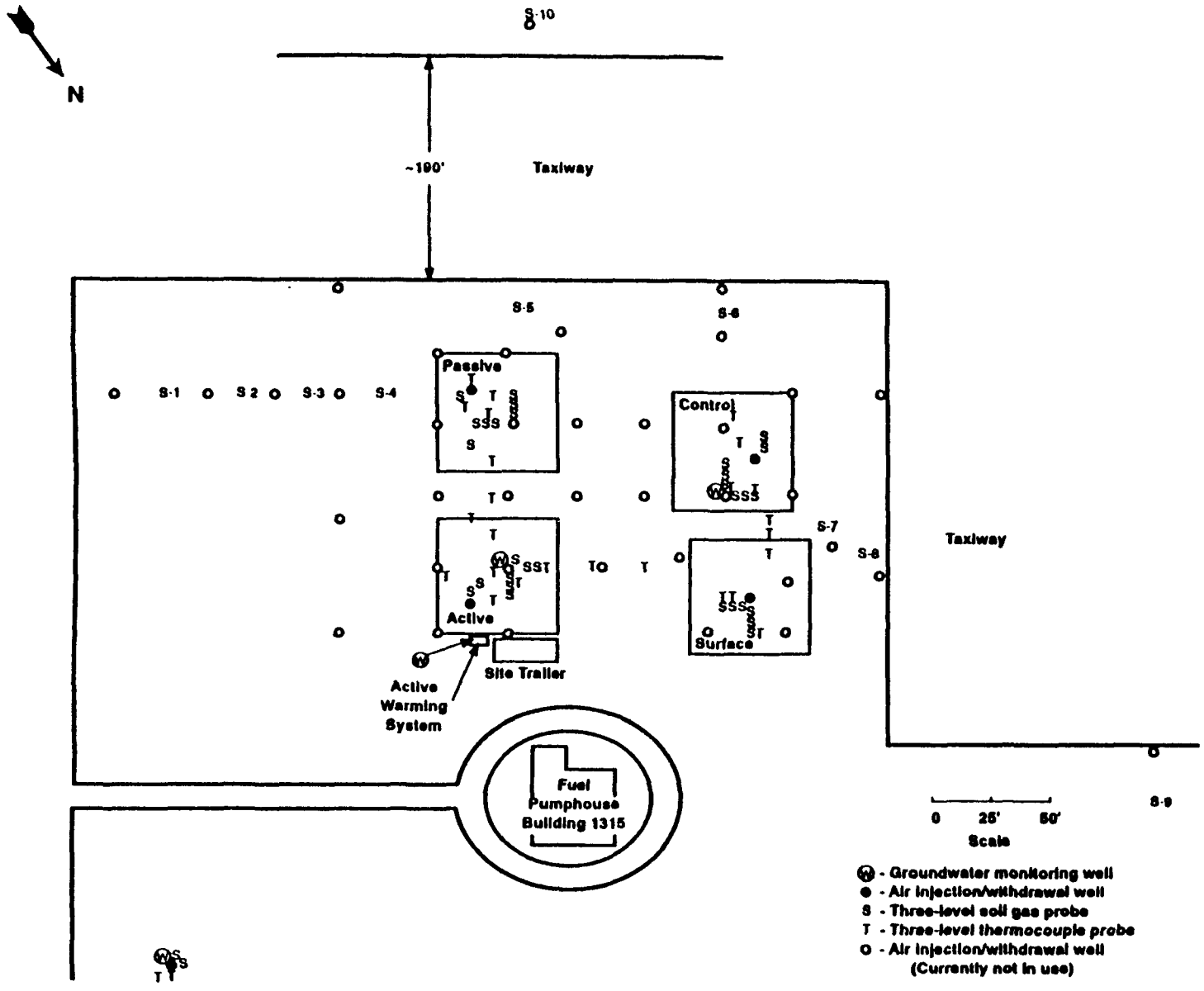

Figure 1. Plan view of the joint U.S. EPA and U.S. Air Force bioventing activities at Eielson AFB, near Fairbanks, Alaska. The "surface" plot is the test plot containing heat tape.

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 clear plastic covering over the plot, and passive heat retention the remainder of the year by applying insulation on 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.

- *Contaminated Control.* Contaminated soil vented with injected air with no artificial method of heating.

The passively heated, actively heated, and control test plots were installed in summer 1991, and the heat tape plot was installed in September 1992.

Periodically, in situ respirometry tests (3) are conducted to measure the 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. Quarterly comprehensive and monthly abbreviated in situ respiration tests are planned.

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

RESULTS

Evaluation of Soil Warming Methods. Table 1 shows soil temperatures in each plot averaged over the plot and over a 3-month period (a season). Each warming method calls for maintaining temperatures higher than the unheated control, despite, for example, mean minimum ambient air temperatures in January of about -20°C. The active and heat tape warming methods involve maintaining summerlike temperatures during the winter.

Biodegradation Rates. Soil warming is only worthwhile if the resulting elevated temperature provides enhanced rates of biodegradation. Figure 2 shows the rate of biodegradation from all test plots, as measured by in situ respirometry, as a function of seasonal averaged temperature. The trend of higher rates at higher temperatures is clear. To clarify this relationship, an Arrhenius plot was constructed (Figure 3) plotting $\log_{10}$(rate) vs. inverse absolute temperature. If an Arrhenius relationship exists, the data should be linear in this plot. The resulting linear fit gives: $\log_{10}$[rate(mg/kg/day)] = -4030.(1/T) +14.6, regression constant R=0.88, where temperature, T, has units of K. The resulting Arrhenius relationship is:

$$\text{Rate (mg/kg/day)} = 3.98 \times 10^{14} e^{-9280/T}$$

Table 1. Temperatures in Each Plot as a Function of Season

	Temperature (°C)			
Season	Active	Passive	Heat Tape	Control
Autumn 1991	10.8	5.7	NA	5.7
Winter 1992	10.1	0.5	NA	-0.2
Spring 1992	17.4	0.7	NA	-0.5
Summer 1992	18.1	15.6	NA	9.6
Autumn 1992	17.0	7.1	8.0	3.8
Winter 1993	15.9	1.3	10.0	0.2

Note: Temperatures are averaged over the plot and over the length of the season.

NA = Not available, prior to installation.

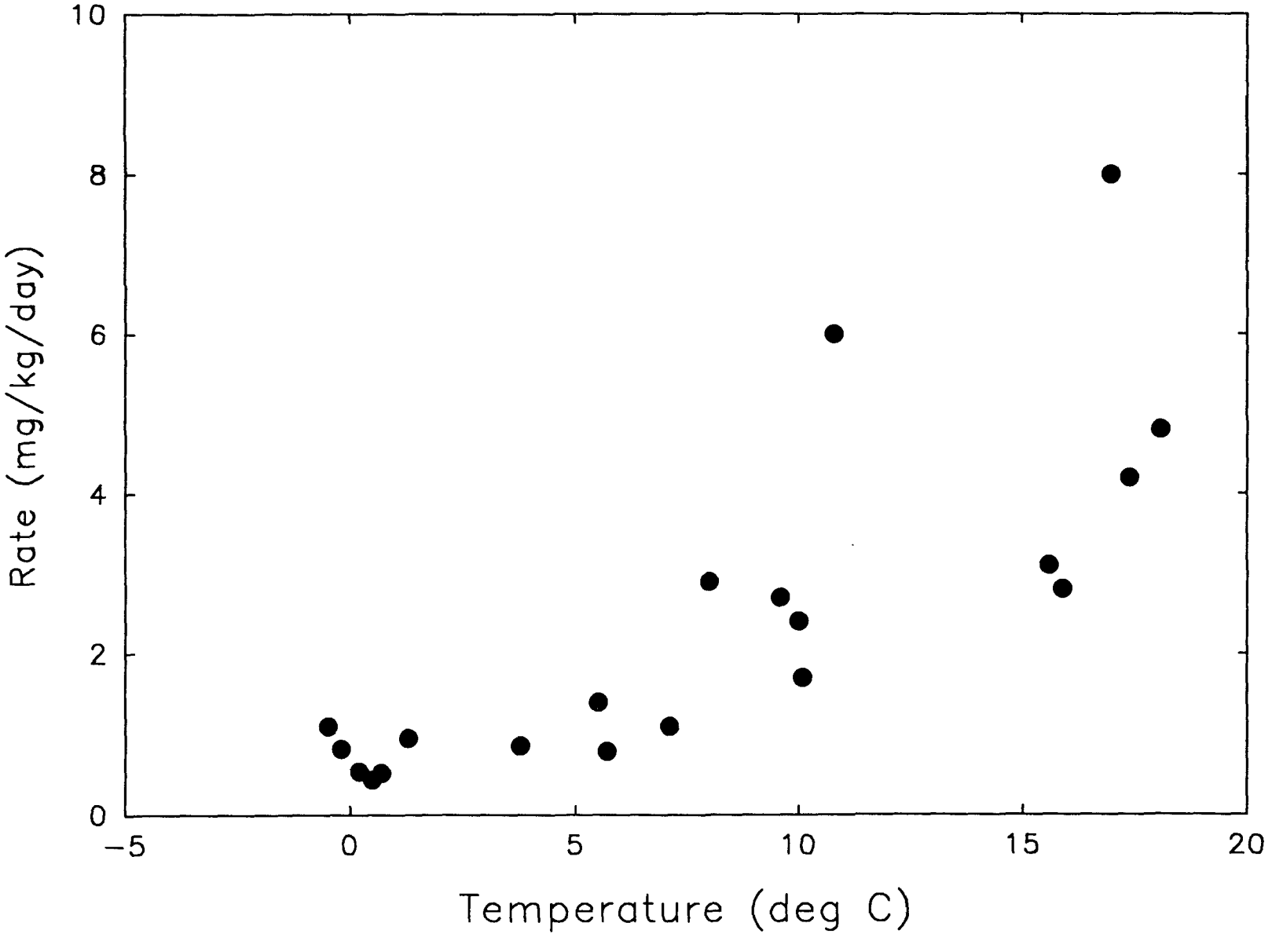

Figure 2. Rate of petroleum biodegradation as a function of temperature averaged over the test plot and averaged over the season (i.e., averaged over winter 1992).

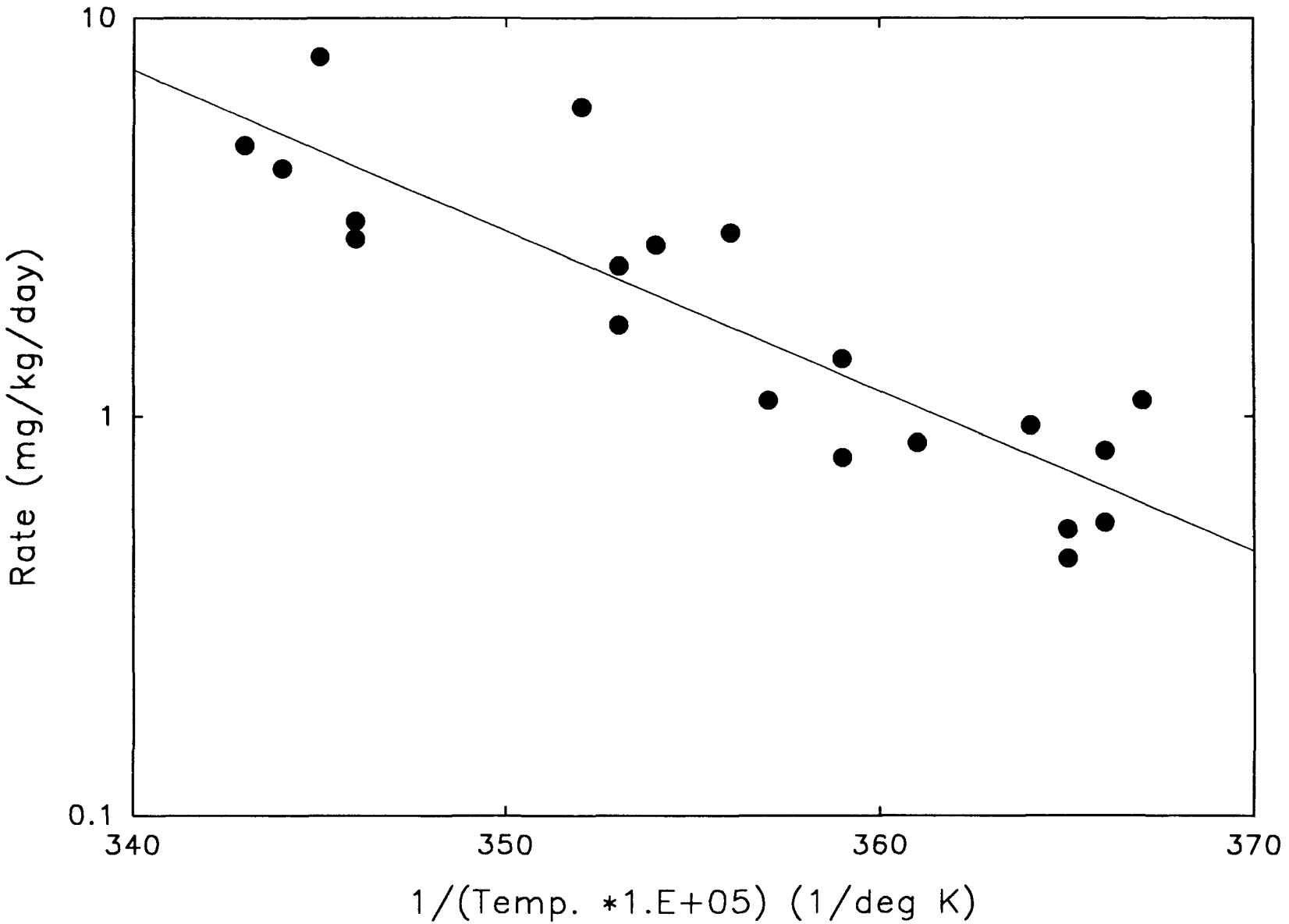

Figure 3. Rate of petroleum biodegradation as a function of inverse temperature. Data are compiled from all test plots. Line is the linear regression fit.

This function is in agreement with previous studies (4).

Soil Sampling. Soil samples for total petroleum hydrocarbon measurements will be taken at the completion of the project and compared to the initial soil analysis to determine net loss of hydrocarbons by bioventing in each test plot.

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DOCUMENTING BIOVENTING OF JET FUEL TO GREAT DEPTHS: A FIELD STUDY AT HILL AIR FORCE BASE, UTAH

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INTRODUCTION

Bioventing is the process of supplying 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 blower, and soil gas monitoring wells) is relatively non-invasive.

Through the Bioremediation Field Initiative, a cooperative program between EPA's Office of Research and Development and Office of Solid Waste and Emergency Response, EPA's Risk Reduction Engineering Laboratory began a 2-year field study of in situ bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Hill Air Force Base (AFB) near Salt Lake City, Utah. 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 Laboratories, Columbus, Ohio. With the pilot-scale experience gained in these studies and others, bioventing should be available in the near future as a reliable, inexpensive, and unobtrusive means of treating large quantities of organically contaminated soils.

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

METHODOLOGY AND RESULTS[1]

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, continuously screened from 30 to 95 ft below grade, is indicated; "CW" wells are soil gas "cluster wells" where independent soil gas samples can be taken at 10-ft intervals from 10 to 90 ft deep; "SMP" wells are shallow soil gas monitoring points at a depth of 2 ft below grade that were installed recently (September 1992) to assist in determining the volatilization rate. No data from these wells are available at this time.

Air Injection. From August 1991 until October 1992, air was injected into the vadose zone at a rate of about 70 ft^3/min. Since October 1993, the injection rate has been about 45 ft^3/min. Biodegradation and volatilization rates as a function of air flow rate will be measured. An optimal air injection that maximizes overall biodegradation rate and minimizes total volatilization rate will be sought.

Soil Gas Composition. Quarterly soil gas measurements during venting are conducted. Soil gas oxygen, carbon dioxide, and total hydrocarbons are measured at each depth in all wells. The radius to which the air injection provides oxygen, and thus the area in which biodegradation is occurring, depends on the air flow rate. This is demonstrated by the data shown in Figure 2 where soil gas oxygen as a function of distance from the injection well is plotted for the two flow rates, 70 and 45 ft^3/min. The oxygen levels at great distance are strongly influenced by the injection rate.

In Situ Respiration Tests. For each flow rate used, an in situ respirometry test (3) is conducted to evaluate the in situ biodegradation rate. Rates are measured at each soil gas monitoring location. Table 1 shows rates at three well locations, averaged over depth, from tests conducted in September 1991 and 1992. The air flow rate was about 70 ft^3/min for both measurements. The lower rates after a year of venting suggest that bioventing is removing petroleum hydrocarbons from the site at a significant rate.

Inert Tracer Gas Study. An inert gas tracer study was conducted in November and December of 1993 to evaluate how uniformly the injected air is distributed at the site. Maintaining a constant flow rate, the air was supplemented with helium, giving a helium feed composition of 5.6 percent. The data are still being analyzed. Figure 3, however, shows the helium concentration at well CW-5, 70 ft deep, as a function of time. Well CW-5 is 50 ft from the injection well. As noted in the figure, the mean time for injected air to arrive to this sampling point was 118 hr.

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[1]See previous report (2) for additional details.

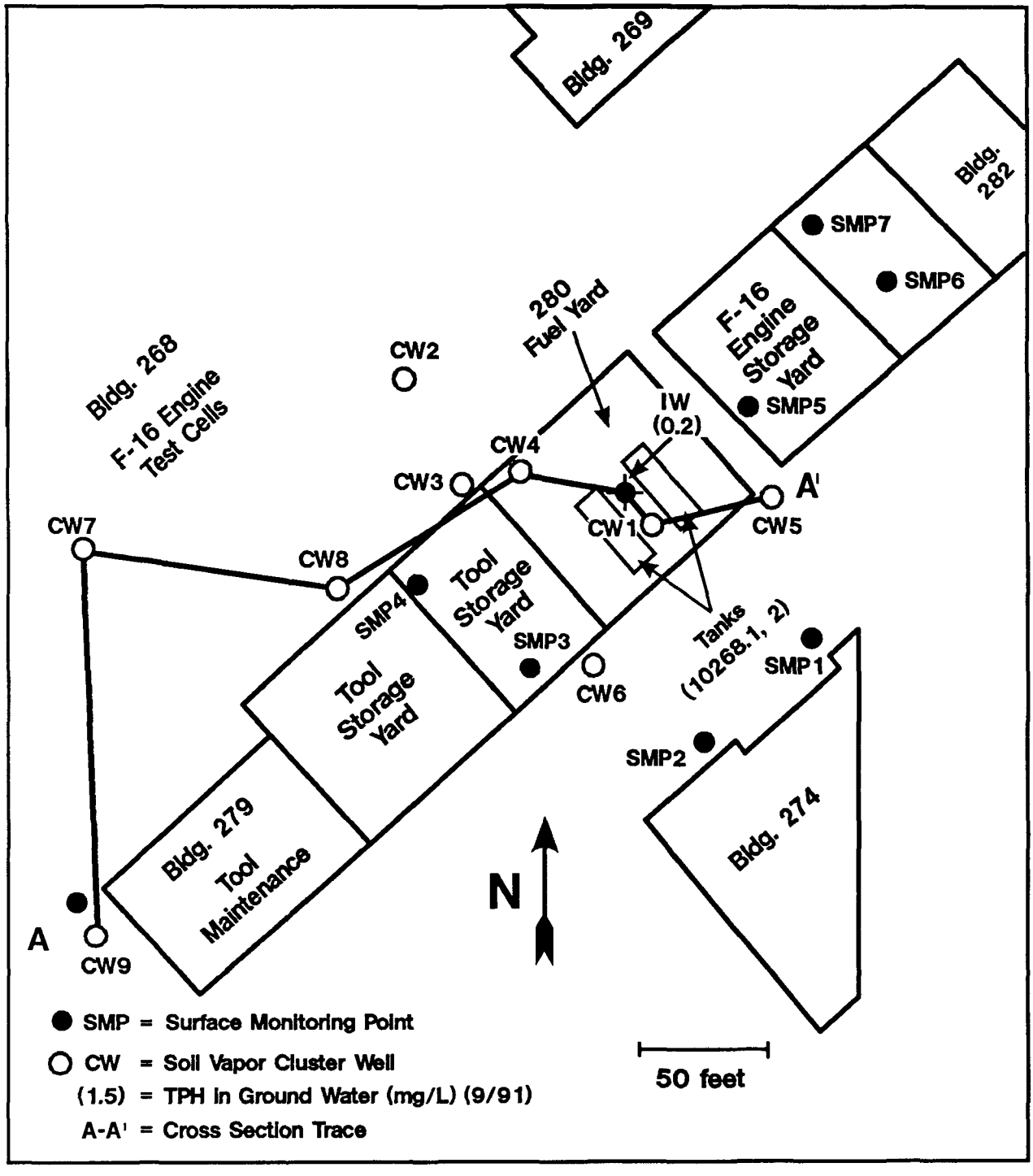

Figure 1. Plan view of the joint U.S. EPA and U.S. Air Force bioventing activities at Hill AFB, near Salt Lake City, Utah. CWs are cluster soil gas monitoring wells; SMPs are shallow soil gas monitoring points.

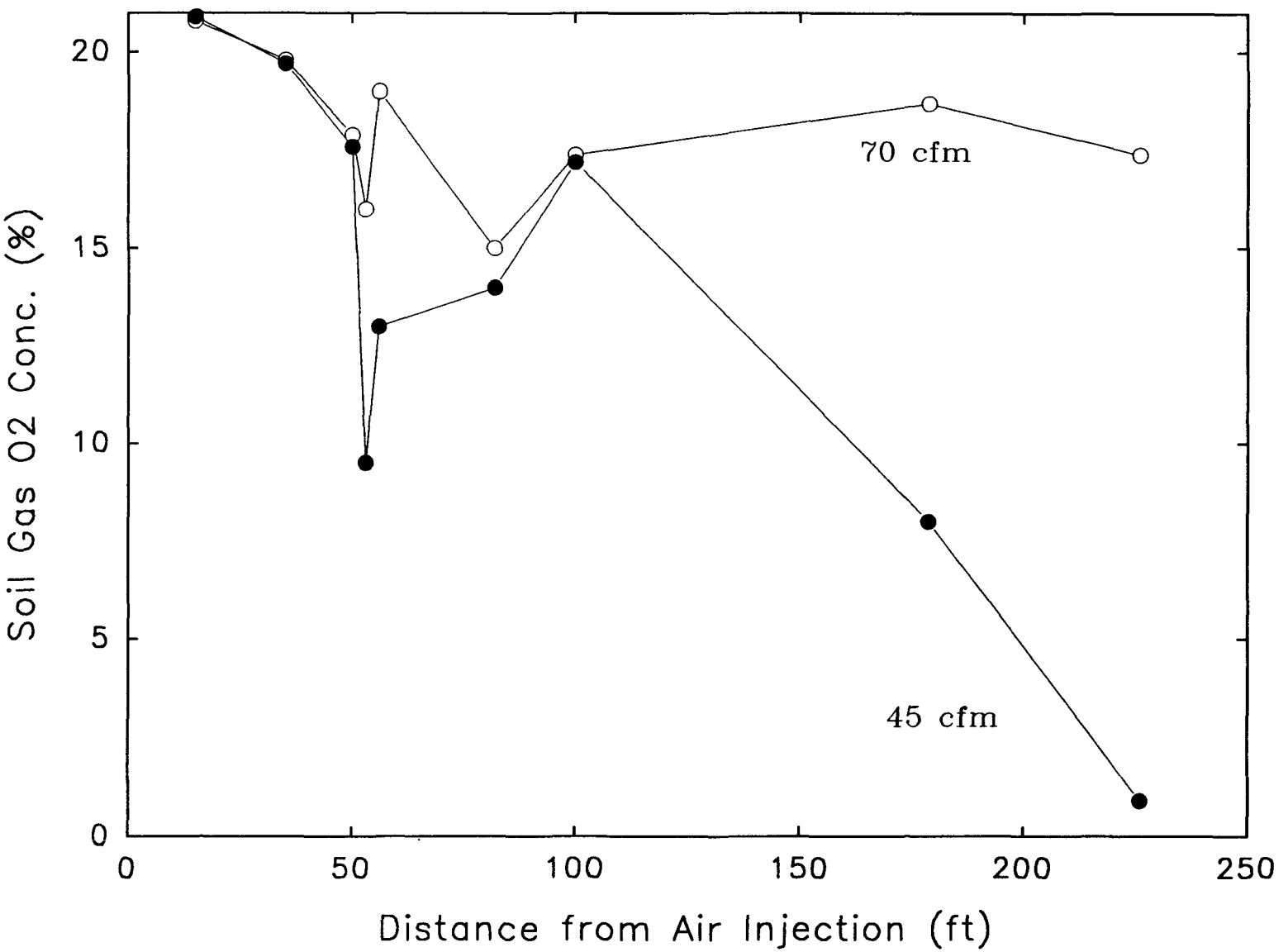

Figure 2. Soil gas oxygen concentrations as a function of distance from the injection well for two air injection rates, 70 and 45 ft^3/min (cfm). Oxygen concentrations are averages of the oxygen level at the 70 and 80 ft depths in a particular well.

Table 1. Rates of Biodegradation, Averaged over the Depth Noted, at Three Wells

Well	Depths (ft)	Rate (mg/kg/day) September 1991	Rate (mg/kg/day) September 1992
CW-1	20-90	0.97	0.30
CW-2	60-90	0.59	0.36
CW-3	10-90	0.56	0.32

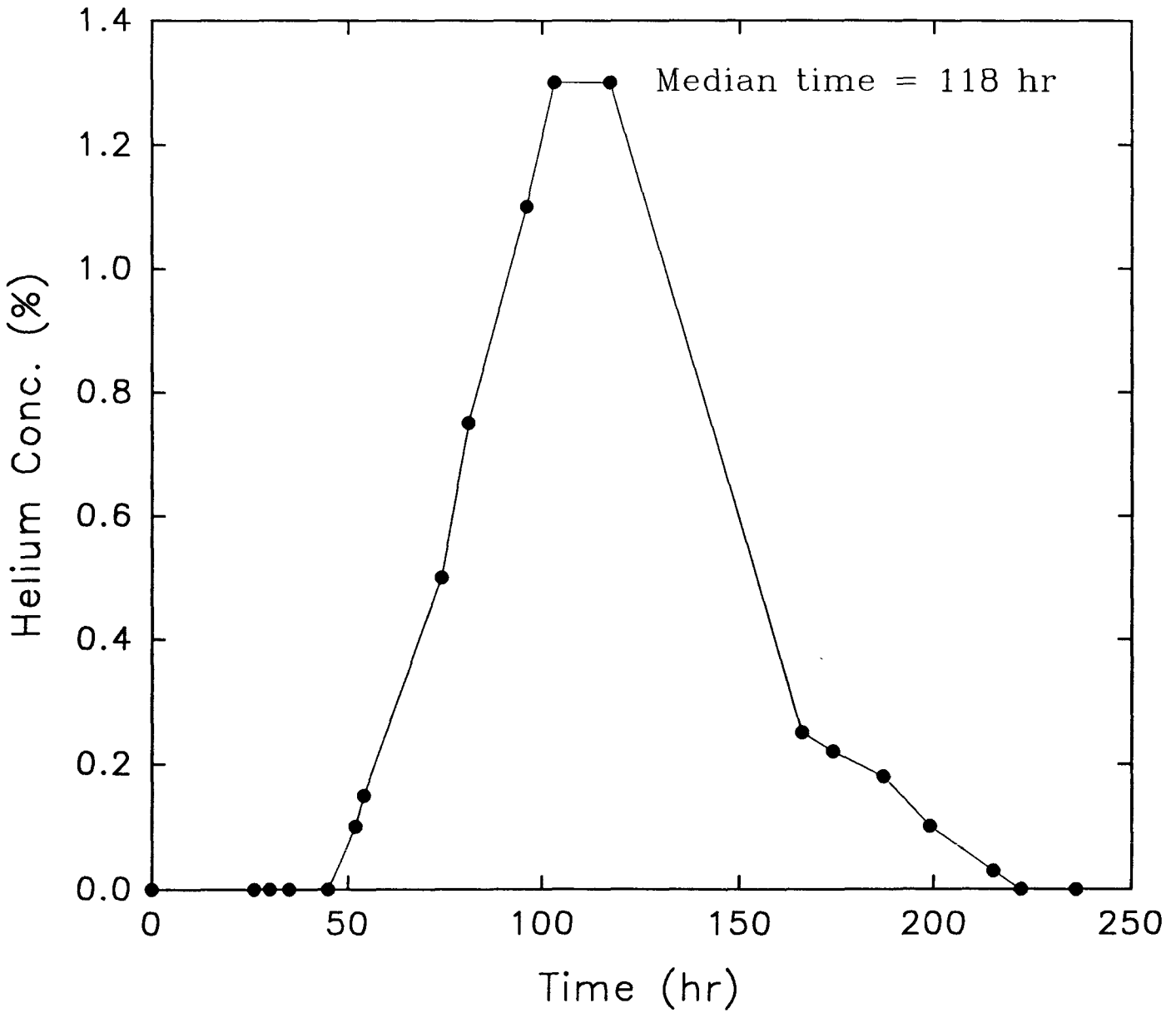

Figure 3. Helium concentration in soil gas during the inert tracer gas study at well C-5, 70 ft below the grade sampling port, versus time after commencement of injection.

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

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APPLICATION OF WOOD-DEGRADING FUNGI TO TREAT CONTAMINATED SOILS

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INTRODUCTION

Past investigations of soil treatment systems using lignin-degrading fungi have largely been confined to laboratory or bench-scale studies (1,2). Recently, a project consisting of two phases, a treatability study in 1991 and a demonstration in 1992, was conducted at an abandoned wood-treating site in Mississippi to evaluate fungal treatment effectiveness under field conditions. The study site in Brookhaven, Mississippi, 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 studies reported on in this paper were conducted at Brookhaven because the site characteristics were suitable for conducting field investigations, not to promote consideration of fungal treatment as one of the treatment options for the site. Treatability results are presented in this paper. Analysis of the demonstration phase results was not complete at the time of this writing and will be reported at a later date.

METHODOLOGY

The treatability study was designed to evaluate the ability of three different fungal species to degrade pentachlorophenol (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 the treatability study.

A test location was constructed on an uncontaminated portion of the wood-treating site. The base for the test plots was formed by changing the elevation with the addition of clean soil to promote better drainage conditions. Soil beds measuring 3 m by 3 m (10 ft by 10 ft) were constructed of galvanized sheet metal. 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 contaminated soil was sized through a 2.5-cm (1-in.) mesh screen using a Read Screen All shaker screen having a 8.4-m^3/hr (10-yd^3/hr) capacity. The soil was deposited in separate piles on a polyethylene tarp. Further homogenization was accomplished by the mixing of different portions of screened soil. The mixed soil was then applied to the treatment plots using a front-end loader. Wood chips were added to the soil plots to provide a substrate that could sustain growth of the fungi.

Inoculum was developed jointly with the L.F. Lambert Spawn Co. of Coatesville, Pennsylvania. The prepared inoculum and inoculum carrier were shipped to the site by refrigerated transportation. A total of 10 plots were used in the study. The experimental design (Table 1) consisted of a randomized complete block (RCB) without replication and a balanced incomplete block (BIB) with treatments replicated four times. Six of the plots were allocated to the RCB design and four to the BIB design. The BIB plots were subdivided into 1.5 m by 1.5 m (5 ft by 5 ft) subplots.

After inoculation with fungi, each plot was irrigated and tilled with a garden rototiller. The tiller was cleaned as it was moved between the plots to prevent cross contamination between treatments and controls. 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 throughout the study daily. 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 then after 1, 2, 4, and 8 weeks of operation.

RESULTS

The treatability study was conducted over a 2-month period between September 18 and November 13, 1991. The greatest removal of PCP (Table 1) was achieved in the plot inoculated with *Phanerochaete sordida*. Over the 42-day study period, this treatment regime produced nearly 90 percent transformation of PCP from the contaminated soil initially having a pH of 3.8 and a PCP concentration of 673 mg/kg.

Table 1. Feasibility Study Experimental Design and Pentachlorophenol Transformation Results[a]

Treatment[b] Regime	Fungus/Control	Inoculum Loading	PCP Transformed (Dry Wgt.)	PCP Initial Conc. (mg/kg)
1	*P. chrysosporium*	5%	15%	576
2	"	10%	67%	1017
3	*P. sordida*	10%	89%	673
4	*P. chrysosporium/* *T. hirsuta*	5% 5%	23%	615
5	Inoculum Carrier Control	10%	14%	687
6	No Treatment Control	--	15%	737
7	*T. hirsuta*	10%	55%	334
8	*P. chrysosporium*	13%	52%	333
9	"	10% (Day 0) 2% (Day 14)	55%	360
10	Wood Chip Control	--	0%	471

[a]The extent of transformation indicated in this table is developed from the 42-day treatability study conducted from September 18 to November 13, 1991.

[b]Wood chips were added to each plot at a 2.5 percent loading level with the exception of the No Treatment Control, which received no amendment.

Removal data for the creosote constituents (PAHs) are presented in Table 2 for the treatment using *P. sordida*. Concentration decreases of the three- and four-ring PAHs were consistently greater for the fungal treatment than for the controls. Larger ring PAHs persisted in both the treatment and control plots.

SUMMARY AND CONCLUSIONS

The superior performance of *P. sordida* in biotransforming PCP parallels previous field study experience with *P. chrysosporium* at a Wisconsin site. At Brookhaven, however, *P. sordida* achieved the greatest percentage removal. *P. sordida* is an organism that resides in the soil under normal conditions. The soil conditions encountered at the Brookhaven site are not optimal for the cultivation of microorganisms, and the concentrations of PCP found in the Brookhaven soil are excessive for sustaining suitable growth of many microorganisms. All of these factors strongly suggest that fungal treatment using *P. sordida* has promise for processing PCP and other difficult-to-degrade pollutants. Future challenges to making the technology more cost effective are developing improved and cheaper inoculation techniques and extending application of the technology to other organic pollutant classes.

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Table 2. Creosote Constituent Transformation Results[a]

Compound	PAH Initial Conc. (mg/kg)	% Decrease		
		No Treatment Plot	Carrier Plot	*P. sordida* Plot
Acenapthene	429	49	68	95
Fluorene	225	75	57	95
Phenanthrene	941	69	49	90
Anthracene	684	57	48	85
Fluoranthene	972	23	42	72
Pyrene	572	10	22	52
Benzo[a]anthracene	74	11	13	24
Chrysene	90	6	14	33

[a]Initial concentrations were based on soil samples taken 1 day after treatment application. Each value for initial concentration and percentage decrease is the mean of 24 observations.

PERFORMANCE EVALUATION

FIELD EVALUATION OF PHENOL FOR COMETABOLISM OF CHLORINATED SOLVENTS

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INTRODUCTION

Aerobic microorganisms grown on phenol or toluene can initiate the cometabolic oxidation of chlorinated aliphatic hydrocarbons (CAHs) to stable nontoxic end products (1,2,3,4). Such microorganisms have good potential for bioremediating aquifers contaminated with CAHs and their anaerobic and abiotic transformation products. Recent in situ studies at the Moffett Field test site demonstrated that 80 percent of the trichloroethylene (TCE) added could be degraded in the 2 m biostimulated zone while injecting 12.5 mg/L phenol, 35 mg/L dissolved oxygen (DO), and 40 μg/L TCE (6). The objective of the current work is to determine how effective the phenol-utilizers were at degrading TCE over higher concentrations ranging from 62.5 to 1,000 μg/L.

METHODOLOGY

The methodology for the in situ tests was similar to that used in our previous studies (5,6). The experiments consisted of a series of stimulus-response tests; the stimulus being the injection of ground water amended with the chemicals of interest, and the response being the concentration history of the chemicals at the monitoring locations. Experiments were performed under the induced gradient conditions of injection and extraction. In this study, TCE was injected along with phenol and DO. This permitted the TCE concentration effect to be studied in greater detail. Phenol was pulse injected at 100 mg/L for 1 hr in an 8-hr pulse cycle, resulting in a time averaged concentration of 12.5 mg/L during the first 1,000 hr of the test. The initial TCE concentration was 62 μg/L. The TCE concentration was gradually raised by doubling the concentration after effective transformation had been achieved at the lower concentration. After 1 week of operation, the injected TCE concentration was raised to 125 μg/L, then to 250 μg/L after another week, 500 μg/L during the subsequent week, and then 1,000 μg/L. The time averaged phenol injection concentration was then raised to 19 mg/L for 1 week, and then increased again to 25 mg/L, or twice the initial concentration, while maintaining a TCE concentration at 1,000 μg/L. Bromide was added as a conservative tracer as a basis of comparison for determining transformation extents.

RESULTS

Figure 1 shows the normalized concentration breakthrough of TCE at the three observation wells SSE1, SSE2, and SSE3, located 1, 2.2, and 3.8 meters from the injection well. The normalized concentration represents the observed concentration divided by the injection concentration at the time of the observation. The smoothed lines through the raw data are running averages. The depiction of data in running averages is especially helpful for well SSE1 where the pulsed addition of phenol induced competitive inhibition causing fluctuations in TCE concentration. The damping effect of transport and sorption combined with the lack of phenol results in much smaller fluctuations at the SSE2 and SSE3 wells.

For TCE injection concentrations ranging from 62.5 μg/L to 500 μg/L, the extent of biodegradation is similar despite the increases in injection concentration. Bromide tracer tests conducted over this period show complete breakthrough of the conservative tracer, C/Co = 1. Approximately 70 and 85 percent TCE removal was observed at wells SSE1 and SSE2, respectively. Upon increasing the TCE injection concentration to 1,000 μg/L, removals were lower.

While injecting 1,000 μg/L TCE, the phenol concentration was increased to 19 mg/L at 1,008 hr and then to 25 mg/L at 1,176 hr. The results are illustrated in Figure 2. TCE removal increased following each phenol increase. Since no controls are available, it is not possible to conclude that the degree of improvement results from phenol concentration increases or other factors such as increased population of phenol-oxidizing bacteria.

Phenol concentration was measured in the injection stream and at all monitoring locations. Phenol was frequently detected at SSE1, but the concentrations found were generally less than 0.5 mg/L while 12.5 mg/L was injected. Detections at SSE2 and SSE3, however, were infrequent. The measurement method at the field site had a visible response to phenol concentration as low as 10 μg/L, but the quantifiable detection limit was about 25 μg/L. The phenol concentrations at the SSE2 and SSE3 monitoring locations were generally too low to give a visible response, and thus are presumed to be below 20 μg/L, and probably below 10 μg/L. Thus phenol removal was excellent and probably greater than 99.9 percent in this system.

The TCE results are summarized in Table 1. The percentage removals listed are based on average values at the end of the period following the changes in concentration. Removal up to 89 percent was observed at the SSE3 well, with 12.5 mg/L phenol added. Removals were similar with up to 500 μg/L TCE injected. This indicates the removal was first order with respect to TCE concentration. Upon increasing the TCE concentration to 1,000 μg/L, the removals decreased to 77 percent with 12.5 mg/L phenol injected. This lower percentage removal may result because TCE concentration is nearer the K_s value resulting in deviations from first-order kinetics, TCE transformation product toxicity is beginning to have a measurable effect, or sufficient reducing power from phenol oxidation is not available.

Transformation yields are also presented in Table 1. The highest yield observed in the field was 0.062 g TCE/g phenol. This yield was obtained while injecting 12.5 mg/L phenol and 1,000 μg/L TCE. Lower yields are obtained at lower TCE concentrations or at higher phenol injection concentrations.

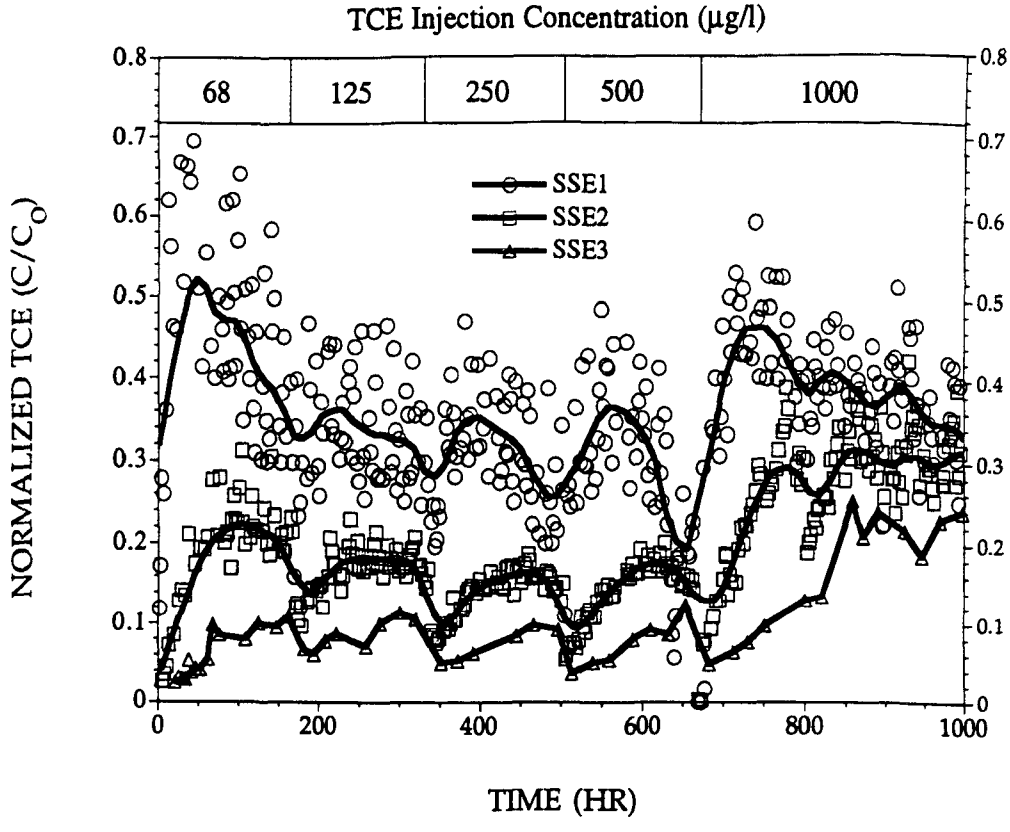

Figure 1. Normalized breakthrough of TCE at observation wells during biostimulation at 12.5 mg/L phenol. TCE injection concentrations range from 68 to 1,000 μg/L.

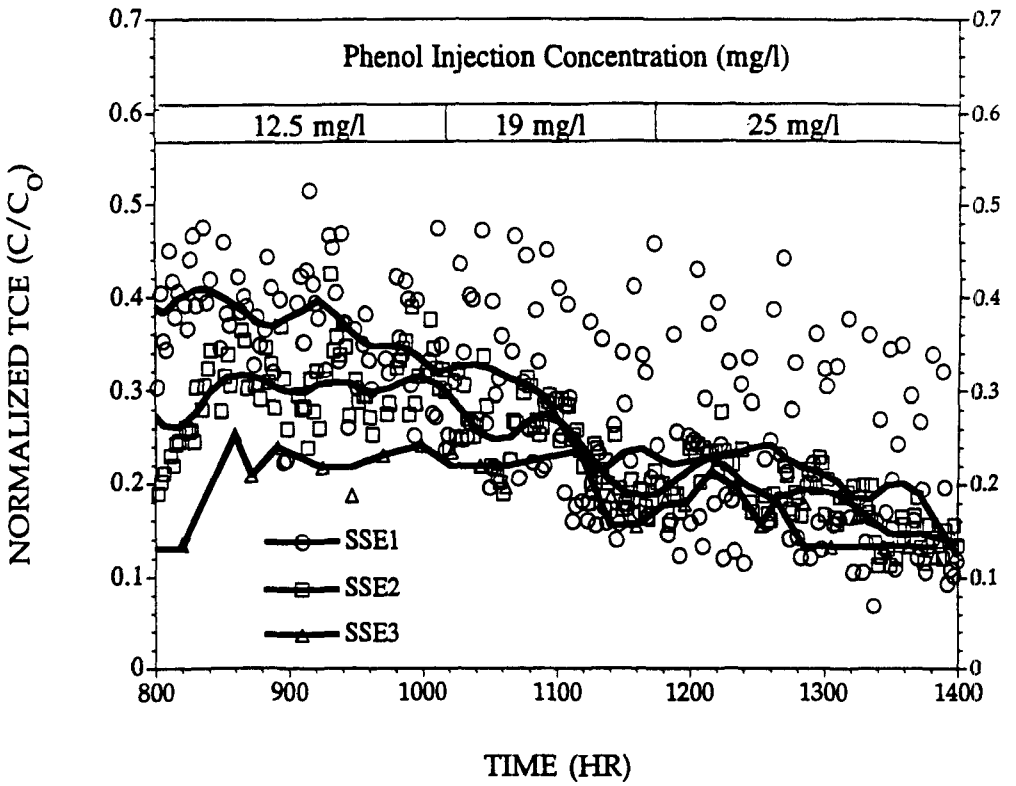

Figure 2. TCE concentration response resulting from increased phenol addition.

Table 1. Average Removal Efficiencies for TCE, at Various Well Locations, and Transformation Yields

Phenol Added mg/l	TCE Added µg/l	Percentage Removal			Transformation Yield g TCE/g phenol
		SSE1	SSE2	SSE3	
12.5	62	60	78	89	0.0044
12.5	125	68	82	87	0.0087
12.5	250	70	82	88	0.018
12.5	500	68	84	88	0.035
12.5	1,000	78	85	90	0.062
19	1,000	75	82	85	0.045
25	1,000	78	85	90	0.036

SUMMARY AND CONCLUSIONS

An indigenous phenol-utilizing population effectively degraded TCE up to 1,000 μg/L. At the highest phenol injection concentration, up to 90 percent of the 1,000 μg/L TCE added was degraded in a 3-m biostimulated zone. With injection of 12.5 mg/L phenol, first-order removal within the test zone of 85 to 90 percent was obtained with TCE concentrations up to 500 μg/L. A phenol concentration of 25 mg/L was required to obtain similar removal efficiency with a TCE injection concentration of 1,000 μg/L. These results suggest in situ biodegradation of TCE with phenol to be quite promising.

Future studies at the site will explore a range of contaminants including vinyl chloride, chloroform, and 1,1-dichloroethylene. The injection of a noncompetitive source of reducing power, such as formate, and its effect on removal efficiency will be explored. Studies with toluene as a growth substrate will also be performed for a comparison with phenol.

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INNOVATIVE BIOREMEDIATION STRATEGIES FOR CREOSOTE: CHARACTERIZATION AND USE OF INOCULA

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New microorganisms possessing novel biodegradative abilities continue to be discovered at a rapid rate. For example, bacteria have been isolated that utilize high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs), such as those found in creosote, coal tar, and crude oil, as sole sources of carbon and energy for growth (1,2). In addition, microorganisms such as *Mycobacterium* sp. strain PYR-1 (Dr. Carl Cerniglia, U.S. Food and Drug Administration [FDA] National Center for Toxicological Research, Jefferson, Arkansas), which co-metabolize mixtures of HMW PAHs and structurally related compounds, have been described (3,4). Concomitantly, our knowledge of physicochemical factors limiting biological activity in the field (e.g., bioavailability) has increased significantly, while innovative methods for assessing and monitoring field performance have been developed.

Successful combination of these technological advances into functional bioremediation strategies could result in reliable, cost-efficient remedial tools for full-scale remediation of soil and water contaminated by compounds notoriously difficult to treat biologically. Despite the significant application potential of these newly described microorganisms, however, the most common, and clearly the most successful, uses of bioremediation in the field remain focused on the treatment of materials contaminated by readily biodegradable organics (i.e., refined petroleum products). One of the primary reasons for this rather narrow application spectrum relates to the general inability to

effectively employ inoculant microorganisms in the field. Thus, as the practical applicability of bioremediation technologies are expanded to include more persistent chemicals, such as those found in creosote (i.e., HMW PAHs), new inoculation procedures and implementation strategies need to be developed.

Expected advantages of augmented or otherwise improved microbiology of contaminated environments include 1) accelerated rates of biodegradation (e.g., shorter treatment time), 2) furthered extent of contaminant removal ("cleaner" end product), 3) reduced probability of undesirable "side-reactions" (e.g., production of more toxic intermediates by indigenous microbes), and 4) ensured consistency and reliability of biodegradative performance in the field thus yielding more cost-effective treatment technologies. The overall effectiveness of microbial modification, however, depends largely on the type of bioremediation technology being employed. Hence, unique tools for modifying and/or managing microbial systems are being pursued for a variety of bioremediation application strategies, including bioreactor operations, solid-phase (landfarming), and in situ applications (5,6).

A review of the scientific literature and experience in the bioremediation industry offer the following scenarios where, in theory, the use of inoculants might be warranted. In the most straightforward scenario, the inability of indigenous microflora to degrade or transform a persistent chemical is known. In this case, the addition of relevant catabolic machinery, in conjunction with requisite chemical resistances (e.g., heavy metals) or physical tolerances (e.g., low temperature), may be accomplished via inoculation. A second scenario considers incidents of sudden exposure (e.g., oil spill) where it is assumed that indigenous microflora have not had time to adapt biochemically to a chemical insult. If such is the case, then the addition of "trained" microbes may facilitate a more rapid increase in degrader biomass, hence potentially accelerating the biodegradation process. Lastly, when indigenous microorganisms effect incomplete or partial catabolism, inoculants can be used to provide alternative catabolic pathways. Of these possibilities, it appears that the most valid use of inoculants is to increase the rate of biodegradation of the targeted substrates. Hence, the efforts of this study have focused on the development of effective inoculation strategies to amplify the number of rare, or relatively scarce, microorganisms with requisite resistances and/or tolerances.

Few reports describe the use of microbial inoculants to positively influence the biodegradation of targeted compounds in the field under solid-phase conditions, but most studies have been conducted under laboratory conditions. Alternatively, the most successful use of inoculants, beyond the laboratory- or bench-scale levels, has been in bioreactor operations. Thus, the present status of inoculation technologies is limited by a general inability to employ them, in a consistent and reliable fashion, to treat soil or ground water outside of a controlled environment, such as a laboratory vessel or a bioreactor in the field.

When inoculant strains are applied to soil or water in the open environment, rarely is a measurable effect clearly associated with their presence. This may be because inoculation is often performed in conjunction with other efforts to modify the chemical and/or physical environment, hence the comparative data necessary to clearly elucidate the response as directly attributable to the inoculant per se are not available. From more of a microbiological perspective, other potential reasons for this lack of a discernible response include 1) inoculant microorganisms may not successfully compete with indigenous microflora for essential micro-sites or ecological niches essential for their survival, 2) inoculant bacteria are particularly prone to predation, and 3) inoculants experience rapid die-off due to desiccation and other physiological shocks (e.g., temperature changes, osmotic changes).

In an effort to overcome factors known to inhibit inoculation procedures for bioremediation applications, a variety of microbial encapsulation and cell immobilization technologies (7,8) similar to some of those previously described (9,10) have been developed. Data from in-house laboratory studies using bacteria active toward HMW PAHs have shown that these technologies provide an effective means of inoculant storage and distribution (Lin et al., unpublished data). Furthermore, newly developed encapsulated cell technologies provide an efficient means of applying high numbers of viable, catabolically active inoculant cells (>1 x 10^{10} bacterial cells/g inoculant) to soil and facilitating their timed release (Lin et al., unpublished data).

Encapsulation technologies currently are being used to improve the solid-phase bioremediation of soils contaminated with organic wood preservatives (i.e., creosote and pentachlorophenol). The importance of the encapsulated cells for these applications is to 1) ensure the consistent presence of catabolically relevant, active biomass; 2) provide for slow-release of essential nutrients and electron acceptor; and 3) offer an ecological niche conducive to microbial growth, proliferation, and catabolism. Results to date have identified effective encapsulation and immobilization technologies for various PAH- and pesticide-degrading bacteria. Depending on the desired end points, this strategy may offer a viable approach for remediating soils contaminated with creosote and similar substances. Similarly, immobilized cells in liquid bioreactor systems (above-ground or in situ bioreactors) are being tested for their ability to treat ground water impacted by related compounds. In addition, research is being conducted to determine the effectiveness of co-encapsulating microorganisms (e.g., HMW PAH-degraders) with nutrients, electron acceptors, and/or electron donors.

ACKNOWLEDGMENTS

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DECONTAMINATION OF PCB-CONTAMINATED SEDIMENTS THROUGH THE USE OF BIOREMEDIATION TECHNOLOGIES

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INTRODUCTION

It is now widely recognized that polychlorinated biphenyls (PCBs) can be reductively dechlorinated in anaerobic environments such as sediments. This process was first suggested by PCB congener distribution patterns observed in sediment core samples from the upper Hudson River (1). Compared to both surficial sediments and the Aroclor 1242 originally input to these sediments, chromatograms for deeper sediment samples showed a depletion of the more heavily chlorinated, later eluting congeners and a corresponding relative increase in lesser chlorinated, early eluting congeners. This transformation process has since been demonstrated to be the result of anaerobic microbial activity (2), and has been observed in PCB-contaminated sediments from a number of other sites (3,4).

The discovery of the anaerobic dechlorination of PCBs has stimulated interest in developing 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 is able to dechlorinate more highly substituted congeners, producing products that are aerobically degradable. Indeed, all products of the anaerobic dechlorination of Aroclor 1254 (5) have been shown to be aerobically degradable by one or more strains of aerobic bacteria (6). Also, the high proportions of mono- and dichlorinated biphenyls that can accumulate as a result of anaerobic PCB dechlorination can serve to induce PCB-degrading enzymes in aerobic microorganisms. Although more highly chlorinated congeners can be aerobically cometabolized, they are not inducing substrates.

To develop a sequential anaerobic/aerobic biotreatment process for PCBs, more about the factors controlling the anaerobic dechlorination process needs to be understood. The extent of in situ PCB dechlorination that has occurred varies among sites (Table 1). This is likely attributable to both the congener specificity of the predominant PCB-dechlorinating microorganisms present and certain environmental characteristics. Recognizing that environmental characteristics may have changed since the time of in situ dechlorination, assays were conducted to determine the ability of each sediment to currently support dechlorination. Sediments from each site were slurried with reduced anaerobic mineral medium and inoculated with Hudson River microorganisms, and Aroclor 1242 (500 μg/g sediment) was added. The amount of chlorine removed from the PCBs in 16 weeks was taken as a measure of the sediment's current ability to support PCB dechlorination. This measure correlated with the extent of in situ dechlorination, except for the Lagoon (A and B), Silver Lake, and River Raisin E sediments. Conditions at the first three sites may have become unfavorable for dechlorination after in situ dechlorination had already begun. The exceptionally high total PCB concentration in the River Raisin E sediments made the detection of assay dechlorination difficult for analytical reasons.

Correlations observed between the extent of assay dechlorination for the other samples and several site characteristics suggested that co-contaminants, notably heavy metals and oil and grease, may be important limiting factors (Table 1). The preliminary results of laboratory experiments designed to better define the impact of these factors on the dechlorination process are summarized below. Research to be undertaken under a cooperative agreement between Michigan State University and EPA will address ways potentially limiting factors, including heavy metals and oil and grease as co-contaminants, can be overcome in a biotreatment process.

RELATED RESEARCH

Oil and Grease

Oil and grease in sediments probably act as a separate phase to which PCBs may partition (7). High concentrations of oil and grease therefore might be expected to limit dechlorination by decreasing the availability of the PCBs to the microorganisms. This hypothesis is being tested using vacuum pump oil and Aroclor 1242 added to sediment slurries inoculated with Hudson River microorganisms.

A dose response between oil concentration and the extent of dechlorination after 8 weeks of incubation has been observed, but dechlorination still occurred even with the addition of 40 mg oil/g sediment, the highest level tested (Table 2). This highest concentration is comparable to the level of oil and grease in the most heavily contaminated sediments examined. The extent of dechlorination during the first 8 weeks of incubation is still approximately one-half of that when no oil is added. Continued monitoring of this experiment for 24 weeks is proposed, but the results so far suggest that oil as a sorptive phase is not extremely important in preventing PCB dechlorination.

Qualitative differences between oils in environmental samples (e.g., additives and polycyclic aromatic hydrocarbons content) might be important in inhibiting PCB dechlorination. Vacuum pump oil was chosen for the above experiment because it does not contain potentially toxic or inhibitory additives. Oils found in environmental samples also are being characterized and their effect on PCB dechlorination is being determined.

Table 1. Extent of Dechlorination and Selected Environmental Parameters for PCB-Contaminated Sediments

Sediment	*In situ* Dechlorination	Assay Dechlorination	Total PCBs (µg/g)	Oil & Grease (µg/g)[a]	Total Metals (µg/g)[b]
Acushnet 17	5	0.3	20	10,730	2,796
Acushnet 19	26	0.5	1,112	11,939	2,790
Hudson H7	74	1.6	74	626	1,143
Lagoon A	37	0	800	17,750	1,934
Lagoon B	38	0	1,113	38,338	3,352
Raisin D	9	0.8	88	7,157	3,919
Raisin E (0-10 cm)	7	0	25,093	3,807	1,152
Raisin E (10-25 cm)	3	0	14,993	3,542	542
Saginaw 8R (10-20 cm)	7	0	323	13,092	5,427
Sheboygan R212	41	0.5	418	1,083	295
Sheboygan R28	63	1.1	395	955	292
Sheboygan R9	67	1.8	319	581	244
Silver L F3	32	0	694	62,856	9,135

[a] Oil and grease concentrations were determined gravimetrically and corrected for PCB concentration.

[b] Total concentration of cadmium, chromium, copper, nickel, lead, and zinc.

Note: In situ dechlorination is defined as the percent decrease in *meta* and *para* chlorines that has occurred naturally in the environment. Assay dechlorination is defined as the µg atoms of chlorine removed from PCBs in a 16-week laboratory incubation after addition of Aroclor 1242 (500 µg/g sediment).

Table 2. Extent of Aroclor 1242 Dechlorination

	Avg m & p Cl's	
Treatment*	Mean	S.D.
0	0.85	0.11
5	1.10	0.06
10	1.16	0.02
20	1.26	0.04
40	1.30	0.03
1242	1.8	

*mg oil / g sediment

Note: Dechlorination is as indicated by the average number of chlorines remaining in the *meta* and/or *para* positions, after 8 weeks of incubation by Hudson River microorganisms with various levels of vacuum pump oil added to the non-PCB-contaminated Hudson River sediments used in the dechlorination assay. No dechlorination from the *ortho* positions was indicated. Addition of 40 mg oil/g sediment reduced the extent of dechlorination by half.

Metals

Additional experiments are directly testing the effects of the more abundant metals found in the contaminated sediments examined. Various concentrations of metal salts (5 to 500 $\mu g/g$ sediment of copper, lead, zinc, or chromium) were added to pre-incubated anaerobic sediment slurries. The slurries were then autoclaved and inoculated with Hudson River microorganisms, and Aroclor 1242 was added. These experiments have been sampled over 8 weeks and preliminary analysis suggests that zinc is the most important metal for limiting dechlorination. Monitoring PCB dechlorination in these experiments will continue for 24 weeks.

In a related experiment, Silver Lake sediments were found not to support dechlorination after oil and grease have been removed by solvent extraction, leaving the metals behind. Additional experiments are being conducted to ensure that the extraction process itself does not adversely affect the ability of the sediments to support PCB dechlorination.

FUTURE RESEARCH OBJECTIVES AND APPROACH

The basic objective of this research to be undertaken under a cooperative agreement between Michigan State and EPA is to find ways of overcoming potential environmental limitations on PCB dechlorination. The general approach will be to select soils or sediments based on the probable factor(s) limiting PCB dechlorination, and then experimentally evaluate means of overcoming those limiting factors. The initial focus will be on overcoming limitations attributable to high levels of oil and grease, low carbon content, low bioavailability, and high redox potential (in soils). These limiting factors may be overcome by aerobically biodegrading the oil and grease, adding nutrients, using surfactants, and chemically or biologically providing more reducing conditions. Subsequently, an attempt will be made to overcome limitations attributable to metal toxicity by chelation, extraction, precipitation, and bioleaching. Research aimed at overcoming limitations relating to the congener specificity of the microorganisms involved currently is being investigated in a separate project funded by General Electric. Together, these efforts represent a comprehensive evaluation of factors limiting PCB dechlorination and approaches for overcoming these limitations.

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NATURAL ANAEROBIC BIOREMEDIATION OF TCE AT THE ST. JOSEPH, MICHIGAN, SUPERFUND SITE

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INTRODUCTION

In a sand aquifer near the town of St. Joseph, Michigan, trichloroethylene (TCE), 1,2-cis-dichloroethylene (c-DCE), 1,2-trans-dichloroethylene (t-DCE), and vinyl chloride (VC) are present at ground water concentrations from 10 to 100 mg/L. In the summer of 1991, a few hundred ground water samples were collected along three transects located near the areas of highest concentrations. The study was originally undertaken to evaluate the potential of in situ treatment by stimulating the growth of a native population of methanotrophic bacteria. The data suggested that natural anaerobic degradation of TCE has been taking place. An analysis of these measurements was undertaken to gain a greater understanding of the distribution of chlorinated aliphatic compounds several years after the contamination and of the natural mixing and transformation processes. This presentation summarizes the methodology and the results of this analysis.

The geologic formation of interest is an unconfined aquifer consisting of a layer of unconsolidated fine sand with some silt. The aquifer is nearly homogeneous, having been formed by eolian sorting of glacial deposits. The thickness of the sand layer is variable, ranging from 12 to 32, as is the elevation of the base of the aquifer that undulates over a range of about 16 m. The aquifer overlays a lacustrine clay unit and at some points the transition from sand to clay is gradual.

The site is bounded by Lake Michigan to the west and Hickory Creek to the east. The hydrology of the sandy aquifer is relatively simple and is dominated by a high recharge rate. The ground water drains into the lake and the creek, which act as constant-head boundaries, at a nearly

steady rate. A hydrologic divide separates the part of the aquifer that drains into the lake from the part that drains into the creek. The aquifer contamination probably took place from TCE that leaked from lagoons located over the hydrologic divide. As a result, one plume was formed that moves toward Lake Michigan and one was formed that moves toward Hickory Creek (1).

Halogenated aliphatic compounds in the subsurface can be transformed biologically in the absence of oxygen (2) and in fact have been shown to do so under a variety of environmental conditions. Common anaerobic electron acceptors and the associated microbial process, in the order of their redox potential, are nitrate (denitrification); Mn(IV) (manganese reduction); Fe(III) (iron reduction); sulfate (sulfate reduction); and carbon dioxide (methanogenesis). Many of the chlorinated aliphatic compounds are highly oxidized and have redox potentials above those of common electron acceptors (3). Generally, the less halogenated components are transformed more slowly; for example, VC is transformed at a rate over two orders of magnitude lower than that of TCE. The reduction reaction rates also are faster the lower the redox state (3,4).

Fortuitous anaerobic transformation of chlorinated ethenes, such as tetrachloroethylene (PCE) and TCE involving substitution of chlorine by hydrogen, has been observed in several field studies (1). The detailed chemical characterization of the St. Joseph site that will be presented supports previous laboratory and field findings. The characterization permits zones to be identified where transformations are occurring, and permits flux estimates of the contaminants and transformation products. The characterization is a joint effort of Allied-Signal Corporation, Engineering Science, U.S. EPA Region 5, U.S. EPA Robert S. Kerr Environmental Research Laboratory (RSKERL), and Stanford University.

DATA ANALYSIS

Three transects were completed with 17 slotted auger borings. Transects 1 and 2 span the width of the plume and Transect 3 is located roughly in the center of the plume. Onsite gas chromatography analysis was performed for TCE and its anaerobic transformation products. The purpose of the onsite analysis was to guide the field personnel in the selection of the next boring location, so that the width of the plume and the center of highest concentration could be found using a succession of boreholes. Samples for volatile organic compounds (VOC) analysis were shipped on ice to RSKERL for measurement of solute concentration using EPA methods.

The data from the water samples were then statistically analyzed to construct contour lines of equal concentration and to estimate flux rates. Contouring and averaging require the interpolation from the data of the concentration of the solutes on a fine regular mesh. Univariate statistical methods of data analysis, which treat the data independently of their location in space, are not applicable. For example, to compute the total mass, assigning equal weights to all measurements would not be reasonable because it is common to have more measurements near the center of the plume than elsewhere; instead, one should assign weights that are representative of the area of influence of each measurement and account for the shape of the concentration surface. Linear geostatistical methods account for spatial variability and are practical tools, but they are not accurate when the distribution of estimation errors is not described adequately by the mean and the mean square value. Estimation errors, however, can vary over orders of magnitude, depending on the proximity of measurements to "hot spots." Also, they are highly skewed (i.e., asymmetrically distributed).

The methodology used produces point estimates (i.e., representative values), as well as confidence intervals (i.e., error bars that indicate the range of possible values), which make it possible to evaluate the accuracy of estimated concentrations and masses. The approach accounts for the skewness in the distribution of estimation errors by adding only one parameter to those used in linear geostatistics (variograms or generalized covariances). This parameter can be determined from the data. The resulting nonlinear estimation method is not substantially more difficult to use than linear geostatistics.

PRELIMINARY RESULTS

The detailed chemical characterization of the St. Joseph site that will be presented will mostly support the previous laboratory and field findings about anaerobic transformation of chlorinated aliphatic compounds. A remarkable result from the transect data was that the concentration of the chlorinated aliphatics compounds was found to vary significantly with depth. This feature was not predictable from the data previously collected from nearby monitoring wells.

Perhaps the most interesting results are the concentration distributions obtained from the two-dimensional contour analysis in the two transects that are roughly perpendicular to the ground water flow. Complete transformation of TCE to ethene was associated with methanogenic conditions in the aquifer. VC and ethene concentrations turned out to be correlated with the highest methane concentrations. Zones of high TCE concentration tended to be associated with zones of depressed methane concentrations. The cause for this absence of methane where TCE concentration is the highest is not evident. It is possible that the high TCE values are actively depressing methanogenesis. Another possibility is that TCE remains in zones where active methanogenesis is not occurring, for example, because of an absence of appropriate bacteria, electron donors, or redox conditions. c-DCE represents most of the DCE present, with t-DCE and 1,1-dichloroethylene present in small amounts. The spatial distributions of the DCE isomers, however, are similar, indicating similar formation process(es). c-DCE was found at a slightly greater depth than TCE, and VC and ethene were located even deeper, and associated with high methane concentrations.

The concentration of sulfate and ammonia as inorganic constituents showed some correlation with the concentration of the chlorinated aliphatic compounds. Sulfate tended to be present at shallower depths and decreased in concentration with depth as methane concentrations increased. Ammonia also decreased with depth, as methane concentration increased, possibly because of the uptake associated with biological growth. The data suggest that c-DCE was formed and tended to persist in transition zones from sulfate reduction to methanogenic conditions. Active VC formation and transformation to ethene occurred under methanogenic conditions that were observed at greater depth.

Flux estimates were made based on the contour average concentrations and an estimated ground water velocity of 25 m/yr. The total flux of chlorinated aliphatics plus ethene was 200 and 490 kg/yr for transects 1 (downgradient) and 2 (upgradient) respectively. On a mole basis, the fluxes differ by a factor of about 2.2. c-DCE represents the greatest mole-flux in both transects followed by TCE, VC, and ethene. The fact that c-DCE has a greater mole flux than its parent TCE indicates that significant anaerobic transformation has already taken place. Ethene represents approximately 8 to 22 percent of the total mole flux, indicating significant dehalogenation to a nontoxic end product. A greater amount of contaminant and transformation products were moving across the upgradient location compared to the downgradient location. The methane flux estimate is a factor

of four greater at transect 2 than at 1, suggesting more highly anaerobic conditions upgradient. The differences between the two transects, however, might be due to the transient nature of transport of contaminants as they move toward Lake Michigan.

Estimates were made of the chemical oxygen demand (COD) reduction required to dehalogenate the chlorinated aliphatic compounds and produce the methane (1). The COD reduction required was 1,180 kg/yr and 310 kg/yr for transects 2 and 1, respectively. Up to 15 percent of the COD reduction was associated with reduction of chlorinated aliphatics, which is high considering that these transformations are presumed fortuitous in nature. The results suggest that it might be possible to manipulate the conditions at the site to enhance anaerobic transformation of TCE to nontoxic end products.

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COMPARISON OF BIOVENTING AND AIR SPARGING FOR IN SITU BIOREMEDIATION OF FUELS

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Bioremediation pilot-scale subsurface venting and sparging systems were operated at a low aeration rate at an aviation gasoline spill site. Bioventing removed 99 percent of vadose zone contamination in 8 months with minimal surface emissions. The biosparging process is presently operating and has removed one-third of oily phase residue below the water table in 1 year. The ground water plume has been cleansed of benzene, toluene, ethylbenzene, and xylene (BTEX) components by sparging.

INTRODUCTION

The failure in 1969 of a buried transfer pipe flange within an underground storage area of a U.S. Coast Guard Air Station resulted in an aviation gasoline (Avgas) spill of about 35,000 gallons. The Avgas migrated downward and laterally to form a plume below a surface area of 260 ft wide and 1,200 ft long. The subsurface to 45 ft was a fairly uniform beach sand with the present water table near 15 ft. Water table fluctuations over the years, as much as 5 ft, formed an oily phase smear of Avgas contamination with about 30 percent in the vadose zone, just above the present water table.

Venting and sparging can be in situ bioremediation processes that provide air flow to vaporize and transport volatile organic pollutants upward to more amenable media for mineralization. Microbial degradation processes also utilize the oxygen provided by the air. Injection

wells can be installed at spill sites to emit air just above the water table for venting and below the water table for sparging. Laboratory soil microcosm studies using surface soil from the site showed that once acclimation occurred the degradation of Avgas vapor was rapid and complete (1).

The objective of the study was to demonstrate by pilot-scale process units that venting and sparging can effectively bioremediate an aviation gasoline contaminated subsurface. Since this report has not been subjected to EPA review, however, official endorsement should not be inferred.

EXPERIMENTAL DESIGN

Turf was established on a 75 by 45 ft rectangular area overlying the plume of contamination. An initial nutrient solution of 64 lb nitrogen, 5 lb potassium, and 13 lb phosphorus was applied for dispersion throughout the unsaturated subsurface. Aeration injection wells screened across the water table were placed 10 ft apart in a 3 by 5 grid. A blower rate of 5 cfm was used, which was estimated to be equivalent to a subsurface air retention time of 24 hours.

Vadose zone soil gas samples were obtained with 1/2-inch diameter stainless steel tubing clusters set at depths of 3.2, 6.5, 9.7, and 13.0 ft. A portable Bacharach TLV combustible gas meter was used to measure subsurface Avgas vapors. An inverted stainless bowl with a nipple outlet at the top was used as a collection device for surface emissions. Vented air was removed from the bowl canopy at the same rate of entrance as determined by a propane dilution test and passed through a cartridge trap. The trapped hydrocarbons were analyzed by gas chromatography.

Vertical profile core samples were obtained using a drilling rig with hollow stem augers and a piston barrel sampler. Core samples were placed in glass jars and capped immediately. Within 15 minutes, jars were uncapped one at a time, and a plug aliquot was removed and preserved for later analysis of total petroleum hydrocarbons. Avgas vapors in the plug-evacuated space were quickly measured using a real-time core assay method (2). Ground water samples were collected from installed monitoring wells and analyzed by EPA standard methods.

One year after installation of the venting process, the apparatus was revised into a sparging process. Eight 2-in. diameter polyvinyl chloride (PVC) sparge wells were installed to a depth of 10 ft below the water table in a 2 by 4 grid 10 ft apart. A minimum radius of influence of 15 ft was determined by pressure changes at distances away from a sparge well (3). A blower rate of 10 cfm was used.

RESULTS

Venting operations started during October 1990. Gasoline hydrocarbon vapor in the vadose zone initially increased to near 5,000 mg/L, then within 3 weeks decreased 80 percent (Figure 1). A gradual decline continued until the operation was shut down in January 1991 because the turf root zone was frozen. Venting was restored, 3 months later, after the soil had thawed. Vapor concentrations increased to near 1,000 mg/L then decreased rapidly. After 8 months of operation, soil gas concentrations were 50 mg/L or less. Surface emissions during mid-term operation of the venting system were less than 10 μg/L, compared to 3.2 ft depth soil gas levels of 163 mg/L. Biomass degradation activity from the turf rhizosphere was greater by a factor of 10 than at the same depth of a barren soil control that was not acclimated to gasoline vapor.

Vertical profile fuel carbons for October 1990 and October 1991 are shown in Figure 2. The water table was at the same level of 15.3 ft for both time periods. A reduction of oily phase residue in the vadose zone occurred in excess of 99 percent, while reduction below the water table was only about 22 percent.

Sparge wells were installed and operation started in November 1991. Initial gasoline vapor concentrations exceeded 6,000 mg/L in the vadose zone (Figure 1), then after 5 months sparging were near 1,000 mg/L. After 1 year, the levels were 50 mg/L or less. Ground water monitoring well samples were collected and analyzed after 7 months of sparge operation (Table 1). Sparging effectively reduced contaminants dissolved in the ground water. Vertical profile core samples were collected and analyzed October 1991 and September 1992. Heterogeneity obtained from vertical profile sampling was partially compensated by averaging replicates to obtain a trend shift in Avgas contamination. Real-time core assays are shown in Table 2. Assay levels of control profiles had a 4 percent reduction during the 12-month period, while sparging reduction was 80 percent. Oily phase residue as total fuel carbon was reduced 39 percent by sparging during the 12-month period (Table 3). The data suggested that inaccessibility of gasoline globule contact with air flow had restricted vaporization and transport upward.

CONCLUSION

The vadose zone contaminated with aviation gasoline was satisfactorily bioremediated by venting in 8 months. Surface emissions of gasoline vapor during process operation were minimal. Sparging of the ground water plume at a low aeration rate has reduced oily phase residue by at least one-third in a 12-month period. The sparging system has been operational for 17 months and will continue.

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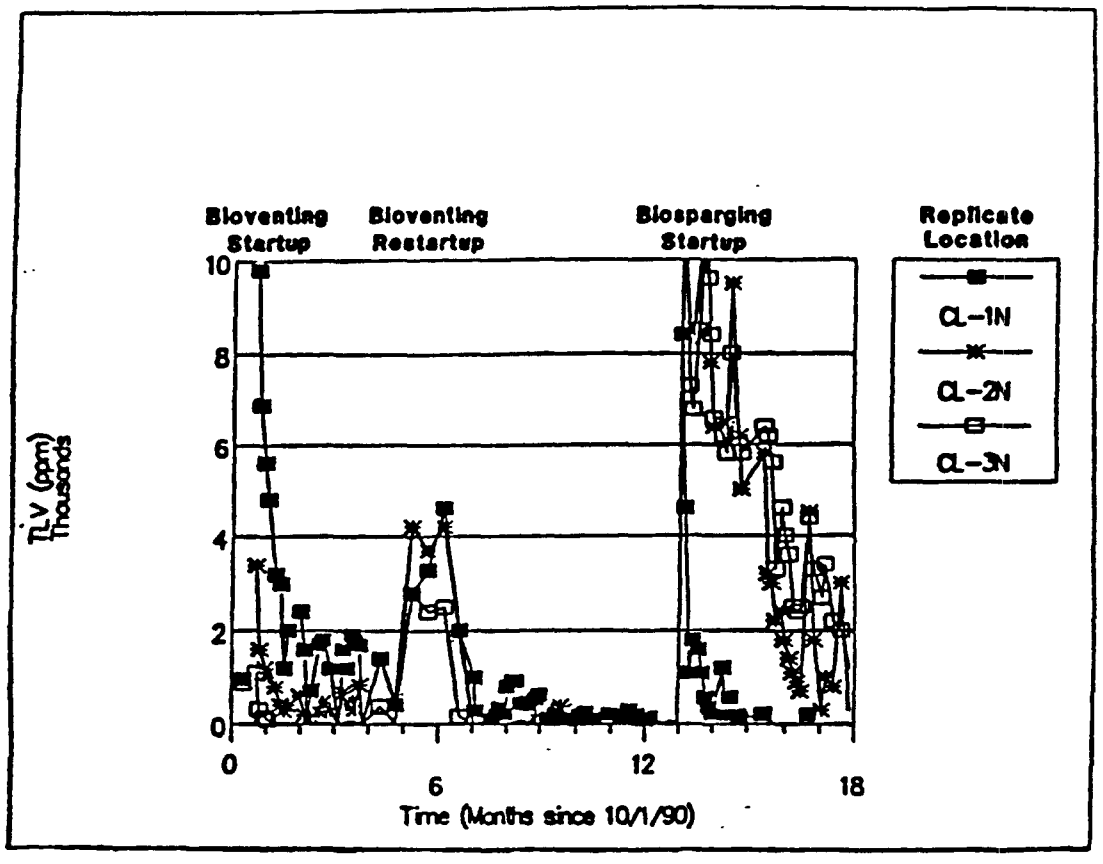

Figure 1. Soil gas hydrocarbons at 4-m depth for injection-only plot.

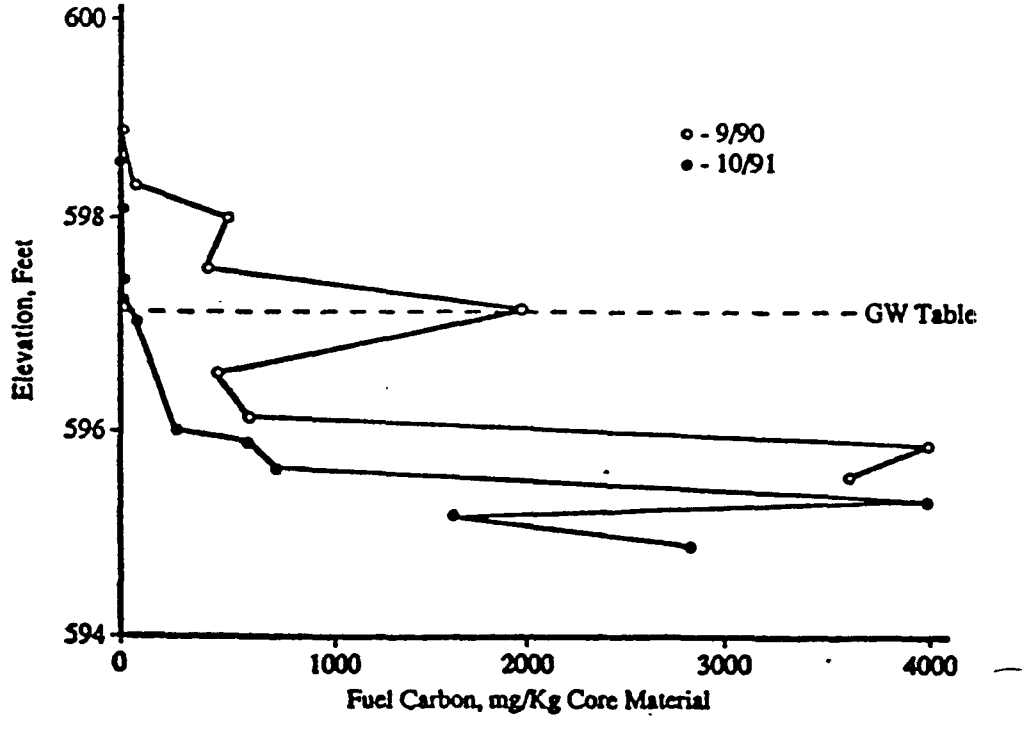

Figure 2. Vertical profile oily phase residue in bioventing of north plot.

Table 1. Ground Water Quality after Seven Months of Biosparging

Monitoring Well	Well Depth	Benzene	Xylenes	Total Fuel Carbon
	-ft-	μg/L		
Control	16	9.9	19	2880
	17.5	228	992	4490
	20.5	70	38	956
	22	57	7.7	783
Sparge Plot	15	1.9	5.3	559
	18	>1	5.0	>6
	19.5	>1	>1	>6
	21	>1	>1	>6

Table 2. Core Assay of Avgas Vapor during Drilling Operations

	Initial	One Year Later	Reduction
	Replicate Average over 40 in. profile, mg Avgas/ft		
Control	4070 (3 reps)	3920 (2 reps)	%
Sparge plot	5980 (3 reps)	1220 (4 reps)	80

Table 3. Total Fuel Carbon in Vertical Profile

	Initial	One Year Later	Reduction
	Replicate average, mg/ft^2 surface area		%
Sparge plot	139,540 (4 reps)	85,230 (5 reps)	39

DETERMINING THE GENOTOXICITY OF BIOREMEDIATION PRODUCTS

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INTRODUCTION

The presence of toxicants in the environment can be determined either by direct analytical chemistry measurements of known toxic substances or by using bioassays. The wide variety of man-made and naturally occurring toxicants in the environment, including carcinogens and mutagens, is well documented. Carcinogens (substances that *initiate* the cancer process) and mutagens (substances that cause heritable effects in cells and organisms) cause "delayed effects." That is, the response to exposures of genotoxic carcinogens and mutagens (collectively called genotoxicants) may not be seen for many years. In contrast to other types of toxic interactions, the action of many genotoxicants is not limited theoretically by threshold limits. In other words, any measurable amount of the substance to which an organism is exposed can potentially cause an adverse effect. The development of methods to detect, measure, and evaluate environmental genotoxicants, therefore, has been one of the primary focuses of the EPA's Health Effects Research Laboratory (HERL). Because environmental samples often contain mixtures of both identified and unidentified genotoxicants, a need to evaluate environmental samples using bioassays exists. Potential health effects can be assessed through the bioassay of samples brought to the laboratory from the environment of interest or through in situ bioassays using either indigenous species or introduced indicator organisms. One potential advantage of biological monitoring is that the lack of hazard

identification by bioassay can lower the level of concern and perhaps help to avoid costly analytical studies. If major toxicants are known to be within the complex mixtures, however, chemical measurements may provide the more cost-effective and rapid approach.

The development of short-term bioassays for genotoxicity in the 1970s led to the use of these assays for evaluating complex environmental mixtures. The genetic assay that has demonstrated the greatest utility is the *Salmonella typhimurium* mutagenicity assay. Many genetic assay systems have now been applied to understanding the toxicity of complex environmental situations. Using some of the studies done at HERL with the salmonella assay (including the semiautomated spiral assay) and the prophage induction assay, this paper illustrates how genetic bioassays can be used to understand the effect of bioremediation on genotoxicity.

LABORATORY-SCALE STUDIES EXAMINING BIOREMEDIATION PRODUCTS

In order to evaluate the potential for both the degradation and production of genotoxicants during the bioremediation of crude oils, biodegradation products of three crude oils metabolized by one of two species of fungi was monitored by analytical chemistry methods and by use of the spiral salmonella assay. The two fungi used, *Cunninghamella elegans* and *Penicillium zonatum*, grow with crude oil as a single carbon source. The three crude oils were chosen to represent complex environmental substances of no, moderate, and high mutagenicity. Oils were incubated with respective fungi for 16 days, and aliquots taken at specified time points were bioassayed for mutagenicity. When the most mutagenic crude oil was degraded by either fungi, its mutagenicity was significantly decreased. The mutagenicity of the moderately mutagenic oil did not change significantly during the 16-day period. The nonmutagenic oil (from Cook Inlet, Alaska), however, showed a mutagenic response after degradation. In all cases, "weathered" control samples (incubated without the fungus), showed little to no change in mutagenicity.

This laboratory experiment illustrates the type of complexity inherent in understanding the toxicity of bioremediated substances and the usefulness of bioassays. Most of the naturally occurring mutagens within the mutagenic oils have not been identified. Likewise, the mutagens produced or concentrated in the nonmutagenic oil during fungal degradation have not been identified. Chemical analysis would not be expected to provide an appropriate approximation of relative genotoxicity for these samples. Bioassays, however, shown to screen for the destruction and creation of genotoxicants, are known to be useful in helping to choose treatment alternatives, and can (on some occasions) be used to evaluate relative toxicity.

EVALUATION OF CONTAMINATED SITES

Spills of crude and processed oils and fuels are among the most common. In a recently initiated study, soil samples were taken at a Wichita, Kansas, location that had experienced an accidental pipeline leak of a refined hydrocarbon product. Samples were taken from "highly" and "moderately" contaminated sites as well as from a "clean" site. The core samples were divided into four sections (two above the water table and two below) for extraction and testing using the salmonella mutagenicity assay. Mutagenicity was demonstrated for all three sites, both above and below the water table. After bioremediation of the site, core samples will be taken so that mutagenicity before and after remediation efforts can be compared.

EVALUATING THE EFFECTS OF MULTIPLE POLLUTANTS

2,6-Dinitrotoluene (DNT) represents a class of compounds that can be found in hazardous waste sites contaminated with munition chemicals. The organochlorine pentachlorophenol (PCP) is a wood preservative found within many sites. The activation of DNT to genotoxic metabolites involves enzymes in both the liver and the intestinal flora. PCP has both bactericidal activity and can induce hepatic mixed function oxidase enzymatic activity in the liver. Studies were done therefore to determine the effect of PCP on the production of mutagenic metabolites from DNT. Results of the study indicated that PCP accelerates the biotransformation of DNT to genotoxic metabolites and potentiates the formation of DNT-induced DNA adducts in the liver.

These types of studies, which typically would be done only for the most commonly co-located pollutants, will help risk managers to understand which unique combinations of pollutants must be closely monitored.

SUMMARY

Although bioassays have been used for many years to evaluate ecotoxicity in complex environmental situations, only in the last decade have major efforts using short-term bioassay for potential human health effects been applied to actual environmental situations. These studies illustrate that short-term bioassays can be applied to bioremediation research to identify toxic pollutants, the loss and/or production of toxic materials during bioremediation, the relative toxicity of contaminants treated by different methodologies, and methods of identifying and evaluating how mixtures of chemicals affect toxicological processes.

FIELD RESEARCH

FIELD DEMONSTRATION OF A CONSTITUTIVE TCE-DEGRADING BACTERIUM FOR THE BIOREMEDIATION OF TCE

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INTRODUCTION

The degree to which trichloroethylene (TCE) has been recognized as a significant environmental pollutant is reflected by the amount of research into methods for its remediation. Despite the demonstrated environmental hazards, its industrial use continues apace because few alternatives exist. TCE owes its environmental behavior partly to its physical properties (i.e., high density and water solubility and low chemical reactivity), and partly to its biological recalcitrance. Both contribute to its notoriety as a persistent point source pollutant, despite numerous reports of both anaerobic and aerobic bacterial transformation capabilities. Aerobic bacteria are more rapid TCE metabolizers, but only in a cooxidative fashion. TCE serves as a cooxidative substrate for various bacterial oxygenases, but not as an inducer of them. These bacteria require co-inducers that include toluene (7,9,10,11), phenol (5,8,10), methane (4,6), ammonia (1), isoprene (2), 2,4-dichlorophenoxyacetic acid (2,4-D)(5), and propane (15).

BACKGROUND

Our research has centered on the miocrobiology of *P. cepacia* G4, which expresses a unique toluene *ortho*-monooxygenase (Tom) in response to various aromatic inducers. Tom carries out the cooxidative metabolism of TCE by this strain (12,13). In this study, a nonrecombinant derivative of G4, called G4 PR1 has been developed that constitutively expresses Tom, and consequently degrades TCE without the need for a co-inducer (14). This paper deals with the characterization, alteration, and application of this constitutive derivative.

RESULTS AND CONCLUSIONS

Bioreactor

A fixed film bioreactor was investigated for the exploitation of PR1 for the degradation of air-entrained TCE. A reactor that would receive air-entrained TCE along with a continuous inflow of nutrient medium (trickle feed) was constructed. Several packing materials were tested for their ability to support growth of a PR1 biofilm capable of TCE degradation: cellite pellets (diatomaceous earth pellets manufactured by Manville Corp.), gravel, glass beads, activated charcoal, sand, and crushed oyster shell. The material exhibiting the greatest degree of TCE removal per gram of colonized material was crushed oyster shell. In addition to performance criteria, the cost and buffering capacity (calcium carbonate) of the oyster shell matrix contributed to its selection for further bioreactor research.

All column designs tested thus far involved the metered application of nutrients (yeast extract, peptone, and glucose or minimal medium with lactic acid, phthalate, starch, or phenol) to a glass or stainless steel column, packed with oyster shell and colonized with PR1. Air-entrained TCE is passed through the column (bottom to top). Strain G4 (the nonconstitutive parent of PR1) had no effect on TCE in a closed bioreactor operated with a 100 percent atmospheric refeed, and no additional nutrient was input beyond initial colonization. PR1, present at $\geq 1.4 \times 10^8$ bacteria/g of the support material, completely removed the 80 μM TCE (10 mg/L air) present in the gas phase under the same conditions, over a 20-hour period.

A 3-L column assembled in this manner, but allowing the continuous application of nutrients and air with TCE, was capable of achieving the complete removal of 7 to 10 μM of air-entrained TCE introduced at a rate of 4 mL/min over a 96-hour period.

A similar design of a 26-liter column reactor (Figure 1) allowed for nutrient (lactate) liquid recycle and a continuous input of freshly grown cells from a lactate-fed chemostat during the inoculation phase. Two separate tests of this reactor system at 0 percent refeed were conducted. One resulted in a 92 percent removal of TCE at an average input concentration of 12.6 μM (2 mg/L) in the air phase over a 72-hour test.

In another test, TCE was introduced at appreciably lower levels and with a significant nutrient/liquid recycle. After a 48-hour inoculation period, air-entrained TCE ($\sim$ 1μM [131 μg/L]) was introduced at 100 mL/min total flow rate (c.a. 58 percent oxygen). Under these conditions, approximately 90 percent of the TCE present in the gas phase was continuously removed during a 4-day test period (Figure 2).

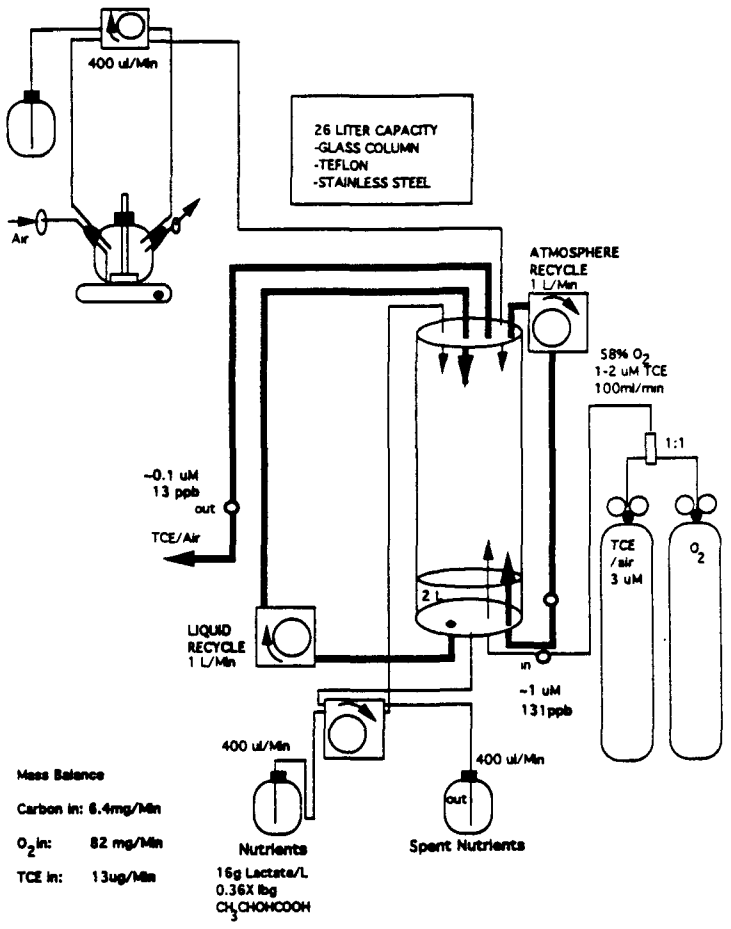

Figure 1. Schematic of 26-liter column reactor.

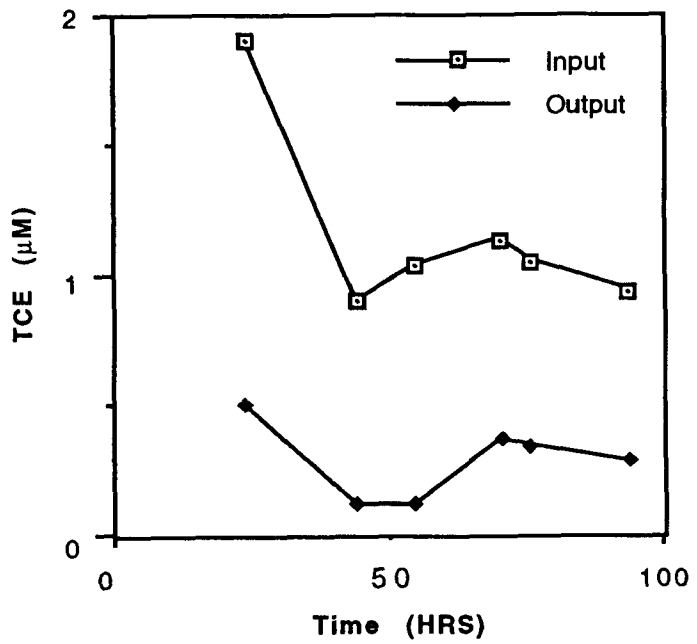

Figure 2. Twenty-six liter biofilter containing crushed oyster shell and colonized with *Pseudomonas cepacia* G4 PR1. Air-entrained TCE was delivered from a compressed gas cylinder and mixed with oxygen to balance nutrient requirements.

Tom expression in liquid culture or as a biofilm attached to an oyster shell surface may be monitored directly by the rate of TCE degradation, or more indirectly by the rate of trifluoromethylphenol (TFMP) conversion to the nonmetabolizable trifluoroheptadienoic acid (TFHA) (a reaction requiring both Tom and catechol-2,3-dioxygenase [C23O]). The 8 nmol TFHA/min/mg protein by uninduced PR1 was similar to the 6.5 nmol/min/mg protein produced by phenol induced G4. Likewise an ~1 nmol/min/mg protein rate of uninduced TCE removal by PR1 grown in batch culture is approximately one-fourth of the average daily TCE degradation rate attained with phenol induced (chemostat grown) G4 (3).

Nonrecombinant Expansion of Catabolic Range and Resistance to Toxic Intermediates

TCE in aquifers is frequently found in combination with other pollutants. Chloro-aromatics like chlorobenzene and chlorophenol are of considerable concern due to their conversion via Tom to toxic chlorocatechols, which are not metabolized by C23O. PR1 metabolizes chlorobenzene to 2-chlorophenol, and 2-chlorophenol to 3-chlorocatechol, but no further. In an effort to extend the range of pollutants degraded by PR1 and offer some protection to metabolic suicide with chloroaromatics, plasmid pRO101 (a tetracycline derivative of pJP4, specifying the degradation of 2,4-D) was introduced into PR1. In so doing, the transconjugant PR1 (pRO101) was created that expresses enzymes for *ortho*-fission (i.e., catechol-1,2-dioxygenase [C12O]) of the resultant 3-chlorocatechol, in addition to the nonproductive *meta*-cleavage mechanism of the C23O of PR1, which will accept 3-chlorocatechol as a substrate, but in so doing inactivates itself. In PR1 this results in the buildup of chlorocatechol that is highly toxic and consequently inhibitory to cellular metabolism, including its ability to degrade TCE. PR1 (pRO101) is capable of growth on 2,4-D, chlorobenzene, or 2-chlorophenol as the sole carbon sources and is also effective in degrading TCE at approximately 20 μm, following a 24-hour pre-exposure to 2-chlorophenol at 1 mM. PR1 is not able to utilize these carbon sources, nor is it able to degrade TCE following exposure to these levels of chlorobenzene or 2-chlorophenol. This brings the established substrate range of PR1 (pRO101) to include 10 aromatic substrates and 5 aliphatics.

CONCLUSIONS

The use of a particular *P. cepacia* strain constitutive for the production of Tom was investigated in a vapor phase bioreactor. Though several toluene oxygenases capable of TCE oxidation have been cloned to *E. coli* and thus no longer require aromatic induction (7, 16, 18, and our *tom* A, *tom* B clone pMS64), this is the first reported bioreactor application of a nonrecombinant bacterium (i.e., PR1) capable of doing so. The capacity of this organism to cause the degradation of TCE in a vapor phase bioreactor under laboratory conditions has been demonstrated at TCE concentrations well above those expected from field air strippers. Current efforts are directed at understanding the microbiological parameters affected by the reactor design and operation. Future considerations will include the organisms ability to metabolize TCE in an in situ bioreactor.

The substrate range of Tom has been established to include TCE, vinyl chloride, *cis*- and *trans*-1,2-dichloroethylene, 1,1-dichloroethylene, benzene, toluene, phenol, *ortho*-xylene, and *ortho*-, *meta*-, and *para*-cresol. *P. cepacia* PR1 carrying pRO101 is capable of using these same substrates in addition to 2,4-D, chlorobenzene, and 2-chlorophenol. Naphthalene has been shown to undergo a single hydroxylation to alpha-naphthol. The efficacy for their treatment in a bioreactor or in situ

system remains to be demonstrated. The introduction of pRO101 into PR1 resulted in expansion of the substrate range of this organism to include chlorobenzene and 2-chlorophenol (determination of the utilization of other chloroaromatics is pending) and an increase in its ability to degrade TCE during prolonged exposure to these chloroaromatics.

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BIOREMEDIATION OF TCE: MONITORING THE FATE AND EFFECTS OF A MICROORGANISM USED IN A FIELD BIOAUGMENTATION STUDY

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INTRODUCTION

The development of a constitutive trichloroethylene (TCE)-degrading *Pseudomonas cepacia* provides us with a unique opportunity to study several microbiological aspects of bioremediation that many believe to be altogether overlooked. Systems for the utilization of such organisms range from contained above-ground bioreactors to more passive in situ processes. Implicit in the understanding of this organism's overall effectiveness in biodegrading a target pollutant like TCE is an exploration of the microbial behavior of such a laboratory construct under anticipated operational conditions. These issues are more readily addressed in a contained bioreactor than in an environmental application. Problems associated with the use of laboratory bacteria in field releases include optimizing the activity of the organism under environmental conditions and defining the risk associated with the introduction of a non-native or genetically altered microorganism. The use of a co-oxidative bacterial pathway does not permit direct selection for the organism because the pollutant cannot be utilized as a carbon and energy source. Therefore, nutrients must be added to the contaminated aquifer to feed the TCE degrader. As a result, a significant shift in the downstream aquifer microbial community is anticipated. The primary purpose of this research is to address not only the fate of the introduced altered bacterium and the specific genetic elements

involved, but also the extent to which this treatment technology may affect the native microbial populations during an in situ bioremediation experiment. The anticipated application of this organism in situ will involve the addition of TCE and nutrients to an aquifer engineered to contain a bacterial treatment system.

Two suites of parameters are critical to assessing the impact of a microorganism released into the environment:

1. Persistence and activity of the introduced cells and their genetic information beyond the spatial and temporal scales of their intended activity.

2. Response of indigenous populations of bacteria and bacteriovores to the added biomass, nutrient enrichment, contaminant, and supplemental substrate metabolites.

PERSISTENCE AND ACTIVITY

P. cepacia G4 PR1 is a nonrecombinant derivative of *P. cepacia* G4 that constitutively expresses toluene *ortho*-monooxygenase (Tom), which in turn co-oxidizes TCE (3). Several methods have come to the forefront of molecular ecology that present the opportunity to track specific bacterial cells regardless of the ability to culture or isolate them. Prior to its release, the identification of DNA sequences unique to an organism will permit tracking of that organism by detection of those sequences in nucleic acid samples extracted from the environment. Our approach centers around determination of the nucleotide sequence of three regions of PR1 DNA:

1. The Tom and catechol-2,3-dioxygenase (C23O) genes responsible for the oxidation of several aromatic chemicals and TCE located on the large plasmid of G4 (1,2).

2. The site of *Tn* 5 insertion in G4.

3. A gene encoding an oxygenase of unknown function, cloned from *P. cepacia* G4, which allows *Escherichia coli* to produce indigo from indole (a trait not demonstrable in G4).

Since the Tom pathway is believed to be broadly distributed, its cloned genes might not provide a specific DNA probe for PR1. Likewise, *Tn* 5 is expected to occur among the aquifer bacteria, though not likely in conjunction with Tom and C23O genes. The junction site of *Tn* 5 insertion in G4, however, should be unique over a 20-to-50 base pair sequence. Another "primer" sequence can be obtained from the published sequence of *Tn* 5, and the two can be used in concert as polymerase chain reaction (PCR) primers. It also would be possible to utilize internal *Tn* 5 sequences, or PR1 sequences, just outside the site of insertion to provide "nested" primers. Because the restriction enzyme map of this region of DNA is known, it will be possible to verify the identity of any amplified fragment by analysis of restriction fragment lengths. Finally, the probe available from the indole oxygenase (indigo-producing *E. coli* clone) would provide additional confirmation.

EVIDENCE FOR TOM BEING PLASMID ENCODED

G4 and its derivatives contain two plasmids of approximately 50 and 112 kb (designated pG4S and pG4L, respectively, for those found in strain G4; pPR1S and pPR1L for those in strain PR1). Following 100 generations of nonselective growth, G4 PR1(pRO101) isolates were discovered that could no longer grow on phenol as a sole carbon source and were unable to degrade TCE. These isolates were found to have retained plasmids pRO101 and pPR1S, but not pPR1L.

Strain G4 (Km^s and phenol inducible for TCE degradation [TCE^{+p}]) was mated to a Km^r, TCE^- derivative of PR1: PR1 (pRO101, pPR1S, $pPR1L^-$). A Km^r transconjugant was isolated that carried pG4L, pPR1S, and pRO101. This strain was found to have regained the ability to degrade phenol. TCE degradation, however, occured only after phenol induction: TCE^{+p}.

The TCE^- strain PR1 (pRO101, pPR1S, $pPR1L^-$) was rendered resistant to nalidixic acid (Nal) and rifampicin (Rif) and mated to PR1 (pPR1S, pPR1L) (Nal^s, Rif^s). The 112-b pPR1L was transferred from the Nal^s, Rif^s strain to the $phenol^-$, Nal^r, Rif^r PR1 (pRO101, pPR1S, $pPR1L^-$) strain to create the Nal^r, Rif^r strain PR1 (pRO101, pPR1S, pPR1L). PR1 (pRO101, pPR1S, pPR1L) also was able to degrade phenol and TCE. However, PR1 (pRO101, pPR1S, pPR1L) degraded TCE constitutively (TCE^{+c}). This is persuasive evidence that Tom and C23O as well as their transcriptional controllers are located on the large indigenous plasmid.

CLONING

The genes responsible for Tom and C23O have been cloned from PR1 into a 2.9-kb *E. coli* cloning vector, pGEM3Z, as an 11-kb *Eco* RI fragment to create the recombinant plasmid: pMS64. *E. coli* JM109 (pMS64) constitutively degraded 20 μM TCE, produced *o*-cresol from toluene and 3-methylcatechol from *m*-cresol and *o*-cresol (all phenotypes associated with Tom activity in G4). In addition, C23O activity (encoded by the Tom pathway in G4) was detectable in this recombinant *E. coli*. Various subclones of this *Eco* RI fragment have allowed us to define an approximately 6-kb fragment that encodes Tom activity (Figure 1).

THE TOM PLASMID AND PROBE DEVELOPMENT

Hybridization of the 11-kb Eco RI fragment of pMS64 with total DNA isolated from G4 (pG4S, pG4L) and its derived strains—PR1 (pRO101, pPR1S), PR1 (pRO101, pPR1S, pPR1L)—took place only with the large, approximately 112-kb plasmid (i.e., pG4L or pPR1L) in each case. We propose that pG4L will serve as the archetype for a new class of catabolic plasmid known as TOM, which encodes an *ortho*-hydroxylation pathway for the degradation of benzene, toluene, *o*-xylene, cresols, and phenol.

This approximately 112-kb toluene degradative plasmid, TOM, is very different from the much better known TOL plasmid (archetype: pWWO) (4), which encodes the oxidation of toluene through a series of oxygenases and dehydrogenases to benzoate and then catechol. Tom is (so far) unique to G4 and TOM. Its existence now poses some provocative possibilities for environmental remediation of several priority aromatic and aliphatic pollutants. This is not only from the point of view of their constitutive degradation, but also is based on the observation that TOM appears to be

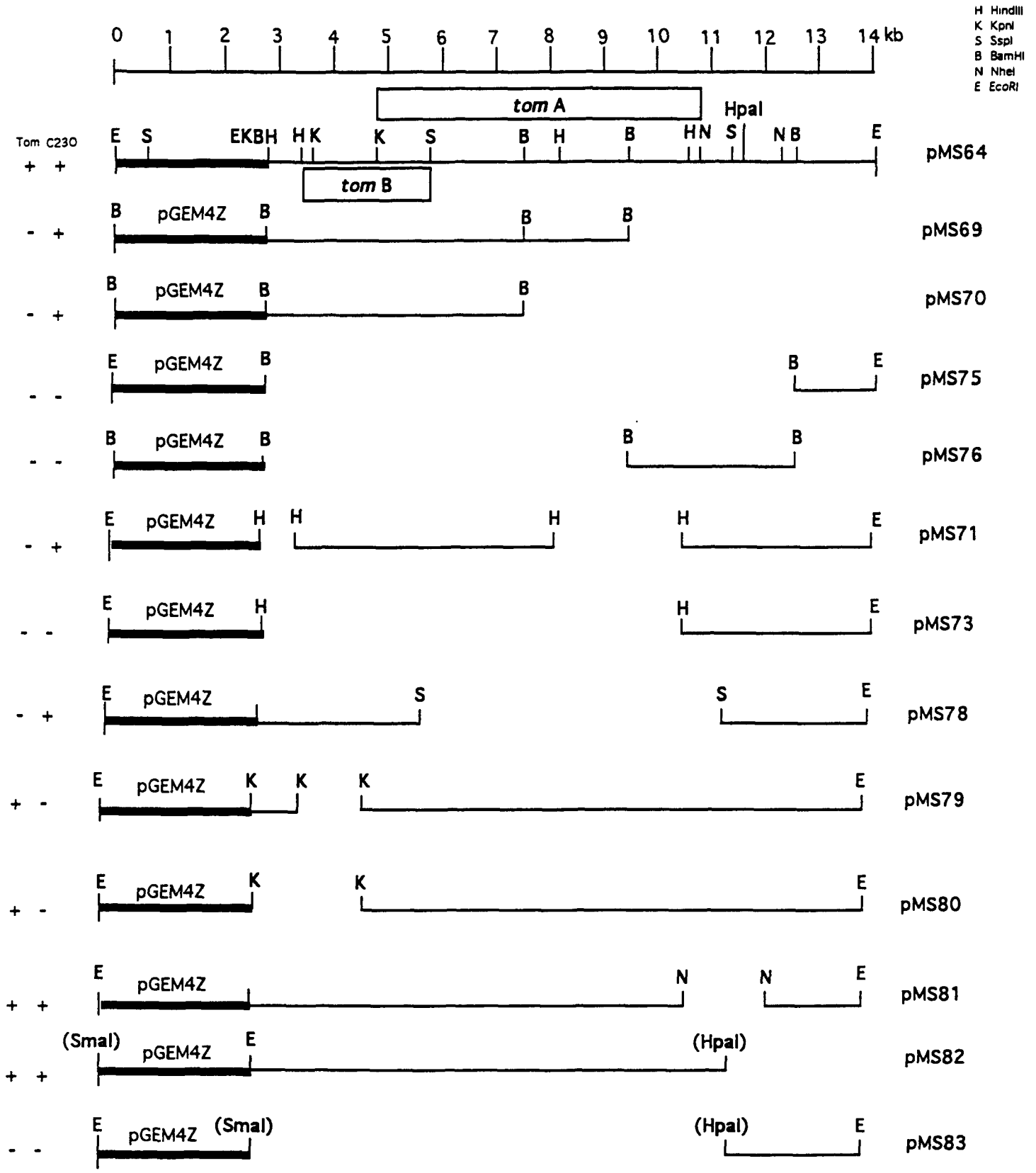

Figure 1. *Eco* RI fragment that encodes Tom activity.

a broad host range, self-transmissible plasmid (a kanamycin-resistant derivative was transferred to *E. coli* where it is currently stably maintained).

The cloning and expression of the genes for Tom and C23O (i.e., *tom* A and *tom* B) in *E. coli* allow study of the effects of expression of these genes in a well-defined genetic and physiological background. In addition, their availability allowed the construction of function-specific probes.

PHENOTYPIC TRACKING

In addition to the genetic probes, G4 is capable of being isolated by making use of its ability to utilize pthalate in the presence of kanamycin. Background numbers of organisms from the aquifer material using this selective medium will be determined. In addition, large numbers of culturable bacteria can be screened for their ability to constitutively transform a fluorinated cresol via the Tom pathway (1). This degree of overlap between the genetic and phenotypic analyses should provide an adequate detection capability to monitor fate of not only the altered organism in the environment, but more important, the fate of the genetic information and its capacity for phenotypic expression.

RESPONSE OF NATIVE POPULATIONS

Monitoring the response of native bacteria and bacterivorous protists to the implementation of this technology will involve a variety of parameters aimed at defining numerical and functional responses of bacteria and protists. Total bacterial numbers will be determined by epifluorescence direct counts using the fluorochrome DAPI (5). Determination of viable bacterial counts will be made using a low nutrient concentration agar in addition to the selective plating for G4 described above. Bacterial activity measurements will be carried out with the incorporation of ^{3}H thymidine into DNA and ^{14}C leucine into protein in a dual label technique (6). Inorganic nutrient analyses will be conducted by standard techniques. Oxygen levels and oxygen consumption will be determined by Winkler titration. Total dissolved organic carbon (Shimadzu TOC) and the carbon-to-nitrogen ratio of particulate material (Carla-Erba CHN) in the ground water will be determined. The ability of G4 to compete with and integrate into the communities of existing microbial organisms in the aquifer material will also be tested by determining the ability of G4 to colonize biofilms developed from aquifer material. This will be tracked with fluorescence techniques, using scanning confocal laser microscopy.

Previous studies of contaminated ground water sites have indicated a significant numerical response of bacterivorous protist populations as part of overall microbial response to carbon enrichment from contaminants (7, 8). The ecological roles of these protists affect the dynamics of bacterial response to organic substrates by limiting bacterial numbers below saturation levels and providing feedback stimulation of bacterial metabolism and growth through excretion of nutrients and labile organic substrates. Protists also might play an important role in the containment and elimination of introduced bacterial cells. Total bacterivorous protist numbers will be determined using a modified MPN technique (9), as well as by direct fluorescent counts.

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FACTORS DETERMINING THE EFFECTIVENESS OF MICROBIAL INOCULATION IN SOILS AND SEDIMENTS: EFFECTIVENESS OF ENCAPSULATION

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INTRODUCTION

Extensive studies have resulted in identification of many microorganisms with unique degradative ability. The potential for inoculating contaminated soils and sediments with the identified microorganisms to effect the degradation of pollutants has been proposed (1). If inoculation is defined as the process of introducing microorganisms into a site in which the inoculated microorganisms survive and significantly affect the fate of a target chemical, then few scientifically documented examples, if any, exist where this process has been successful on a significant scale. In most cases to date, bioremediation has depended on the abilities of naturally existing microbial communities to degrade toxic wastes under environmental conditions that have been managed to enhance their activities. This approach has succeeded for certain types of contaminants, such as petroleum products, low-molecular-weight polycyclic aromatic hydrocarbons (PAHs), and nonchlorinated solvents. As other complex chemicals (e.g., chlorinated solvents, pesticides, high-molecular-weight [HMW] PAHs, and PCBs) come more into focus for biotreatment, however, the sophisticated approaches to bioremediation will undoubtedly include inoculation procedures to introduce unique and specialized metabolic capacities into a contaminated matrix. Furthermore, inoculation also is important in enhancing the consistency and controllability of a bioremediation process.

Effectiveness of microbial inoculation in soils and sediments for bioremediation and pollution control may be determined by the following factors: 1) concentration of active inoculants, 2) interaction between added microorganisms and indigenous populations, 3) nutrient (including electron acceptor) supplies to the target microorganism, 4) availability of target compounds to the added microorganism, and 5) effects of heterogenous matrices on the biodegradation process. Manipulation of these factors has become a critical issue in an inoculation practice.

For this study, the use of cell encapsulation technologies was proposed to overcome some of the existing difficulties associated with inoculation for bioremediation. To understand the effect of encapsulated microbial inoculants on a biodegradation process, several encapsulation technologies were established or evaluated. The use of these technologies for biodegradation of HMW PAHs and pesticides in soil-associated systems was explored. The outline of this work follows.

TECHNOLOGY DEVELOPMENT

Several encapsulation technologies (2-4) have been developed for this study. They are similar to or are modifications of those previously described (5-7) for using identified microorganisms for bioremediation and pollution prevention in soils and sediments (Table 1). These include polyvinyl alcohol (PVA) capsules, vermiculite formulation, and polyurethane pellets, representing controlled-release, solid-particle-supported, and polymer-immobilized inoculants, respectively. Four microbial strains—strain CRE 7, *Pseudomonas paucimobilis* EPA 505, *Mycobacterium* sp. PYR-1, and *Alcaligenes eutrophus* AEO 106 (pRO 101)—were encapsulated with these technologies. Various additives, such as adsorbents, solid nutrients, and densification agents, were also included in the capsules when required. In addition, this study also established several processes, such as a soil-slurry reactor system, solid-state remediation (landfarming or composting), and a washing-water bioreactor system that used the developed encapsulated microorganisms. Three kinds of encapsulated inoculants were used in different treatment processes depending on the following factors: 1) solubility of target compounds, 2) treatment purposes (remediation or prevention), and 3) properties of treated matrices and treatment processes (Table 1).

EXPERIMENTS AND RESULTS

Experiments were performed to determine the following characteristics of the developed encapsulated microorganisms: 1) viability of encapsulated microorganisms under actual storage and use conditions, 2) activity of encapsulated microorganisms under a defined condition and in microcosms containing samples from contaminated sites, and 3) degradation kinetics in conjunction with a treatment system. The treated samples included phenanthrene, fluoranthene, creosote, and 2,4-dichlorophenoxyacetic acid-contaminated soils.

Experiments showed that the encapsulation technologies maintained a high viability of inoculants for at lease 2 months, while the non-encapsulated inoculants lost their viability within 2 to 3 weeks. When the encapsulated inoculants were used for biodegradation in various test systems, they resulted in the degradation of target compounds. Moreover, polymer immobilization of cells could facilitate the reuse of inoculants in continuous-flow systems and provide microenvironmental conditions suitable for their metabolic activity. Co-encapsulation of various additives with cells added another advantage that enhanced the degradative activity over the non-encapsulated inoculants.

Table 1. Summary of Developed Encapsulation Technologies

Encapsulation technology	Properties	Potential applications	Microorganisms encapsulated	Examples of test
Polyvinyl alcohol (PVA) capsules	- capsules are water soluble and their dissolution controllable - additives co-encapsulated - maintain viability at 4°C for > 2 months	- as carriers to deliver inoculants to a site - timed release of inoculants and additives for remediation and pollution prevention	- Strain CRE 7 (Phenanthrene degrader) - *P. paucimobilis* EPA 505 (Fluoranthene degrader) - *Mycobacterium* sp. PYR-1 (HMW PAHs degrader) - *A. eutrophus* AEO106 (pRO 101) (2,4-D degrader)	- soil slurry reactor (3,8) - solid-state remediation (landfarming or composting) (3,8) - controlled release in soil for preventing pollution by pesticides (9)
Vermiculite formulation	- powdered solid support - supplement of medium supports growth of inoculants (no additional fermentation needed) - maintains viability at room temperature for > 2 months	- carriers to deliver inoculants to a site - forms nuclei of inoculants	same as above	- soil slurry reactor (8) - solid-state remediation (8)
Polyurethane pellets	- immobilize inoculants and additives (nutrients, adsorbents, and densification agents) in the polymer matrix	- repeatedly use the inoculant - remove contaminants rapidly from the mobile phase - provide microenvironmental conditions suitable for the inoculant's activity	- *P. paucimobilis* EPA 505 (Fluoranthene degrader)	- bioreactors in conjunction with soil washing and groundwater cleanup (8)

SUMMARY

Encapsulation represents a practical means of storage, distribution, and application of identified microorganisms for inoculation in soils. The optimization and application of encapsulation technologies in a variety of contaminated systems need to be investigated further. Various factors influencing the effectiveness of encapsulated inoculants in a contaminated site need to be identified.

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COMBINING TREATABILITY STUDIES AND SITE CHARACTERIZATION FOR RATIONAL DESIGN OF IN SITU BIOREMEDIATION USING NITRATE AS AN ELECTRON ACCEPTOR

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INTRODUCTION

Rational design relates laboratory treatability studies at field scale to the distribution of contaminants and to the residence time of remedial fluids. The electron acceptor is usually the limiting factor in bioremediation. Ideally, the electron acceptor should not be depleted as water or air moves across the region contaminated with oily phase material. When all of the contaminated mass receives adequate supplies of electron acceptor, the course of remediation should parallel that established in the laboratory study. If regions of the contaminated mass are not adequately supplied, the course of remediation at field scale is not predicted in any straightforward way from the laboratory study. Rational design compares the residence time and concentration of electron acceptor at field scale to the demand demonstrated for the electron acceptor in the laboratory to ensure that the engineered implementation of in situ bioremediation is adequate.

This approach will be used to predict, a priori, the course of remediation of a spill of refined petroleum products from a pipeline near Park City, Kansas. Figure 1 relates the lithology to the

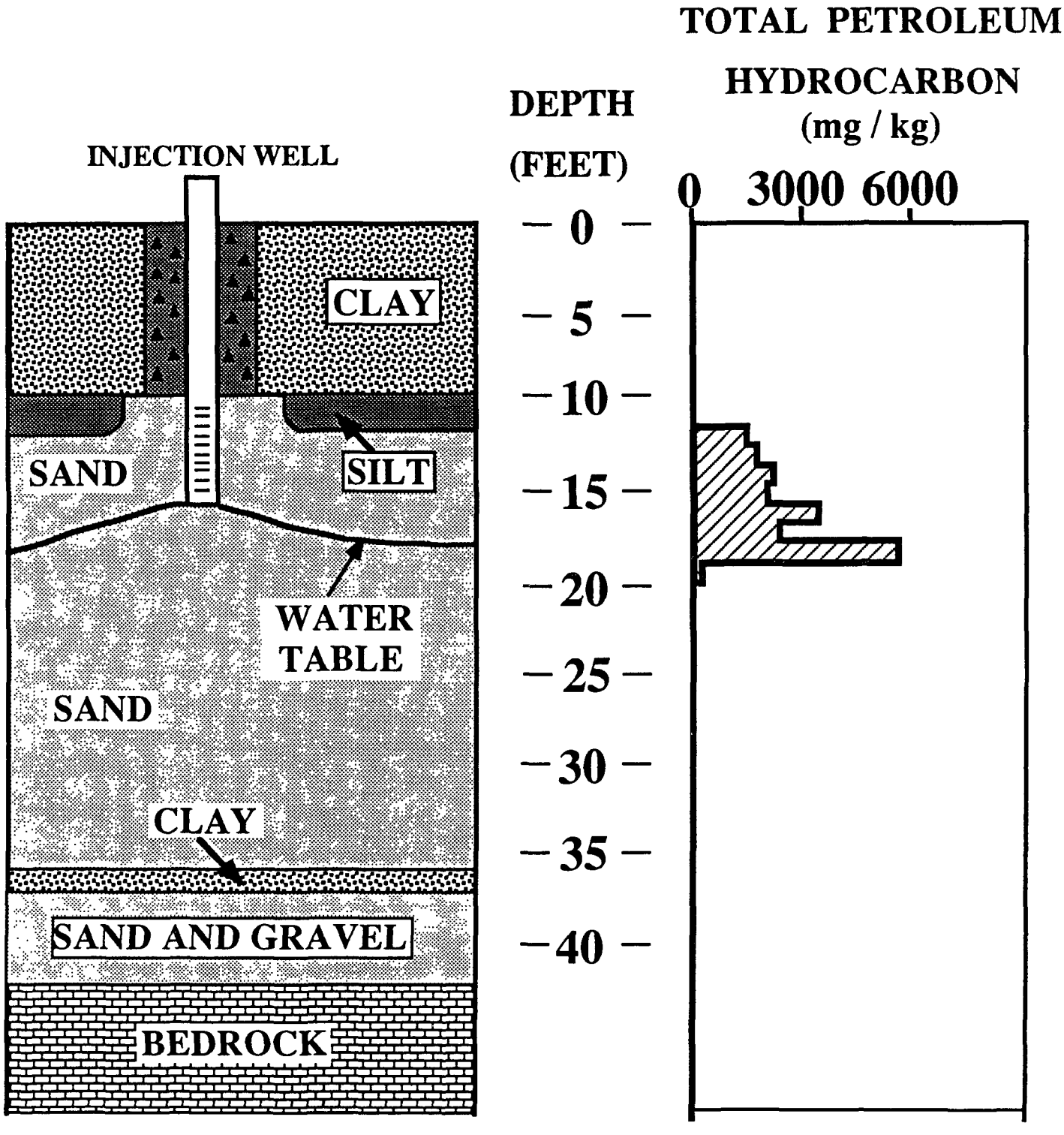

Figure 1. Relationship between the lithology, water table, and vertical interval contaminated with oily phase hydrocarbon at a pipeline spill undergoing in situ bioremediation.

vertical extent of hydrocarbon contamination. A system of 530 shallow injection wells have been installed above the spill. They are arranged in a grid on 6-m centers. These wells will circulate ground water through the spill. Additional site information has been published by Kennedy and Hutchins (1).

The site has been selected for an evaluation of the relative contribution of the various components of in situ bioremediation. Nitrate has been selected as the primary electron acceptor. The demonstration also evaluates a potential synergism between nitrate and low concentrations of oxygen. The water circulation system isolates the site into six cells of roughly equivalent size (Figure 2). Ground water without additional electron acceptors will be circulated to Cell # 4, ground water amended with 8 mg/L nitrate-nitrogen will be circulated to Cell #2, and ground water amended with 8 mg/L nitrate-nitrogen and 1 mg/L oxygen will be circulated to Cell #3. Cells #1, #5, and #6 will not receive ground water and will serve as controls for Cells #4, #2, and #3, respectively.

This study has three components: 1) a treatability study to determine the rate of nitrate consumption in core material, 2) a bromide tracer study of the vertical flow of water at field scale, and 3) a modeling exercise validated by a bromide tracer study to evaluate the horizontal flow of water.

TREATABILITY STUDY

Continuous core samples were acquired from boreholes 60N and 60O in Cell #2. The cores were collected in vertical intervals of 0.5 to 1.0 ft from 9 to 25 ft below land surface. Subsamples were extracted and analyzed for benzene, toluene, ethylbenzene, and xylenes (BTEX) by gas chromatography (GC)/mass spectrometry (MS), and for total petroleum hydrocarbons (TPH) by GC using JP-4 jet fuel as a reference standard.

Microcosms were prepared in an anaerobic glovebox using 60-mL serum bottles with 75 g of aquifer solids and sterile diluted spring water, and amended with ammonium and phosphate salts as described previously (2). No exogenous carbon source was added. Each microcosm was then spiked with 2.21 mg nitrate as nitrogen as a concentrated aqueous solution to yield initial concentrations of 50 to 80 mg/L nitrate-nitrogen.

For each core sample, three viable microcosms and one control were prepared. Controls contained 500 mg/L sodium azide and 250 mg/L mercuric chloride as biocides. Microcosms were sealed without headspace using Teflon-faced septa and incubated in an anaerobic glovebox at 20°C. Periodically, 2-mL samples were obtained from each microcosm and the volume was replaced with sterile glass beads. Samples were analyzed for nitrate, nitrite, ammonia, phosphate, and pH. Those microcosms exhibiting complete removal were respiked with nitrate.

Rates of nitrate depletion were plotted for each microcosm. The maximum zero-order rate constants were calculated from the initial linear portions of the graphs (Table 1). The data indicate that, despite variability in the actual rate of nitrate removal, the entire core profile generally exhibited denitrifying activity, and that there was no significant effect of depth interval on the rate of removal. Further, no correlation with either BTEX concentrations or concentration of TPH was evident.

The average nitrate removal rates were 1.47 $\pm$ 1.09 mg/L/day at borehole 60N and 2.85 $\pm$ 1.87 mg/L/day at borehole 60O. Taking 2.85 mg/L/day as an estimate of the nitrate consumption rate,

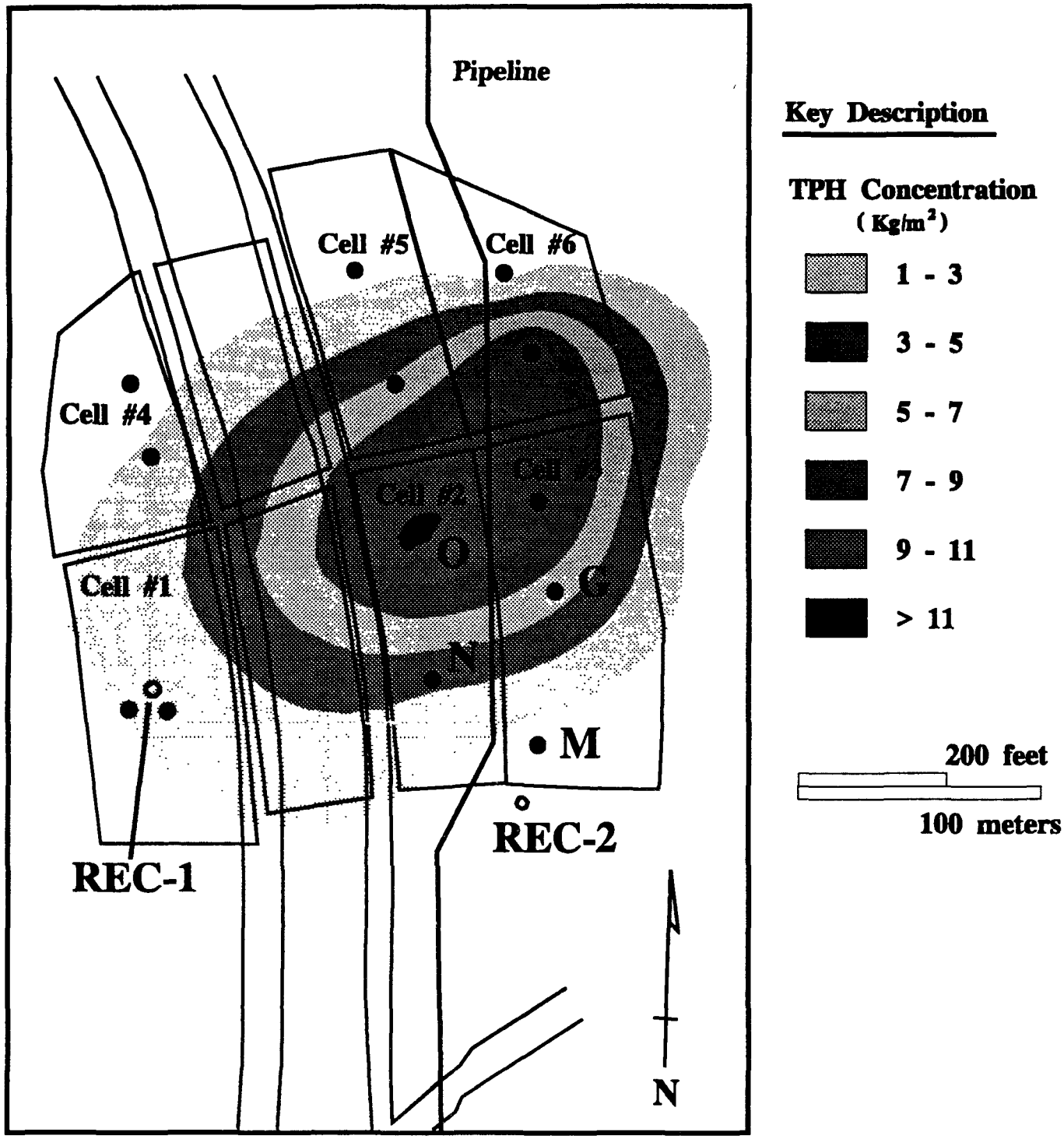

Figure 2. Relationship between treatment cells, monitoring wells, boreholes, and water circulation wells at a pipeline spill undergoing in situ bioremediation. Open circles are pumped wells, closed circles are passive monitoring wells. Recharge was distributed evenly to the cells. The infiltration wells are too numerous to display.

the circulated concentrations of 8 mg/L would be consumed within 67 hours at field scale.

Table 1. Rate and Extent of Nitrate Removal in Core Material from Locations 60N and 60O

Core Sample	Depth (ft)	BTEX (mg/kg)	TPH (mg/kg)	NO_3-N Removal Rate (mg/L/day)	Nitrate-N Removed (mg/kg)
60N-C	16.0 - 16.4	1.6	40	4.37±0.46	69.5
60N-B	16.4 - 17.2	1.4	112	1.30±0.13	22.8
60N-A	17.2 - 18.0	2.2	256	0.83±0.18	23.1
60N-E	18.0 - 19.4	1.4	95	1.21±0.46	26.9
60N-D	19.4 - 20.0	11.3	567	1.21±0.23	24.8
60N-H*	20.0 - 21.2	32.7	695	0.50±0.46	26.0
60N-G	21.2 - 22.0	249	3760	2.59±1.39	25.3
60N-F	22.0 - 22.5	264	3620	1.05±0.46	18.8
60N-J	22.5 - 23.6	331	4530	1.16±0.07	28.1
60N-I	23.6 - 25.0	3.1	38	0.63±0.11	36.8
60N-L	25.0 - 25.4	0.6	8	2.09±0.09	29.3
60N-K	25.4 - 26.2	0.4	14	0.69±0.31	23.9
60O-E	8.1 - 8.6	0.1	18	0.73±0.06	29.5
60O-D	8.6 - 9.0	0.1	12	1.06±0.55	29.1
60O-C	9.0 - 9.8	0.1	14	1.74±0.01	29.3
60O-B	9.8 - 10.6	0.1	15	1.59±0.15	42.5
60O-H	11.5 - 12.3	0.3	18	2.14±0.42	29.3
60O-G	12.3 - 13.1	0.2	16	0.15±0.08	15.6
60O-F	13.1 - 14.0	1.4	57	4.84±0.77	57.7
60O-L	14.0 - 14.4	1.1	84	5.61±0.54	102.3
60O-K	14.4 - 14.8	2.5	245	7.06±0.31	28.9
60O-J	14.8 - 15.6	4.1	389	5.32±0.43	28.4
60O-I	15.6 - 16.5	8.8	1060	3.53±0.71	102.3
60O-O	16.8 - 17.2	17.9	179	3.34±0.91	102.3
60O-N	17.2 - 18.0	9.1	334	2.11±0.34	45.5
60O-M*	18.0 - 19.0	31.9	1090	2.90±0.37	34.5
60O-R	19.0 - 20.0	45.7	1420	3.47±0.21	43.2
60O-Q	20.0 - 21.0	127	3160	1.94±1.15	28.7
60O-P	21.0 - 22.0	132	3110	0.32±0.09	21.5
60O-T	22.0 - 23.0	13.5	219	0.53±0.03	28.9
60O-S	23.0 - 24.0	0.5	9	4.69±0.12	68.9
60O-V	24.0 - 24.9	0.5	9	3.01±0.58	72.8
60O-U	24.9 - 25.3	0.2	7	2.33±0.56	53.1

* Water table

TRACER TEST OF VERTICAL FLOW

The pipeline spill is within alluvial deposits of the Little Arkansas River. A clay and silt layer extends 10 to 15 ft below land surface. Below the clay, a sand extends down to about 35 ft below grade. The deeper regions of the sand are more coarse and have higher hydraulic conductivities. The water table averages about 17 to 20 ft below land surface. The oily phase spill extends from the bottom of the clay layer through the water table to about 20 ft below land surface.

A bromide tracer study was conducted in Cells #2, #3, and #4. Ground water was produced from recovery well REC-1, amended with 45 mg/L bromide, and injected back into the cells through the infiltration wells installed just below the first clay layer. Breakthrough of the tracer was monitored in two sets of cluster wells that were installed in Cell #3, the most contaminated cell. Cluster G was installed in the center of the cell, and cluster M was installed at the downgradient margin of the cell, close to the second recovery well, REC-2 (Figure 2).

Both sets of cluster wells showed the same pattern: breakthrough in the second and third well were appropriately staggered, suggesting the flow was primarily vertical in the depth interval from 20 to 30 ft (Figure 3). Shortly after breakthrough at the third level, tracer broke through at the fourth level. This would be expected if flow at the 30 to 35 ft level was primarily horizontal toward the recovery well REC-2 and the bromide originated from water that had penetrated to this level from upgradient.

The average flow of water pumped to each cell was 120 gal/min, which averaged 40,000 ft^3 in surface area. This flow would produce 0.578 ft of recharge each day; at a porosity of 0.3, this would advance the tracer 1.93 ft/day. In cluster 60-G, the tracer moved 5 ft through the second well in 50 hours, and 10 ft to the bottom of well G-3 in 100 hours. In cluster M, the tracer moved 5 ft to the bottom of well M2 in 100 hours, and 10 ft to the bottom of M3 in 200 hours. The vertical component of flow was 2.2 ft/day in cluster G and 1.1 ft/day in cluster M. The vertical rate of advance of the tracer is close to that predicted from recharge and is consistent with the hypothesis that flow is primarily vertical in wells 60-G2, 60-G3, 60-M2, and 60-M3.

TRACER TEST AND COMPUTER MODELING OF HORIZONTAL FLOW

Ground water was pumped from well REC-1 and circulated to the cells at a total flow of 360 gal/min (see Figure 2). A flow of 120 gpm was delivered to each cell. To maintain hydraulic control, ground water was pumped from well REC-2, located just below Cell #3, at 200 gal/min, and discharged after treatment to surface flow. Flow was modeled in two dimensions using the public domain code RESSQC, which is a module of WHPA, version 2.0, a modular semianalytical model for the delineation of wellhead protection areas (U.S. EPA, Office of Ground Water Protection, 1991).

The model was run forward in time until it approached an equilibrium. It predicts two interesting properties of the engineering design. The ground water recirculation well recruits water from Cell #4, which is much less impacted, and to a lesser extent from Cell #2, which is marginally impacted; however, most of the water is uncontaminated water recruited from the aquifer (cf. Figure 2 and Figure 4). REC-2 captures water from heavily contaminated Cell #3, and part of Cell #2, and relatively little uncontaminated water from the aquifer. When the demonstration goes on line, it will operate as a combination of pump-and-treat and in situ bioremediation.

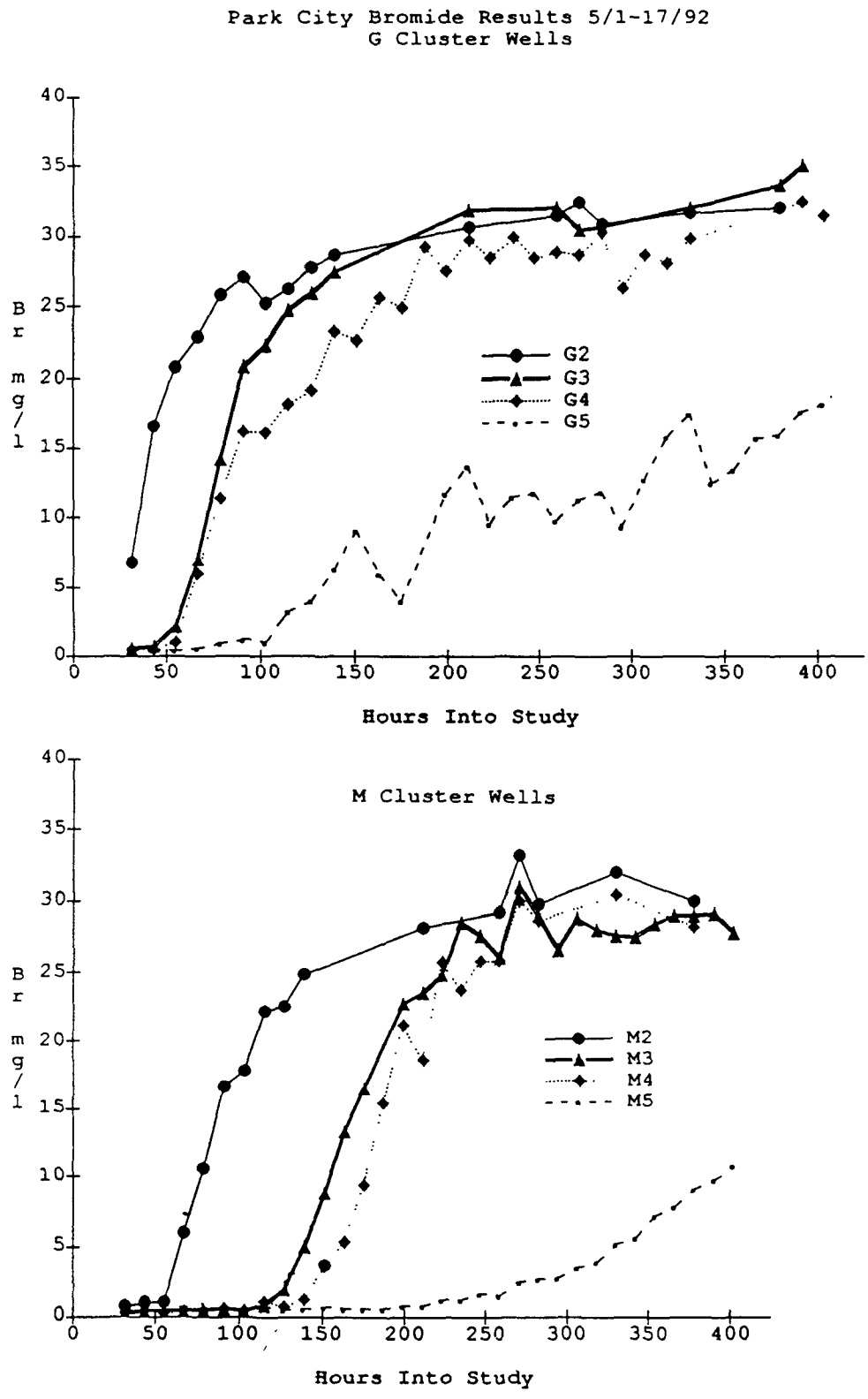

Figure 3. Breakthrough of a bromide tracer in stacked cluster wells. Cluster wells G2 and M2 from 20 to 25 feet, G3 and M3 from 25 to 30 feet, G4 and M4 from 30 to 35 feet, and G5 and M5 from 35 to 40 feet below grade. Compare Figure 1. The shallow wells sampled the sand containing the water table aquifer, but G5 and M5 also sampled water from the sand and gravel unit below the deep clay layer.

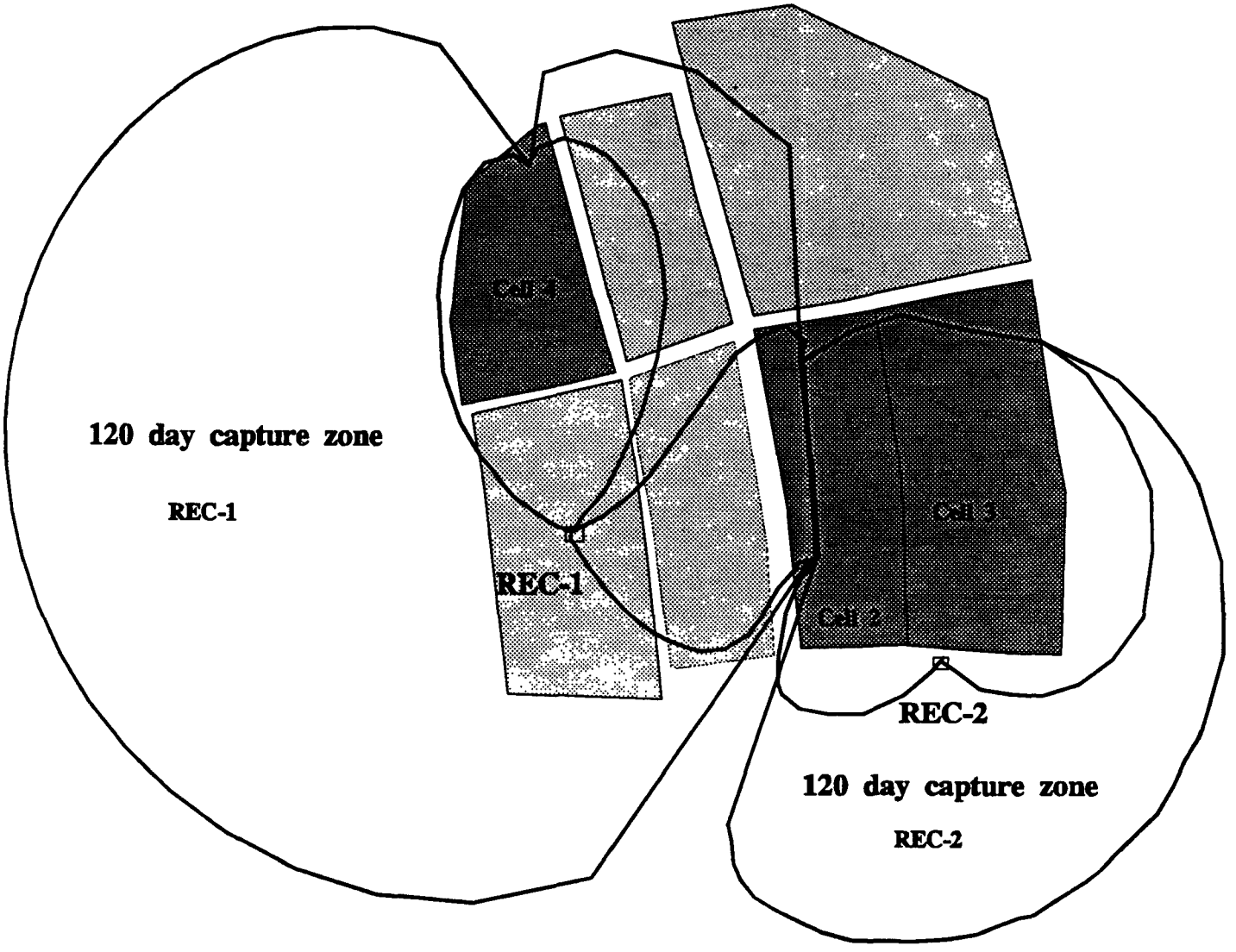

Figure 4. Capture zones of circulated water and uncontaminated water predicted for ground water circulation wells.

The predictions of the models were evaluated by comparing the predicted concentration of bromide tracer discharged from REC-2 to the actual concentrations. Over a 20-day tracer test, there was considerable agreement between the prediction of the model and the concentrations of tracer in the ground water from the discharge well (Figure 5).

CONCLUSIONS

If nitrate survives 67 hours in water moving 2.2 ft/day, it should move at least 6.1 vertical feet after recharge. This should be adequate to carry it below the oil contaminated layer (cf. Figure 1), and there should be no limitation on the supply of nitrate.

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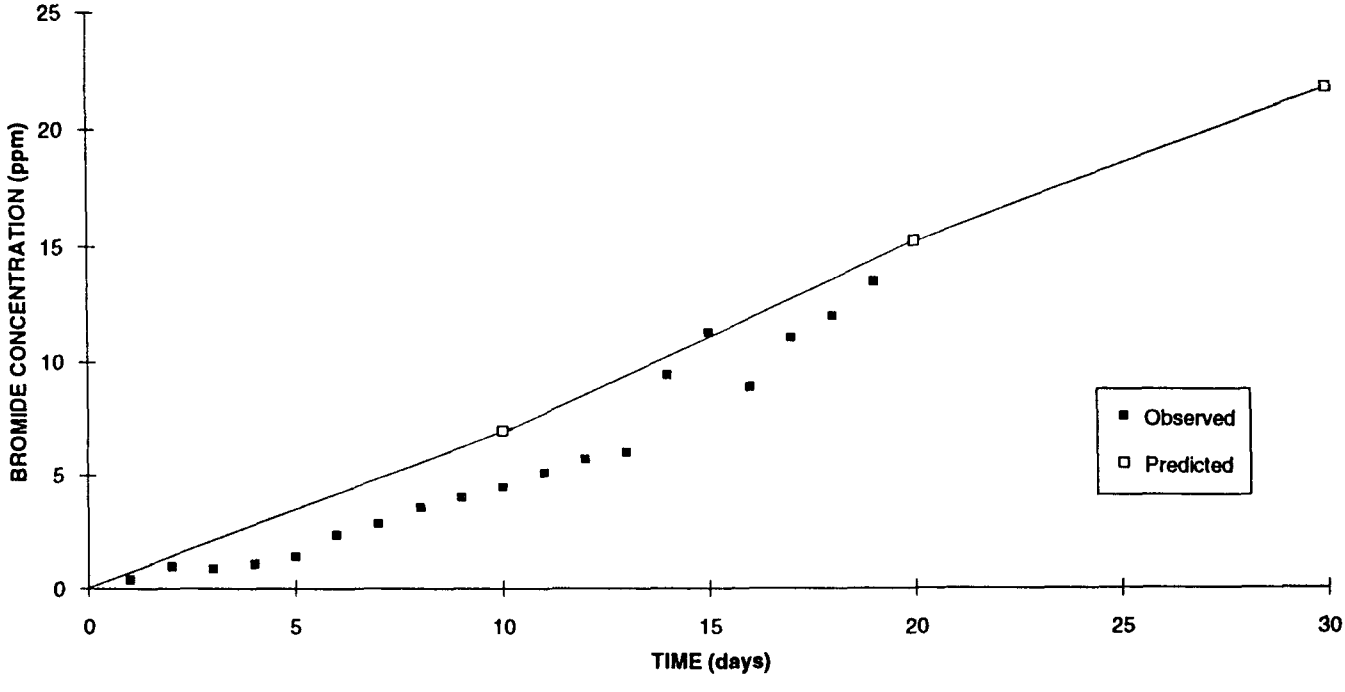

Figure 5. Correspondence between recovery of bromide tracer in circulation well REC-2 and the predictions of the model.

RATE AND EXTENT OF NATURAL ANAEROBIC BIOREMEDIATION OF BTEX COMPOUNDS IN GROUND WATER PLUMES

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INTRODUCTION

A combined field and laboratory study has been conducted on the natural bioremediation of alkylbenzenes released from a petroleum spill at the Sleeping Bear Dunes National Lakeshore near Empire, Michigan. The water table aquifer at the site is in highly transmissive glacial outwash. In December 1989, three underground storage tanks were excavated and removed from the site. Soil surrounding the tanks smelled of gasoline, indicating a leak had occurred. In February 1991, a detailed site investigation was initiated to define the horizontal and vertical extent of soil and ground water contamination and the extent of natural bioremediation of the dissolved benzene, toluene, ethylbenzene, and xylene (BTEX) isomers. This investigation included a detailed hydrogeologic characterization, a soil gas survey, and vertical coring to define the area containing oily phase hydrocarbons. Ground water was monitored at multiple depths over time for BTEX, electron acceptors, donors, and nutrients. Laboratory microcosm experiments were conducted to determine the anaerobic biodegradation of BTEX under controlled conditions.

FIELD CHARACTERIZATION

Ground water flow is from the contaminant source area toward the Platte River, approximately 70 ft away. Ground water flow at the site is complicated by seasonal fluctuations in the water table due to discharge from the Platte River to the aquifer. Contaminant residence time in the aquifer ranges from 5 to 53 weeks. Ambient concentrations of oxygen, nitrate-nitrogen, and sulfate are 2.4, 15.3, and 20 mg/L, respectively.

Dissolved BTEX declines rapidly along a streamline passing through the most contaminated zone. In the most contaminated interval immediately beneath the source, dissolved benzene, total

BTEX, methane, nitrate, sulfate, oxygen, and iron II are 3.12, 51.8, 0.31, 4.6, 8.5, 0.3, and 6.3 mg/L, respectively. Seventy feet downgradient, benzene, total BTEX, nitrate, and sulfate have declined to maximum concentrations of 0.45, 2.0, 0.10, and <0.05 mg/L, respectively, while methane and iron II have increased to 3.1 and 5.2 mg/L, respectively. Nitrate reduction, sulfate reduction, methanogenesis from carbonate, oxygen respiration, and iron reduction accepted 5.5, 1.7, 1.7, 0.3, and 0.5 mmol of electrons per liter, respectively.

Biotransformation rates for BTEX were calculated after first correcting for dilution of 2,3-dimethylpentane (DMP). DMP was assumed to be refractory under the anaerobic conditions present in this plume. First-order bioattenuation rates were estimated by determining the change in the natural logarithm of concentration at two adjoining wells and dividing by the residence time of water flowing between these wells. Results of this analysis are shown in Table 1. The rates vary by about threefold. There was no apparent biodegradation of benzene under the anaerobic conditions present in this plume.

MICROCOSM STUDIES

Microcosm studies were conducted using aquifer material collected 0, 30, and 70 ft from the source to evaluate the extent of biodegradation under ambient conditions. The aquifer material was collected aseptically and anaerobically using methods developed by Robert S. Kerr Environmental Research Laboratory personnel. Ground water was collected anaerobically through a 0.45-μm filter from monitoring wells adjacent to the core locations.

The microcosms were prepared in 15-mL serum bottles and constructed to simulate in situ conditions as closely as possible. The experimental procedure followed was essentially identical to the EPA protocol for estimation of anaerobic microbiological transformation rate data (*Fed. Reg.* Vol. 53, No. 115). BTEX was added to the microcosms in concentrations similar to in situ conditions. This was necessary since some BTEX was lost during ground water collection and preparation of the microcosms in the laboratory. The microcosms were constructed in an anaerobic glovebox with added sodium sulfide and resazurin to ensure reducing conditions. Triplicate live and abiotic controls were sacrificed at selected time points and analyzed to determine the loss of BTEX and production of dissolved methane.

Figure 1 shows the variation in dissolved toluene and methane in live and control microcosms from the 30 ft location. Average concentrations are plotted as a solid line. The dashed line is the first-order linear regression. At day 60 there was a rapid increase in methane in the live microcosms. This increase greatly exceeded the BTEX loss, indicating that other undefined substrates were being biotransformed.

Toluene declined gradually until day 115 when there was a rapid decline to less than 20 μg/L by day 192. After this rapid drop, toluene concentrations plateaued at between 5 and 15 μg/L with no additional removal. Biotransformation of the m+p-xylene, o-xylene, and ethylbenzene was similar although it lagged the toluene results somewhat. The lag in ethylbenzene and xylene removal coincided with a drop in toluene below 20 μg/L, suggesting that biotransformation is sequential, with toluene being the first to degrade. There was no apparent removal of benzene in any of the microcosms.

First-order removal rates for toluene, ethylbenzene, and xylene isomers are shown in Table

Table 1. First-Order Decay Rates (d^{-1}) Estimated from Field Data

	30 Feet	70 Feet
Benzene	0.002 to 0.004	-0.004 to 0.002
Toluene	0.053 to 0.067	0.023 to 0.026
Ethylbenzene	0.003 to 0.010	0.003 to 0.011
p-Xylene	0.005 to 0.009	0.002 to 0.010
m-Xylene	0.005 to 0.014	0.004 to 0.008
o-Xylene	0.009 to 0.016	0.004 to 0.011

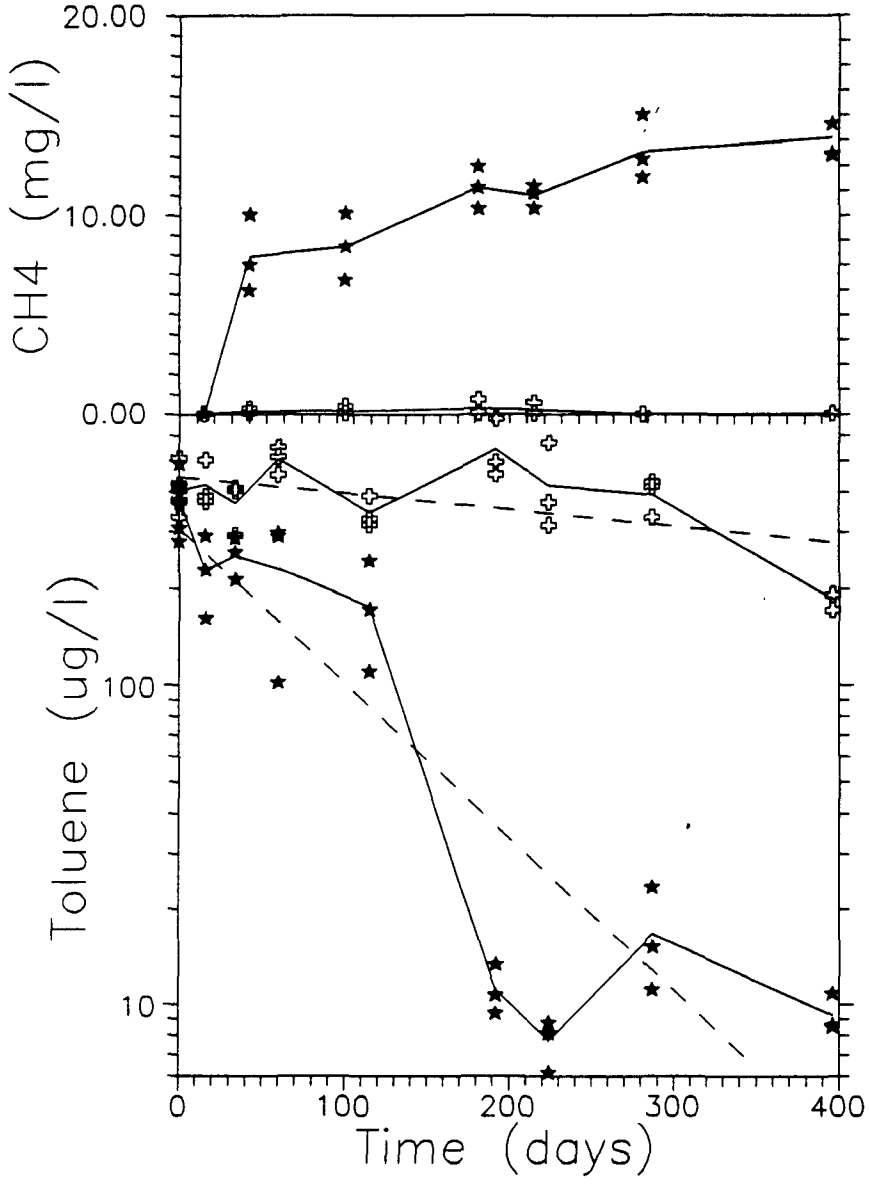

Figure 1. Biotransformation of toluene and production of methane in laboratory microcosms from 30 ft location (stars - live, crosses - abiotic).

2. Toluene biodegradation was the most rapid, followed by ethylbenzene and xylenes. The biotransformation rates shown are for the entire data set and do not reflect the much higher rates observed after the end of the lag period and before a plateau is reached. First-order biodegradation rates were higher in the 30 ft and 70 ft microcosms. This may be due to the lower initial concentrations present in these microcosms. In the 0 ft microcosms, toluene is being removed but has not yet been reduced to below 20 μg/L. In these microcosms, ethylbenzene and xylene biodegradation has been significantly slower.

COMPARISON OF FIELD AND LABORATORY RESULTS

Both the field monitoring and laboratory microcosm results indicate that benzene is not biotransformed under anaerobic conditions at this site. Toluene, ethylbenzene, and the xylene isomers were all transformed under anaerobic conditions in the field and laboratory microcosms. The match between field and laboratory rates is not exact but is good considering the inherent differences in the techniques employed. Laboratory biotransformation rates were typically a factor of 2 to 10 lower than the field rates. This could be due to two factors. The soil-to-water ratio in the microcosms is about a factor of 3 lower than in the field. If most of the microorganisms are associated with the aquifer material, this would result in a lower microorganism-to-water ratio and lower biotransformation rate. Also, since the data from the lag and plateau periods were included in our calculations, the estimated rates would be lower.

Table 2. First-Order Biotransformation Rates from Sleeping Bear Microcosms (d^{-1})

Compound	0 ft	30 ft	70 ft	Average
Toluene - Live	0.0108	0.0111	0.0122	
Toluene - Abiotic	0.0021	0.0012	0.0016	
Toluene - Net	0.0087	0.0099	0.0106	0.0098
Ethylbenzene - Live	0.0037	0.0036	0.0103	
Ethylbenzene - Abiotic	0.0034	0.0026	0.0029	
Ethylbenzene - Net	0.0003	0.0010	0.0074	0.0029
m&p-Xylene - Live	0.0042	0.0040	0.0041	
m&p-Xylene - Abiotic	0.0039	0.0030	0.0035	
m&p-Xylene - Net	0.0003	0.0010	0.0006	0.0006
o-Xylene - Live	0.0036	0.0037	0.0033	
o-Xylene - Abiotic	0.0035	0.0026	0.0028	
o-Xylene - Net	0.0001	0.0011	0.0005	0.0006

PILOT-SCALE RESEARCH

TREATMENT OF PCP-CONTAMINATED SOILS BY WASHING WITH ETHANOL/WATER FOLLOWED BY ANAEROBIC TREATMENT

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Pentachlorophenol (PCP) has been used as a wood-preserving compound since the 1930s. PCP-contaminated soils can be found at abandoned and existing wood preserving sites, and at PCP manufacturing facilities that are currently Resource Conservation and Recovery Act (RCRA) and Superfund sites (1,2,3). PCP is considered a priority pollutant by the EPA, and its removal from contaminated soils has been mandated through the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA).

Among the technologies employing physical/chemical processes for removal of pesticides from contaminated soils, solvent washing of soil can be considered an economical option. Factors that can influence the effectiveness of solvent washing include the solubility of the pesticide in the wash solvent, sorption capacity of the soil for the pesticide, soil/solvent contact time, soil particle size distribution, the hydrophobic nature of the pesticide (and the solvent pH), the pesticide application matrix, soil moisture, the age of contamination, and solvent cost and recovery.

Mueller et al. (4) found that ethanol was effective in extracting polycyclic aromatic hydrocarbons (PAHs) from wet contaminated soils. Traditionally, soil moisture prevented the cleanup of the soil by using more active solvents such as dichloromethane. Peters and Luthy (5) demonstrated that water-miscible solvents such as n-butylamine, acetone, and 2-propanol were capable of removing PAHs from soils contaminated with coal tar. The solvents considered for this study were acetone, for above-ground soil washing, and ethanol, for both in situ and above-ground soil washing. A PCP loading of 100 mg/kg soil was used as representative of PCP-contaminated sites. The solvent washing procedure was used for the removal of PCP from contaminated soils for which both in situ and above-ground solvent washing of soil were feasible. The in situ solvent washing (flushing) of soil was simulated by continuously flushing solvent through a packed bed of

soil until PCP concentration in the effluent did not decrease any further. The above-ground (ex situ) soil washing was simulated by batch tests (reverse isotherms) conducted on PCP-contaminated soil.

Expanded-bed granular activated carbon (GAC) anaerobic bioreactors (6) were used to treat the extracts (spent solvents) from the soil solvent washing process. The spent solvent from the soil washing tests was fed to GAC bioreactors, where the PCP content of the wash fluid was the biodegradable cometabolite and ethanol served as the primary substrate.

For similar solvent throughputs, acetone was found to be less effective than ethanol in flushing PCP from contaminated soil. Thereafter the solvent washing tests were conducted with ethanol. Solvent flushing was conducted on 20 x 40, 60 x 80, and 100 x 140 U.S. mesh soil fractions. The flushing solvent, 95 percent ethanol, was applied at three different flow rates. Lower solvent flow rates were more effective for the same solvent throughput than higher flow rates in extracting PCP from the soil, suggesting that PCP desorption kinetics may limit soil cleanup. The 20 x 40 U.S. mesh soil was flushed with various ethanol/water mixtures, resulting in higher PCP removal efficiency of the 50 percent and 75 percent ethanol solutions at lower solvent throughputs.

The data in Figure 1 show the results of the above-ground soil washing test (reverse isotherm) on 100 x 140 U.S. mesh soil. The results indicate that during batch extraction of soil, the 50 percent and the 75 percent ethanol solutions achieved higher PCP removal efficiencies than the other ethanol solutions. Similar results were obtained for the 20 x 40 U.S. mesh and the clay fraction of the same soil, indicating that the superior removal efficiency of the 50 percent and 75 percent ethanol solutions is not completely attributable to the solubility of PCP in ethanol, but rather partially to the greater desorption of PCP from the soil in the presence of water. In addition, the results from Figure 1 indicate that the desorption of PCP was nearly independent of the soil-mass-to-solvent ratio for the 50 percent and the 75 percent ethanol solutions. The results from Figure 2 indicate that the 50 percent and 75 percent ethanol/water mixtures obtained greater PCP recoveries from 20 x 40 U.S. mesh soil at higher soil-mass-to-solvent volume ratios. Similar results were obtained for the 100 x 140 U.S. mesh soil and the clay fraction.

The effect of time on the removal efficiency of PCP from 20 x 40 U.S. mesh soil was studied (Figure 3). The results from Figure 3 indicate that the maximum removal of PCP from soil with 75 percent ethanol solution occurred within a day, and that no additional removal was obtained after longer periods of contact. The results from a shorter study revealed that maximum removal of PCP occurred within 1 to 2 hours of the batch extraction experiment.

The extract (spent solvent) from the solvent washing process was treated in expanded-bed GAC anaerobic bioreactors. Two reactors were operated under similar conditions during acclimation of methanogenic cultures and initial loading of PCP on the carbon. After stable performance was attained, the PCP/ethanol feed rates and the empty-bed contact times (EBCT) were altered to study the chemical oxygen demand (COD) removal and the reductive dechlorination of PCP. The effluent COD and VFA (volatile fatty acids), and the effluent concentrations of PCP and its biodegradation compounds, were routinely monitored. After breakthrough of PCP biodegradation products from the GAC occurred, the high concentrations of para-chlorophenol (p-CP) proved to be inhibitory to the methanogenic culture. A practice of partial column GAC replacement with fresh carbon followed the breakthrough period in order to diminish p-CP inhibition. After a further breakthrough of p-CP in the reactors, the PCP feed rate was lowered. A gradual reduction of the carbon replacement rate commenced after attaining stable reactor operation. The experimental data demonstrated an incomplete mineralization of pentachlorophenol to carbon dioxide and methane, and an equimolar conversion of PCP into monochlorophenols. This

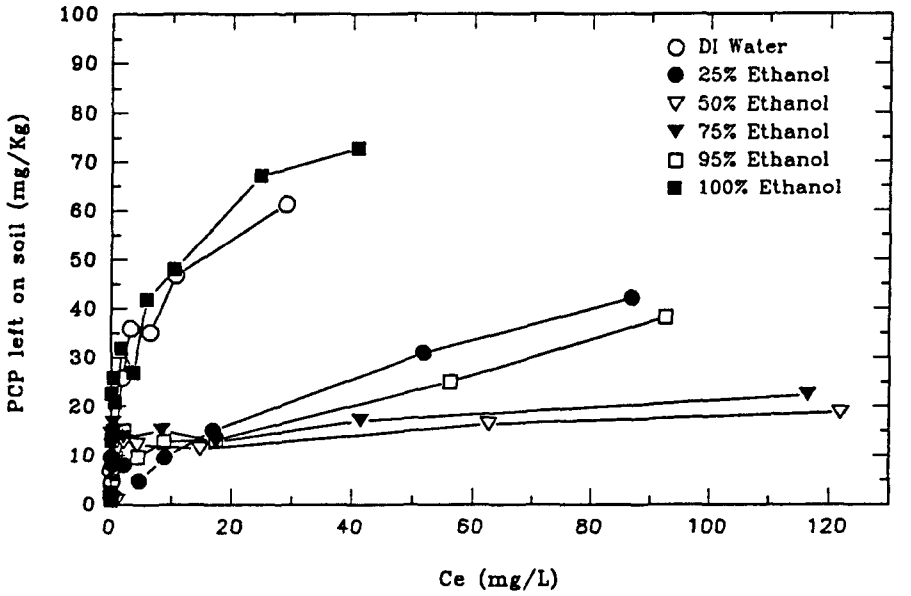

Figure 1. Reverse isotherm for 100 x 140 U.S. mesh soil.

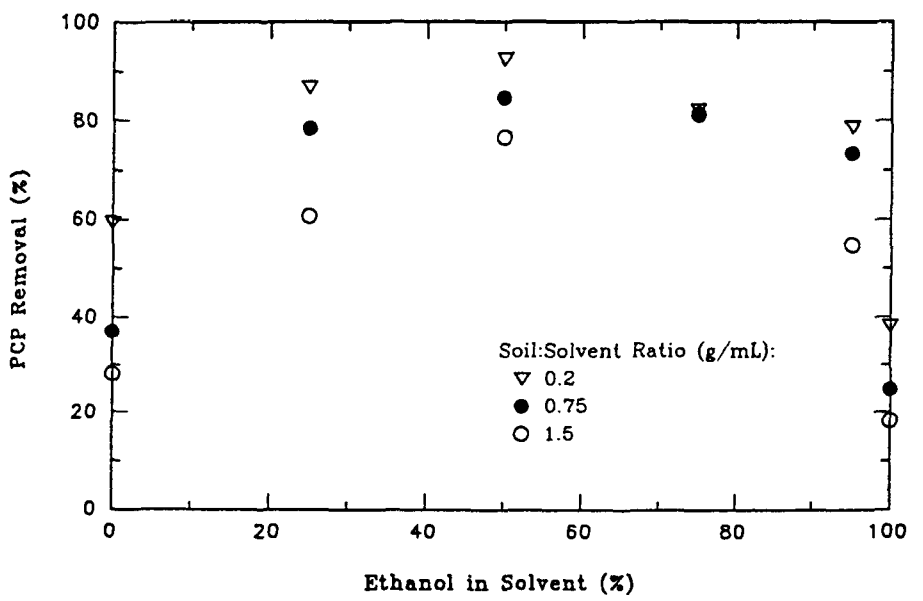

Figure 2. PCP recovery from 20 x 40 U.S. mesh soil at high soil:solvent ratios in batch extraction tests.

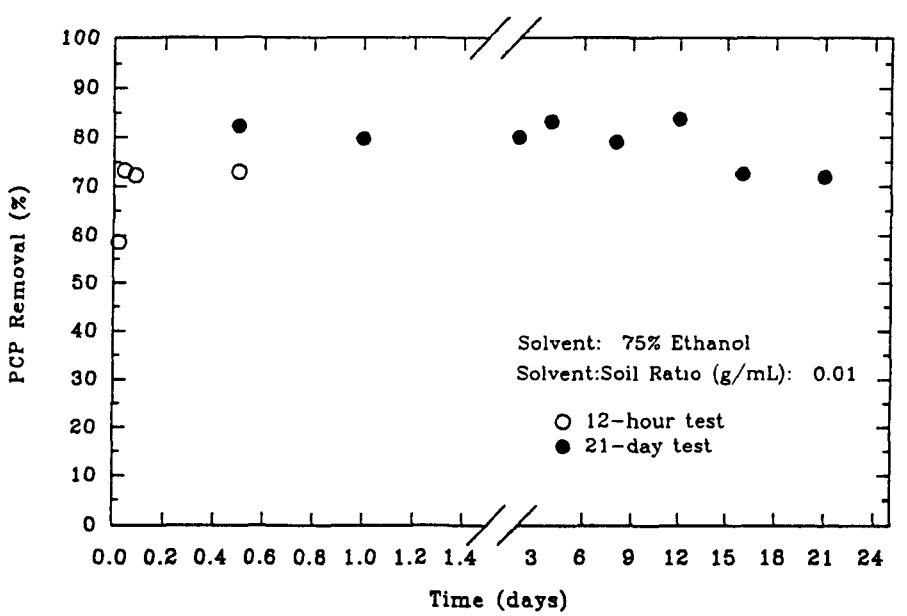

Figure 3. PCP removal from 20 x 40 U.S. mesh soil with time.

indicates that the PCP feed rate was the critical control parameter that effected reactor toxicity via the accumulation and inhibitory role of p-CP (Figure 4).

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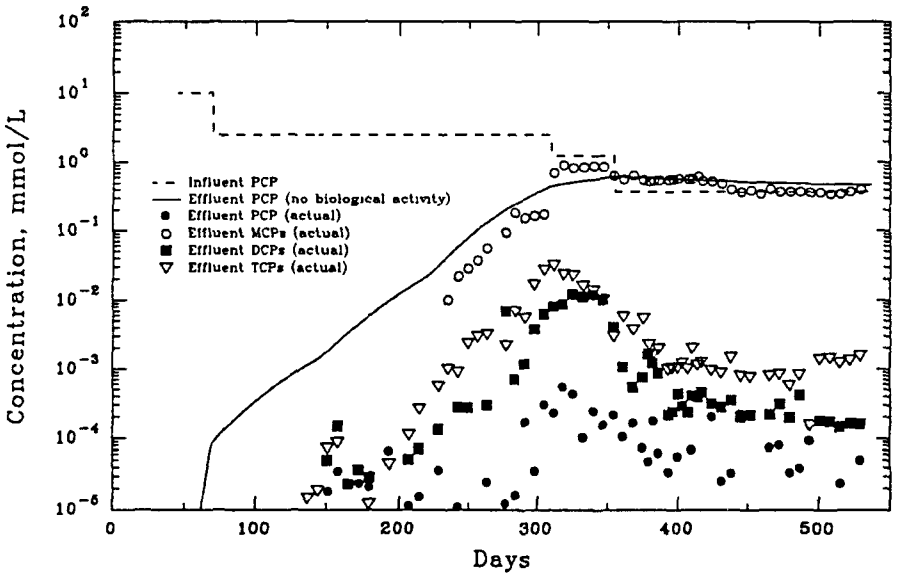

Figure 4. Effluent quality of chlorophenols in reactor A.

PRELIMINARY EVALUATION OF ATTACHMENT MEDIA FOR GAS PHASE BIOFILTERS

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INTRODUCTION

The control and removal of volatile organic compounds (VOCs) from contaminated air streams has become a major air pollution concern since the enactment of the 1990 amendments to the Clean Air Act (1). For other contaminated materials, such as water and soil, the use of biological processes has become an accepted practice. More recently, biofiltration for treating contaminated air has emerged as a practical technology for removal of many VOCs. In comparison with other VOC control technologies, such as carbon absorption and incineration, biofiltration can be more cost effective, particularly for treatment of large volumes of air with low concentrations of biodegradable VOCs (2). The low annualized cost is due to the utilization of ambient temperature microbial oxidation, rather than oxidation by thermal or chemical means. Essentially, biofiltration utilizes ambient temperature, self-regenerating, enzymatic catalysis. Biofiltration consists of contacting a contaminated air stream with a moist film of microbes attached to a stationary synthetic or natural support material. The microbes then oxidize the VOCs to simple end products, such as carbon dioxide and water. By logical extension, biofiltration as a hazardous VOC control technology has been the subject of extensive research, and the process design criteria have been identified (2-6).

This paper discusses the preliminary research performed utilizing trickle bed biofilters with monolithic channelized (as well as pelletized) microbial support media for treatment of VOCs that are typical by-products of landfill leachate stripping. Until now only toluene has been tested, to characterize the trickle biofilter system. In the future, this system will be tested for degradation of ethylbenzene, chlorobenzene, trichloroethylene, and methylene chloride. The objectives of the experiment, relative to most biofilter research to date, are to investigate the use of such biofilters

for treating chlorinated compounds, while achieving high removal efficiencies at high feed concentrations. Such compounds can be encountered in air streams from industrial processes as well as environmental cleanup processes, such as soil washing. The further research objective is to reduce to practice biofiltration for the treatment of such VOC containing air streams.

EXPERIMENTAL APPARATUS

The trickle bed biofilter apparatus used in this study consists of three independent, parallel trains, each containing 4 ft of attachment media: Biofilters A, B, and D. Biofilters A and B are filled with Corning Celcor channelized media, and D is filled with Manville Celite pelletized media. The air supplied to each biofilter is highly purified, for complete removal of water, carbon dioxide, VOCs, and particulates. After purification, the air is split off to each biofilter, humidified, injected with VOCs, and fed to the biofilters. The air is mass flow controlled, and the VOCs are fed by syringe pumps. Each biofilter is independently temperature controlled to 14°C.

Buffered nutrient solutions are fed to each biofilter. Each biofilter independently receives a nutrient solution that contains all the necessary macro- and micronutrients, with a sodium bicarbonate buffer. Biofilters A and B are also equipped with effluent recirculation in order to provide even distribution of the biomass throughout the Corning Celcor channelized medium. Biofilter B is operated in a countercurrent mode (i.e., with the air flow fed to the bottom). Biofilters A and D are operated in a cocurrent mode.

RESULTS AND DISCUSSION

During startup each biofilter was fed 50 ppmv toluene at a 12-minute residence time, and a nutrient solution feed of 20 L/day. Each biofilter was maintained at a constant temperature of 14 ± 2 °C and 7.7 ± 0.2 pH range.

Biofilter A: The mass flow of toluene was increased steadily up to 400 ppmv at a residence time of 12 minutes. The maximum percent removal of toluene that could be obtained at 400 ppmv was only 80 percent. In order to improve the performance, the effluent was recycled to provide for even distribution of the biomass throughout the support media. On day 127 after initial startup, this recycle was started and the percent removal increased to about 90 percent. The mass flow of toluene was then increased to 500 ppmv at 12 minutes of residence time. When the percent removal of toluene was stable at 99 percent, a residence time cycle test was performed, with the residence time being varied from 12 to 1 minutes and then back to 12 minutes. This was done while holding constant the total mass of toluene fed per day. Figure 1 shows the performance of the biofilter during the residence time cycle test. Note that the toluene removal stabilized at better than 96 percent up to 4 minutes of residence time. At 2 minutes of residence time the removal efficiency dropped to about 90 percent, and the performance dropped further to 65 percent at a 1-minute residence time. At a 1-minute residence time the pressure drop was about 1 in. water.

Biofilter B: The mass flow of toluene was increased steadily up to 200 ppmv at a residence time of 12 minutes. The performance was particularly poor compared to Biofilter A. The removal of toluene did not exceed 70 percent, and after day 87 the performance started to drop. On day 113 the removal of toluene dropped to about 57 percent, and at this point a decision was made to introduce effluent recycle to the biofilter. On day 114 this recycle was started, and by day 122 the

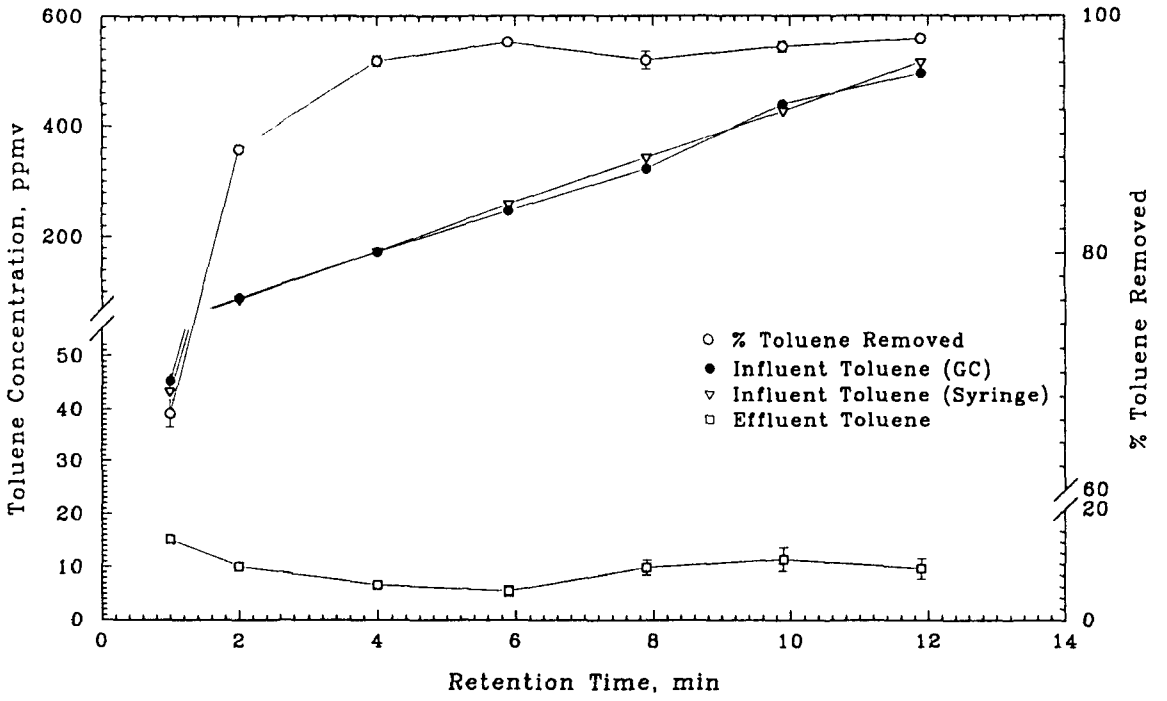

Figure 1. Biofilter A performance w.r.t. toluene removal during a residence time cycle.

removal of toluene was 85 percent. At this point the mass flow of toluene was increased steadily until it reached 500 ppmv at a residence time of 12 minutes. The biofilter was maintained at these conditions until a stable effluent was obtained. At this point a residence time cycle test was started and conducted in a manner similar to that for Biofilter A. Figure 2 shows the performance of the biofilter during the residence time cycle test. Note that toluene removal during the cycle test stabilized between 87 and 89 percent up to 6 minutes of residence time, then 72 percent at 4 minutes, 45 percent at 2 minutes, and 30 percent at 1 minute. At a 1-minute residence time, the pressure drop was about 1.75 in. water.

Biofilter D: The mass flow of toluene was increased steadily up to 500 ppmv at a residence time of 12 minutes and a 99+ percent removal efficiency. At this point a residence time cycle test was started and conducted in a manner similar to that for Biofilter A. Figure 3 shows the performance of the biofilter during the residence time cycle test. Note that toluene removal during the cycle test stabilized above 99 percent up to 2 minutes of residence time, then 95 percent at 1 minute. The pressure drop ranged from 4 in. water at 12 minutes to 22 in. water at a 1-minute residence time.

CONCLUSIONS AND FUTURE WORK

The removal efficiency for each residence time was similar, for both increasing and decreasing residence times. It appears, however, that when increasing the residence time, which causes an increase in the VOC concentration at constant mass loading, more time is required by the biofilter to achieve maximum efficiency. Effluent recycle, to establish even distribution of biomass throughout the media, was necessary to achieve maximum efficiency with the channelized support media.

Biofilter D showed the highest VOC removal efficiencies. The efficiencies ranged from 99+ percent to 95 percent for residence times of 12 to 1 minute. Biofilter A, also operated cocurrently, showed somewhat lower removal efficiencies of from 99 percent to 65 percent for times of 12 and 1 minute, respectively. Finally, Biofilter B, operated countercurrently, showed efficiencies less than 90 percent of from 90 percent to 30 percent for times of 12 minutes and 1 minute, respectively.

The pressure drops for both A and B were quite low, with a maximum of 1 in. and 1.75 in. of water, respectively. This low pressure drop, even for the countercurrent mode, is very promising since it indicates that the major operating cost for this biofilter design (i.e., blower motor power) will be lower than for most typical biofilter designs. The pressure drop for D was as high as 22 in. water. Further investigation will evaluate techniques for removing excess biomass from the pelletized support media in order to achieve high removal efficiencies at acceptably low pressure drops.

Continuing work will include investigating the effect of the recycle flow rate on the performance of each biofilter. The effect of increasing the mass loading to 500 ppmv at lower residence times will also be investigated. After finishing the characterization of the biofilters with toluene, feeding the other VOCs will be initiated.

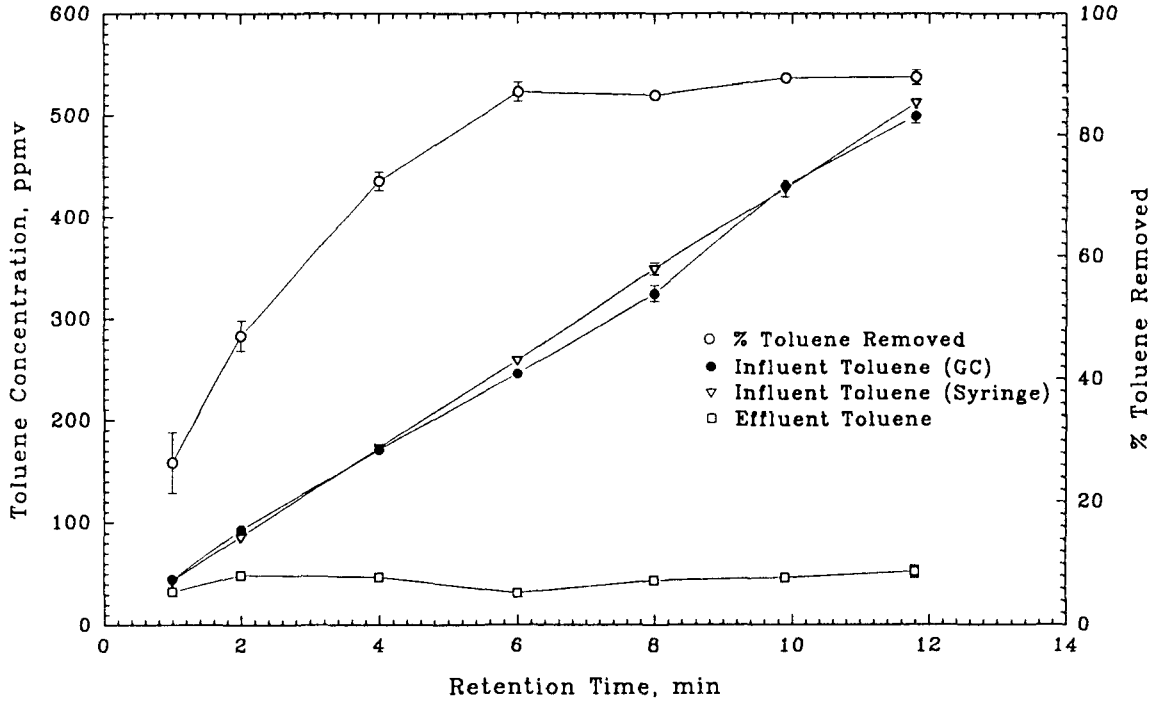

Figure 2. Biofilter B performance w.r.t. toluene removal during a residence time cycle.

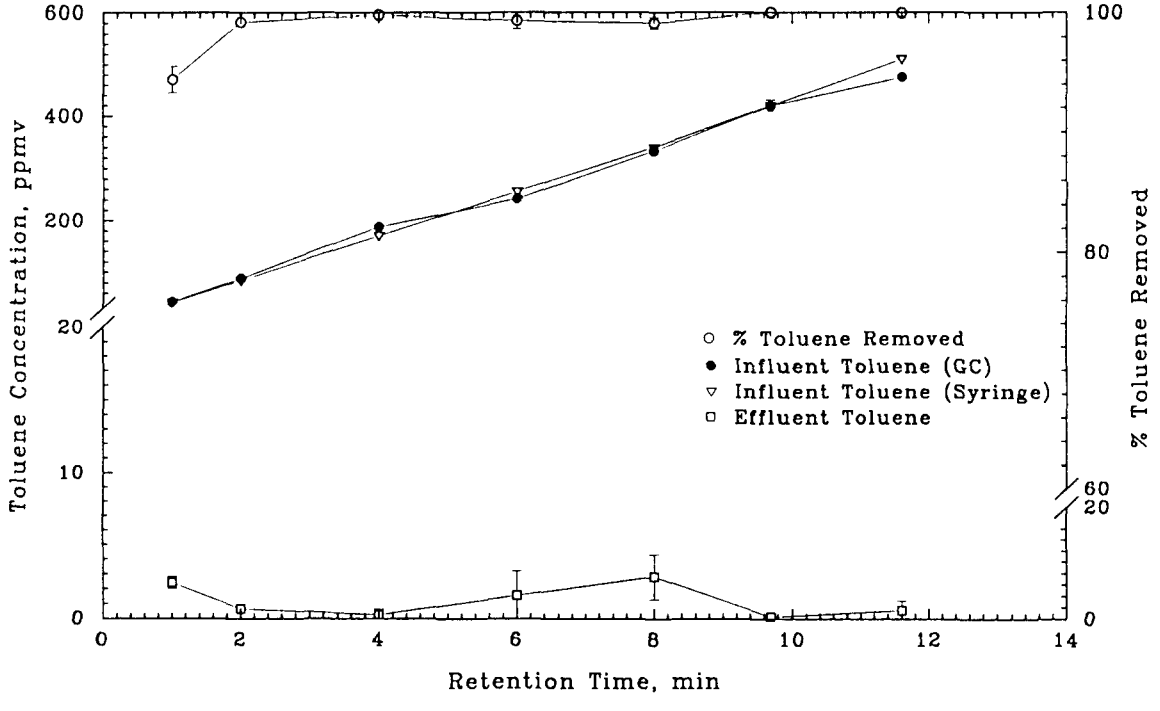

Figure 3. Biofilter D performance w.r.t. toluene removal during a residence time cycle.

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PROCESS RESEARCH

METHANOGENIC DEGRADATION KINETICS OF NITROGEN AND SULFUR CONTAINING HETEROCYCLIC AROMATIC COMPOUNDS IN AQUIFER-DERIVED MICROCOSMS

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INTRODUCTION

The fate of nitrogen and sulfur containing heterocyclic aromatic compounds in subsurface environments is controlled by various transport and transformation processes. The most important, but least understood, process affecting ground water quality is biotransformation of these compounds by indigenous microorganisms. Heterocyclic compounds are frequently encountered in the environment because they are major constituents of both fossil and synthetic fuels and many pesticide mixtures (e.g., creosote).

This study presents the Monod-decay kinetics for the conversions of benzothiophene, oxindole, 2(1H)-quinolinone, and 1(2H)-isoquinolinone to methane and carbon dioxide by a complex aquifer-derived methanogenic microbial consortium. The stoichiometric oxidation of indole, quinoline, and isoquinoline to the persistent compounds oxindole, 2(1H)-quinolinone, and 1(2H)-isoquinolinone, respectively, requires an input of energy. A thermodynamic explanation is presented to rationalize the persistence of the oxidized intermediates.

BACKGROUND

The source of contaminated aquifer material is located adjacent to an abandoned wood-preserving plant within the city limits of Pensacola, Florida (1). The wood-preserving process consisted of steam pressure treatment of pine poles with creosote and/or pentachlorophenol (PCP). For more than 80 years, large but unknown quantities of waste waters, consisting of extracted moisture from the poles, cellular debris, creosote, PCP, and diesel fuel from the treatment processes,

were discharged to unlined surface impoundments that were in direct hydraulic contact with the sand-and-gravel aquifer.

To model the degradation kinetics, laboratory microcosms were prepared in 4-L glass bottles. The microcosms contained approximately 3 kg of aquifer material anaerobically collected from the approximate centroid of the active methanogenic zone (1). Compounds of interest were added to a mineral salts solution (1) at concentrations similar to those present in the aquifer. Subsamples for substrate utilization determinations were removed from the microcosms at approximately 3-day intervals and analyzed by high pressure liquid chromatography (HPLC) and verified when necessary by gas chromatography (GC)/mass spectrometry (MS). Concentrations of methane and carbon dioxide in the microcosms were determined by GC (1). Total biomass concentrations in the microcosms at the onset and at the end of incubations were determined by total protein analysis using the Coomassie brilliant blue staining technique of Bradford (2) as described by Gälli (3).

Substrate depletion curves were fitted to the Monod substrate utilization and growth with decay equations (4):

$$-\frac{dS}{dt} = \frac{\mu_m X_a S}{Y(K_s + S)}$$

$$\frac{dX_a}{dt} = \frac{\mu_m X_a S}{K_s + S} - k_d X_a$$

where:

μ_m = maximum specific growth rate, day^{-1}
K_s = half-saturation constant, mg L^{-1}
Y = yield coefficient, mg cells per mg substrate utilized
S = substrate concentration at time t, mg L^{-1}
X_a = active biomass at time t, mg L^{-1}
k_d = specific bacterial decay rate, day^{-1}

The method of Marquardt (5) was used for the determination of parameter values (μ_m, K_s, and k_d) that best fit the experimental substrate depletion data by minimizing the residual sum of squares using NLR (non-linear regression) techniques.

RESULTS AND DISCUSSION

The bacterial substrate utilization for all of the compounds tested was modeled successfully using the Monod-decay equations with the exception of the initial oxidation of indole, quinoline, and isoquinoline. These compounds were stoichiometrically oxidized with the intermediates persisting for particularly long, variable, and unreproducible times from the initial oxidation to final mineralization to methane and carbon dioxide (up to 184 days elapsed after the oxidation of isoquinoline and the start of methanogenesis). The mass balances on the degradation of benzothiophene and the oxidized nitrogen intermediates were 86.4 percent of theoretical methane and carbon dioxide production for benzothiophene, 87.6 percent for oxindole, 91.7 percent for 2(1H)-quinolinone, and 88.5 percent for 1(2H)-isoquinolinone. A stable oxidized intermediate similar to oxindole or 2(1H)-quinolinone that would be formed from the oxidation of benzothiophene to 2-hydroxybenzothiophene was not observed in this study or in a previous study (6).

The oxidation of the nitrogen heterocycles is endergonic and must be coupled with some other reaction as shown in Table 1; however, the nature of the couple is not clear at this time. The reactions are endergonic even if the reactions presented in Table 1 are coupled to methane, carbon dioxide, and /or H_2 production; however, the overall conversion of the nitrogen sulfur organic (NSO) compounds to methane and carbon dioxide provides a negative Gibbs free energy (Table 2). It can be reasoned that the electrons produced during the oxidation of parent compounds are very likely being used as a source of reducing power, perhaps as a source for the reduction of other aromatic ring compounds present in complex mixtures such as the contaminated ground water (e.g., the reduction of phenol to cyclohexanone). In the laboratory microcosms, where indole, quinoline, or isoquinoline are the only major carbon and energy sources, the complete oxidation of the parent compound may supply necessary reducing power required for the subsequent ring reduction and ring cleavage.

Monod-decay kinetic constants for the anaerobic degradation of sulfur and oxidized nitrogen containing heterocyclic compounds have not been reported in the literature under methanogenic conditions as exist in the aquifer at the study site (Table 3). The kinetic constants for all of the compounds are quite similar and may represent the same rate-limiting bacterial population; however, there is no statistical test for this hypothesis when the parameters are generated by NLR techniques (9). Although acetate was not measured in this study, in a previous study of the contaminated ground water at the research site (1), acetate concentration increased concomitantly with the degradation of individual phenolic and nitrogen and sulfur heterocyclic compounds, suggesting that acetate utilization was a rate-limiting step. This limitation may explain why the values for μ_m and K_s are essentially the same for each of the compounds tested. The value of the kinetic parameters agree with previously published values for acetate utilization by well-acclimated acetate-fed enrichment cultures of methanogenic bacteria (10).

The apparent low biomass yield suggests that the microbial community from this ground water environment has adapted to low nutrient conditions by utilizing a major portion of the available energy for maintaining cellular integrity in a relatively hostile environment (11). It is unlikely that other explanations (i.e., inefficiency at capturing the free energy available or storing carbon as intracellular storage products) would account for the low Y values.

The biomass yields obtained by protein determination for the compounds tested were compared to theoretical values calculated on thermodynamic principles (12). Experimental and theoretical values of Y for the compounds tested are given in Table 4. The theoretical values are greater than the values obtained from the measured protein increase in the microcosms by approximately an order of magnitude.

The bacterial decay term ($-k_dX_a$) in the biomass equation is apparently not required to describe substrate utilization and/or biomass increase in the batch growth microcosms. The values determined by NLR are such that $\mu_m >> k_d$, and as a result k_d can be neglected. NLR analyses of the compound disappearance data using Monod equations without the decay term resulted in essentially the same kinetic constants. Using the Monod equations without decay should, however, alleviate the problem of increased uncertainty associated with fitting three parameters versus two.

Table 1. Gibbs Free Energy Changes During Oxidation of Nitrogen Compounds at 25°C and pH of 7

	$\Delta G^{o'}_{(aq)}$ kJ mol^{-1}
Indole to Oxindole	
$C_8H_7N + H_2O \rightarrow C_8H_7NO + 2\,H^+ + 2\,e^-$	168
Quinoline to 2(1H)-Quinolinone	
$C_9H_7N + H_2O \rightarrow C_9H_7NO + 2\,H^+ + 2\,e^-$	84
Isoquinoline to 1(2H)-Isoquinolinone	
$C_9H_7N + H_2O \rightarrow C_9H_7NO + 2\,H^+ + 2\,e^-$	56

$\Delta G^{o'}_{(aq)}$ calculated using values from Stull *et al.* (7) or estimated using the method of Jobak in Reid *et al.* (8) for compounds not found in the literature.

Table 2. Gibbs Free Energy Changes During Methanogenesis of NSO Compounds at 25°C and pH of 7

	$\Delta G^{o'}_{(aq)}$ kJ mol^{-1}
Indole to CH_4 and CO_2	
$C_8H_7N + 7\,H_2O + H^+ \rightarrow 3.5\,CO_2 + 4.5\,CH_4 + NH_4^+$	- 205
Quinoline to CH_4 and CO_2	
$C_9H_7N + 8\,H_2O + H^+ \rightarrow 4\,CO_2 + 5\,CH_4 + NH_4^+$	- 245
Isoquinoline to CH_4 and CO_2	
$C_9H_7N + 8\,H_2O + H^+ \rightarrow 4\,CO_2 + 5\,CH_4 + NH_4^+$	- 231
Benzothiophene to CH_4 and CO_2	
$C_8H_6S + 7\,H_2O \rightarrow 3.5\,CO_2 + 4.5\,CH_4 + 0.5\,H_2S + 0.5\,HS^- + 0.5\,H^+$	- 185

$\Delta G^{o'}_{(aq)}$ calculated using values from Stull *et al.* (7) or estimated using the method of Jobak in Reid *et al.* (8) for compounds not found in the literature.

Table 3. Kinetic Constants Determined for Methanogenesis of the Heterocyclic Compounds Tested ± 95% Confidence Intervals

	μ_m (day^{-1})	K_s (mg L^{-1})	k_d (day^{-1})	Y (mg mg^{-1})[a]
Benzothiophene	0.117± 0.136	0.80 ± 5.06	0.000 ± 0.129	0.025
Oxindole	0.160± 0.002	1.10 ± 2.10	0.000 ± 0.012	0.029
2(1H)-Quinolinone	0.089± 0.058	11.41 ± 0.06	0.000 ± 0.049	0.033
1(2H)-Isoquinolinone	0.099± 0.342	5.00 ± 22.0	0.000 ± 0.027	0.035

[a] Values for Y are determined from average of three protein determinations.

Table 4. Experimental and Theoretical *Y* Values for the Compounds Tested in Microcosms

Compound	Experimental *Y* (mg mg^{-1})[a]	Theoretical *Y* (mg mg^{-1})
Benzothiophene	0.025	0.14
Oxindole	0.029	0.35
2(1H)-Quinolinone	0.033	0.21
1(2H)-Isoquinolinone	0.035	0.21

[a] Based on the average of three protein determinations each before and after growth.

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ANAEROBIC DEGRADATION OF HALOGENATED AND NONHALOGENATED PHENOLIC COMPOUNDS

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Pentachlorophenol (PCP) and creosote have been widely used as wood preservatives. Improper use and accidental spills can cause serious contamination of soil and water at wood-preserving sites. Approximately 10 percent of creosote consists of phenolic compounds. Given their water solubility, these phenolics as well as PCP can leach from soil with subsequent transport to ground water.

To better understand the fate of mixed creosote and PCP wastes in soil and ground water, interactions between the anaerobic dechlorination of PCP and the biodegradation of phenol or methylphenols (*ortho-*, *meta-*, and *para*-cresol) were examined. Methanogenic enrichment cultures were established and fed phenol or one of the methylphenols at a concentration of 500 μM (see 1 and 2 for methodology). In addition, a parallel series of cultures was established to which 50 μM PCP was added together with each of the phenolic compounds. The source of inoculum was sediment from the Hudson River, mile point 145, near Albany (3).

Our results indicate that PCP was reductively dechlorinated via 2,3,4,5-tetra- and 3,4,5-trichlorophenol to 3,5-dichlorophenol within 60 to 100 days, with 3,4-dichlorophenol observed as a minor product. These two dichlorophenols were not substantially dechlorinated further within 160 days. The addition of propionate, phenol, or cresol did not stimulate dechlorination, and thus does not appear to serve as an electron donor for reductive dechlorination.

In the absence of PCP, phenol, *m*-cresol and *p*-cresol (500 μM) were utilized within 30 days, while degradation of *o*-cresol took over 60 days. Methane was produced in excess of background controls, indicating that the phenolics were mineralized. On the other hand, the presence of 50 μM of PCP completely inhibited degradation of the phenolic compounds and methanogenesis.

Degradation of phenol and the cresols took place only after PCP had been dechlorinated to 3,5-dichlorophenol. Thus in the presence of PCP a 60- to 100-day lag was observed before degradation of phenol and cresols could proceed.

Toxicity of PCP and its dechlorination products was substantiated with experiments in which PCP, 2,3,4,5-tetrachlorophenol, and 3,4,5-trichlorophenol were added at concentrations of 10 to 100 μM to active phenol-degrading cultures. PCP and the tetra- and trichlorophenols at a concentration of 10 μM and above exhibited toxicity, while 10 to 25 μM of 3,5-dichlorophenol did not inhibit degradation of phenol. Therefore, high concentrations of PCP might inhibit the anaerobic degradation of creosote-derived phenolic compounds in ground water and soil.

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ANAEROBIC BIODEGRADATION OF 5-CHLOROVANILLATE AS A MODEL SUBSTRATE FOR THE BIOREMEDIATION OF PAPER-MILLING WASTE

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The anaerobic biodegradation of 5-chlorovanillate (5CV; 5-chloro-4-hydroxy-3-methoxybenzoic acid) was investigated. 5CV was selected as a model compound for studying the biodegradation of paper-milling effluents because it contains the methoxy, chloro, and carboxyl side groups representative of those present on aromatic chlorinated compounds released in paper-milling effluent. Using sediment from a river receiving discharge from a paper-milling plant, an anaerobic enrichment culture was developed that degraded 5CV. The major pathway of 5CV degradation in this enrichment culture was concluded to be stepwise demethoxylation to 5-chloroprotocatechuate (5CP; 5-chloro-3,4-dihydroxybenzoic acid), decarboxylation to 3-chlorocatechol (3CC; 3-chloro-1,2-dihydroxybenzene), and dechlorination to catechol, which was completely degraded (Figure 1). Dechlorination of 3CC was the rate-limiting step of degradation (Figure 2).

Degradation of 5CV was investigated using a sequential inoculation approach designed to avoid the formation of 3CC (Figure 3). In lieu of the 5CV enrichment described above, the 5CV medium was initially inoculated with an anaerobic bacterial co-culture that dechlorinates 3-chlorobenzoate (3CB). This co-culture had been previously shown to sequentially dechlorinate and then demethoxylate 5CV to form protocatechuate, which was not degraded further. Upon formation of protocatechuate, this culture was inoculated (indicated with an arrow in Figure 3) with a vanillate-degrading bacterial consortium that had been derived from the above-mentioned 5CV enrichment and is known to perform the degradative steps downstream from dechlorination (i.e., sequential demethoxylation to protocatechuate, decarboxylation to catechol, and complete degradation of catechol). As expected, protocatechuate was decarboxylated to catechol, which was then rapidly degraded. Under these conditions, 5CV was completely degraded without formation of 3CC, as predicted (Figure 4). The time required for complete dechlorination increased, however, from 4 to 17 weeks. Subsequent transformation of vanillate to protocatechuate took 29 weeks, in contrast to less than 1 week in the original enrichment. In a sequential inoculation study currently under way, the co-culture was adapted to degrading 5CV before inoculation, which resulted in reducing the time required for dechlorination and demethoxylation to 10 and 6 weeks, respectively. Inoculating the sequential experiment with the vanillate consortium as soon as dechlorination is complete might reduce complete degradation of 5CV to less than 3 months—something that is currently under investigation.

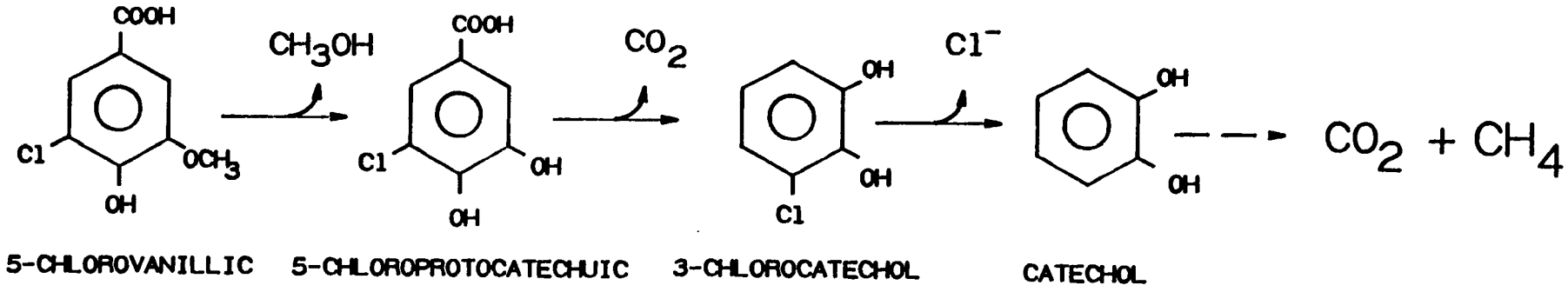

Figure 1. Pathway for the complete degradation of 5-chlorovanillic acid in a primary freshwater sediment enrichment.

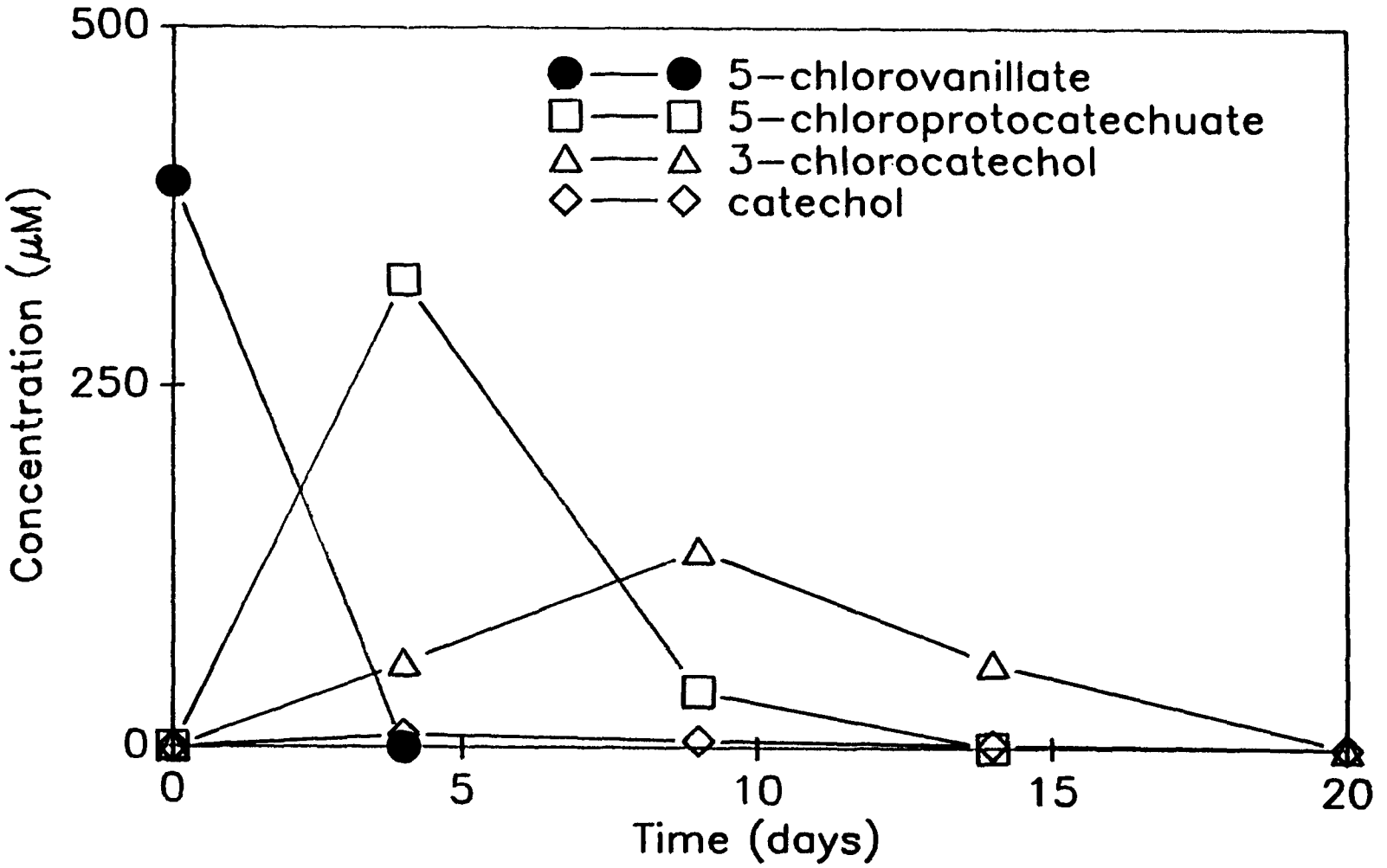

Figure 2. Anaerobic degradation of 5-chlorovanillate in a freshwater sediment enrichment.

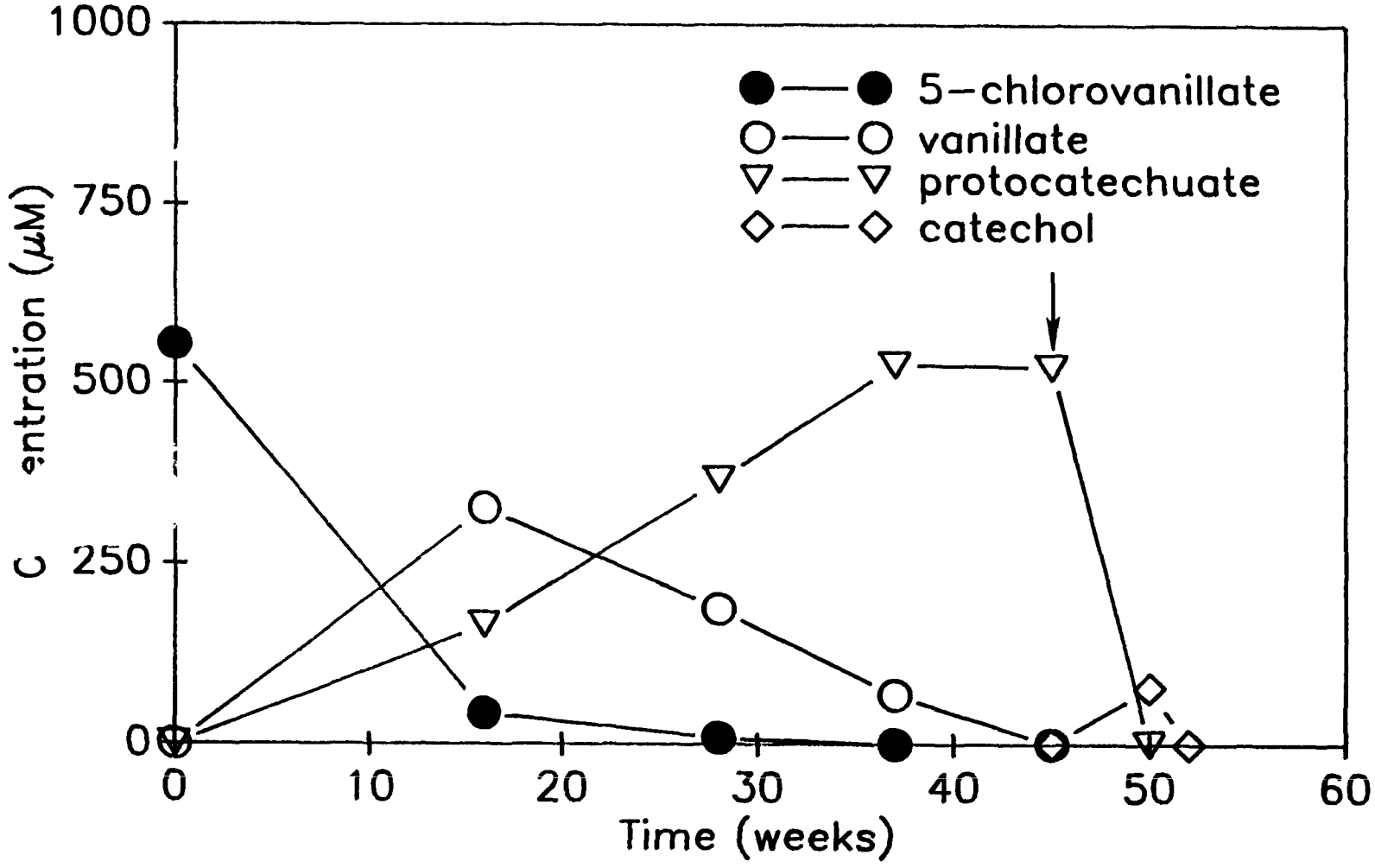

Figure 3. Anaerobic degradation of 5-chlorovanillate using sequential inoculation.

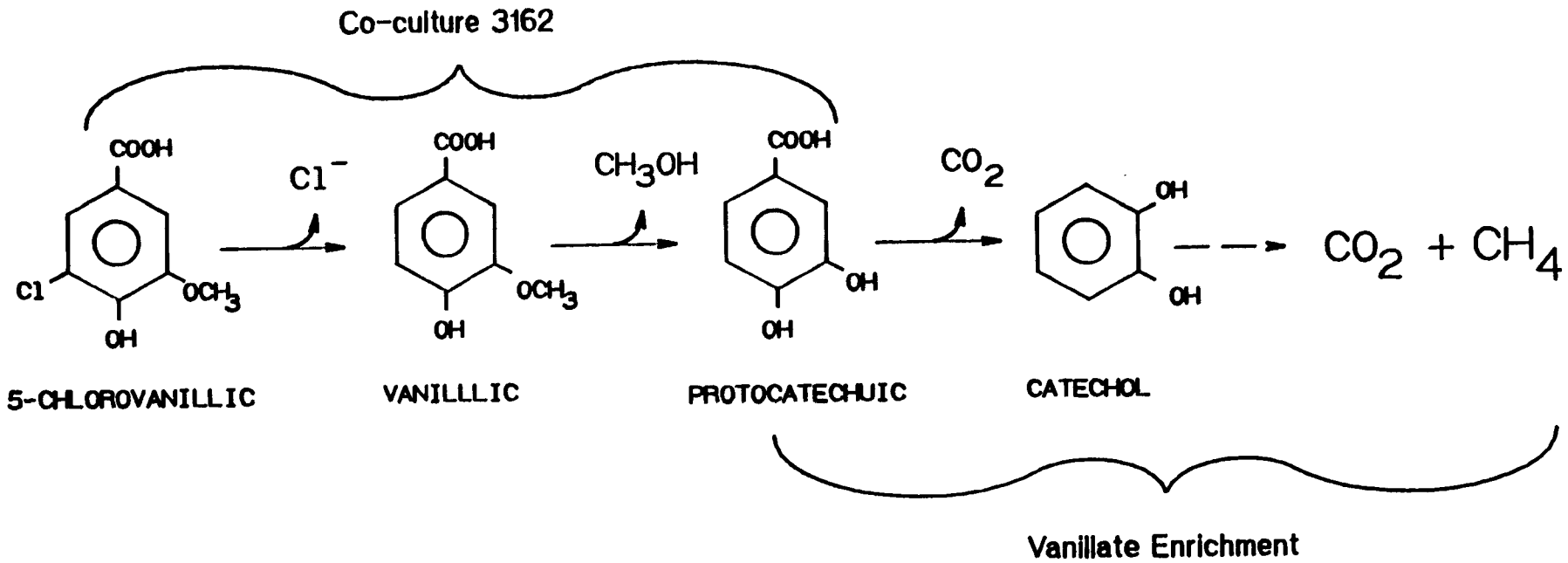

Figure 4. Pathway for the complete degradation of 5-chlorovanillic acid using sequential inoculation.

To further investigate the anaerobic degradation of 5CV, the 3CB co-culture and the vanillate consortium used in the sequential inoculation experiment were inoculated into several media to enrich for bacterial species performing the individual transformation steps. The 3CB co-culture was inoculated into 5CP and vanillate medium to enrich for dechlorination and demethoxylation, respectively. The vanillate consortium was maintained in vanillate medium and inoculated into guaiacol (2-methoxyphenol) medium to enrich for the demethoxylation, protocatechuate medium to enrich for the decarboxylation, and catechol medium to enrich for the catechol degradation. Transformation of target compounds in these enrichment cultures was followed closely using high pressure liquid chromatography (HPLC) analyses. Immediately upon completing the transformation of interest, the cultures were passed to fresh medium. Complete degradation of vanillate takes less than 1 week, while demethoxylation and decarboxylation take 3 and 4 days, respectively.

While studying the capacity of the co-culture to dechlorinate 3CB, a sulfate-reducing bacterium was isolated and identified as a new species, *Desulfomicrobium escambium*. This bacterium is responsible for dechlorination of 3CB and is currently being tested for dechlorination of 5CV and 5CP and demethoxylation of vanillate. Isolation and identification of the bacteria able to decarboxylate protocatechuate and degrade catechol currently are under way.

Anaerobic degradation of chloroaromatic compounds may prove useful in bioremediation of paper-milling effluents. An appropriate toxicity test currently is being devised to assess the toxicity of various untreated paper-milling effluents. Effluents found to be toxic will be "bioremediated" by inoculating culture media that contains increasing percentages of effluent with the bacterial cultures developed in these studies. Cultures will be monitored using HPLC analyses to determine biotransformation and biodegradation of the aromatic compounds in the effluents. Toxicity tests will be repeated on filter-sterilized supernatant from these cultures to determine the effect of this treatment on toxicity.

BIOREMEDIATION OF SOILS AND SEDIMENTS CONTAMINATED WITH AROMATIC AMINES

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INTRODUCTION

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 the compounds can be toxic and/or carcinogenic to animals. Aromatic amines can enter the environment from the degradation of textile dyes, munitions, and numerous herbicides, including the phenylureas, phenylcarbamates, and acylanilides. Because these chemicals are synthesized from aromatic amines, loss of aromatic amines to the environment may also result from production processes or improper treatment of industrial waste streams. The high probability that aromatic amine contamination of soils, sediments, and ground water aquifers will occur necessitates the development of in situ bioremediation techniques for treatment.

This study has demonstrated by sequential extraction of sediments treated with ^{14}C-aniline and ^{15}N-NMR analysis of dissolved organic matter treated with ^{15}N-aniline that aromatic amines become covalently bound to the organic fraction of soils and sediments through oxidative and/or nucleophilic coupling reactions (1). It generally is accepted that, once covalently bound, the bound residue is less bioavailable and less mobile than the parent compound. Thus, procedures that enhance the irreversible binding of aromatic amines to soil constituents could potentially serve as a remediation technique (2).

The general approach to bioremediating soils and sediments contaminated with aromatic amines is to utilize the naturally occurring phenol oxidases in soil that are thought to play a significant role in catalyzing the formation of bound residues (3). It is thought that the binding capacity of sediments and soils for aromatic amines can be increased by the activation of indigenous enzymes (e.g., peroxidase) through the addition of hydrogen peroxide and readily oxidizable substrates such as phenolic compounds. Oxidation of the phenolic substrates by activated peroxidase results in the in situ generation of oxygenated polymers, which provide additional binding sites for aromatic amine pollutants (4).

RESULTS AND DISCUSSION

Initial experiments have been conducted with pond sediments that were collected in the Athens, Georgia, area. Concentration-dependent studies with aniline in sediment-water slurries (5 percent solids) demonstrated that, at low aniline concentrations (1×10^{-6} M), approximately 90 percent of the aniline was irreversibly bound in 48 hours; however, as the initial concentration of aniline was increased, the fraction of aniline that was bound became increasingly less, suggesting that there was either a kinetic limitation to covalent binding or a limit to the number of reactive binding sites. In an attempt to increase the binding capacity of these systems, the sediment was treated with peroxidase, hydrogen peroxide, and ferulic acid, a readily oxidizable phenolic compound. In a typical experiment, a sediment-water slurry (5 percent solids) was first treated with aniline at an initial concentration of 5.5×10^{-5} M. Within 48 hours, only 15 percent of the aniline had bound to the sediment. Upon subsequent treatment of the sediment-water slurry with activated peroxidase and ferulic acid, the aqueous concentration of aniline decreased to below detectable levels within several hours.

In other studies, ^{15}N-NMR is being used to make a comparative study of phenoloxidases concerning 1) the types of covalent linkages formed in their presence, and 2) the degree to which they increase binding capacity. Initial studies have focused on horseradish peroxidase and tyrosinase. In both peroxidase- and tyrosinase-catalyzed binding of ^{15}N-aniline to fulvic acid, INEPT and ACOUSTIC ^{15}N-NMR spectra exhibited resonances that indicated the formation of imine, anilide, anilino-quinone, and anilino-hydroquinone covalent linkages. Although the distribution of covalent linkages was quite similar for the two enzymes, peroxidase proved to be a much more effective catalyst for binding; the binding capacity of the fulvic acid was significantly greater for the peroxidase-catalyzed reaction than for the reaction catalyzed by tyrosinase.

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ANAEROBIC BIOTRANSFORMATION OF MUNITIONS WASTES

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INTRODUCTION

2,4,6-Trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) are commonly found together in soil where munitions loading and packing operations have contaminated large amounts of soil in lagoons. These contaminants leach into the surrounding soil and have been known to contaminate ground water (10). Although this disposal approach is no longer practiced, large quantities of soils contaminated in this manner still must be cleaned up. Incineration is the only proven technology available for the remediation of soils contaminated with explosives. Unfortunately, this technology is expensive for small locations, and only 40 percent less expensive for especially large sites, with estimates approaching $800/ton (12).

The approach described in this study is based on the similar application of anaerobic treatment procedures that have been shown to be effective with the nitroaromatic compound dinoseb (2-*sec*-butyl-4,6-dinitrophenol) (6,7,12) to a soil contaminated with 12,000 mg/kg TNT, 3,000 mg/kg RDX, and 300 mg/kg HMX as the major contaminants.

BACKGROUND

The literature on the biological degradation of TNT and other hazardous energetic nitroaromatic compounds reviewed by Kaplan (8) shows that under both aerobic and anaerobic culture conditions the initial step in the metabolism of nitroaromatic compounds is typically a reduction of the nitro constituents to amino groups. This reduction usually proceeds in steps with

the para-nitro group being the most susceptible to reduction. The reduction of the para-nitro group appears to be nonspecific and will be performed by most cells and some cell extracts as long as growth is exhibited or sufficiently reduced conditions are used (unpublished data). The next reduction usually occurs at one of the ortho groups, producing diaminonitrotoluene isomers. The reduction of the third nitro group occurs only under anaerobic conditions (11).

Under aerobic conditions the production of unstable hydroxylamino intermediates during the reductive process can lead to the formation of azo or azoxy linkages with other intermediates, followed by dimerization or polymerization (2). Under anaerobic conditions, the reductions occur more rapidly and the hydroxylamino intermediates do not accumulate or have as much opportunity to form linkages. The production of diaminonitrotoluenes and aminodinitrotoluenes does not completely reduce the toxicity of the TNT molecule (5,11). Amerkhanova and Naumova (1) found that the amino derivatives of TNT were less toxic to some organisms but more toxic to other organisms. Complete remediation of a TNT-contaminated soil would require not only conversion of TNT to its amino derivatives, but the further conversion of these derivatives to nontoxic products.

Although research using *Phanerochaete chrysosporium* has shown microbial mineralization of ^{14}C-ring-labeled TNT to $^{14}CO_2$ in aqueous culture (3,4), complete inhibition of growth was found and no degradation of TNT was observed when 0.02 percent (weight/volume) of soil was present in the growth medium of *P. chrysosporium* (13).

This paper reports on the application of a procedure for the strictly anaerobic microbial treatment of nitroaromatic-contaminated soils to the remediation of munitions-contaminated soils. The soil examined in this study was heavily contaminated with munitions compounds and is representative of the types of soils found at munitions loading, handling, and packing (LHAP) sites. The procedure used for these soils has effectively treated soils contaminated with the herbicide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) in the laboratory (7) and in field studies (6,7,12). Anaerobic metabolism was found to occur in two stages. The first stage is a reductive stage in which TNT is reduced to its amino derivatives as described by most researchers. The second stage begins after the reduction of the third nitro group. For this, the report describes the optimization of the reductive stage of TNT metabolism. This optimization leads to the removal of the toxic amino intermediates in a short period of time.

RESULTS

Anaerobic conditions were established in soil cultures by providing potato starch to indigenous aerobes, which then utilized all the oxygen, creating anaerobic conditions. The redox potential in the cultures usually dropped from initial values of +250 mV to approximately +50 mV within 1 to 2 days of incubation. Although the cultures were anoxic, the nitrotoluenes kept the redox potential above 0 mV. The redox values remain constant at this value until after the 4-amino-2,6-dinitrotoluene is removed from the culture supernatant, when they then drop to approximately -200 mV during the remainder of the incubations. When grown under optimal conditions, the cultures regularly demonstrated the reduction of TNT to 4-amino-2,6-dinitrophenol (4A26DNT) and then to 2,4-diamino-6-nitrotoluene (24DA6NT) as shown in Figure 1. The reductive stage of the remediation of munitions-contaminated soil to the elimination of 24DA6NT was accomplished by about 25 days of incubation in the optimized cultures. RDX was removed to below detection limits in the cultures in as little as 24 days of incubation with no identifiable intermediates detected, although no radiolabeled tracer studies have been performed. Visual

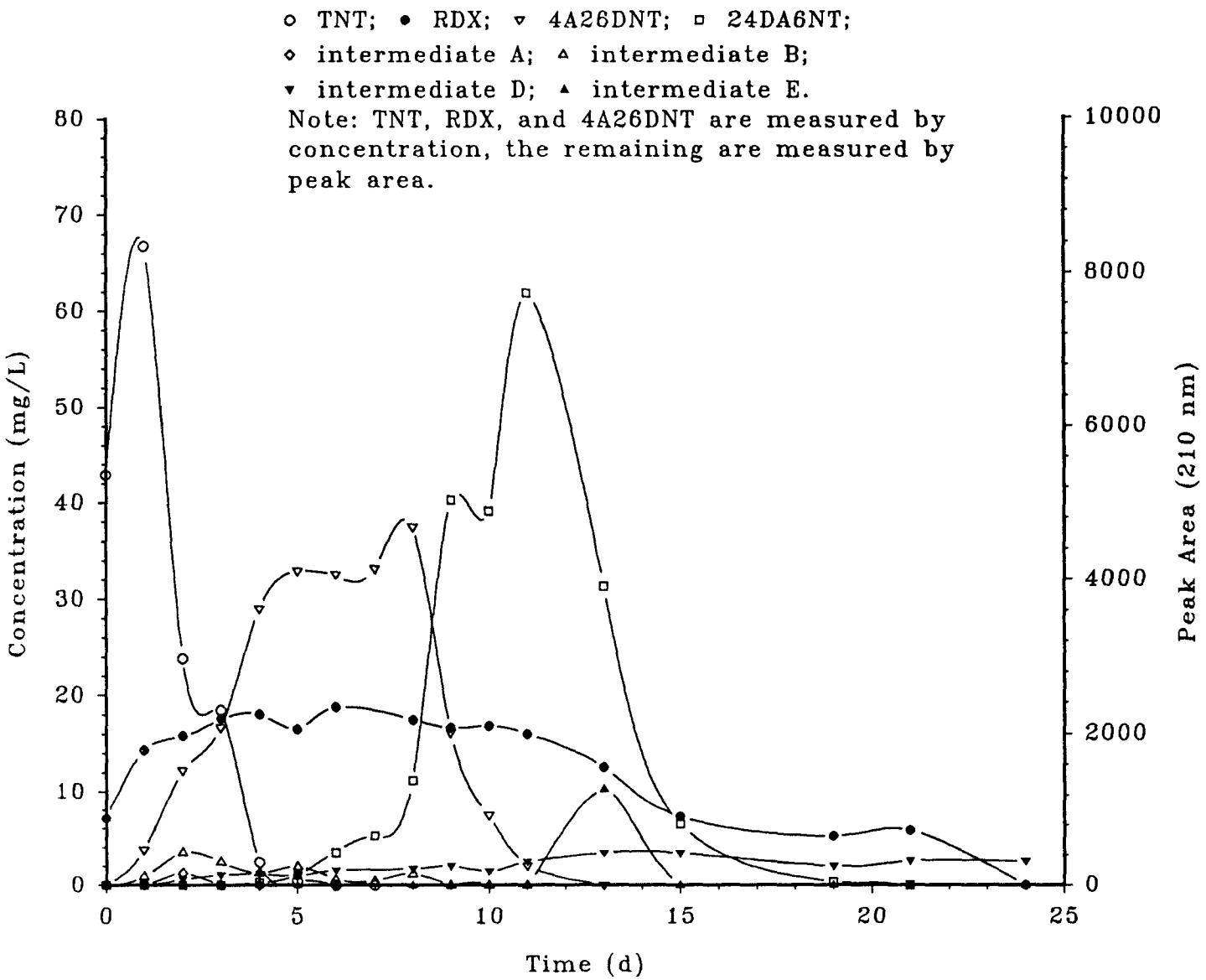

Figure 1. Biodegradation of TNT and TNT biotransformation products under optimized anaerobic conditions.

observation of these cultures showed the presence of a fungal mat on the surface of the cultures after the completion of the reductive stage of TNT metabolism.

A summary of the optimal conditions as determined to date for the first stage of munitions-contaminated soil remediation is presented in Table 1. Although TNT removal rates were more rapid at pH 8, a separate experiment showed that up to 60 percent of the label from ^{14}C-TNT was incorporated into nonfilterable material (Table 2), which may be dimerized or polymerized azoxy compounds as described by Kaplan (9). In a separate experiment performed at pH 6, the majority of the label from ^{14}C-TNT was recovered as the intermediates 4A26DNT and 24DA6NT after 24 days of incubation. Further experiments to define the pH optima revealed that biological activity was optimal between pH 6.5 and 7. A 50 mM ammonium phosphate buffer (pH 7.0) completely inhibited TNT reduction. This also was seen in phosphate-buffered cultures receiving 50 mM ammonium chloride. TNT reduction rates were found to rise with increasing amounts of ammonium up to 25 mM ammonium chloride (1.33 g/L), which was optimal.

SUMMARY AND CONCLUSIONS

The research described here has allowed the optimization of the first phase of the remediation of munitions compounds from munitions-contaminated soils. This phase of remediation has been shown to be complete within 24 days of incubation. The completion of the first, or reductive, phase of TNT degradation from these soils results in an aqueous phase that contains very little organic material and coincides with the removal of RDX from the supernatant. The resultant aqueous phase from the completed first phase cultures has been shown to support the growth of fungi present in the soil. It appears that the toxicity of the cultures is reduced by the removal of TNT, the amino-intermediates, and RDX. Toxicological studies with completed first stage supernatant are planned.

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Table 1. Optimal Conditions for the Anaerobic Remediation of Munitions-Contaminated Soils

Condition	Optimal	Range	Comments
ph	6.5	6.5-7.0	Polymerization at alkaline pH
Temp. (°C)	35	25-37	
Buffer	50 mM KH_2PO_4		
N supply	25 mM NH_4	15-30	50 mM inhibits TNT degradation

Table 2. Carbon Label Distribution in the Aqueous Phase* (% of T=0 (+/-sd))

Culture Conditions	Retained on Filter	4A26DNT	24DA6NT	Fractions 1-3	Total Recovery
Anaerobic pH 8	56.8 (2.2)	5.8	3.2	39.7	105.5
Anaerobic pH 6	nd	54	38.8	5.7	98.5
Aerobic	71 (10.6)	15.3	5.2	7.4	98.9

*Includes polar intermediates and volatile organic acids
nd = not detected

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COMETABOLIC BIODEGRADATION OF 2,4-DINITROTOLUENE USING ETHANOL AS A PRIMARY SUBSTRATE

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2,4-Dinitrotoluene (DNT) is a major by-product of the manufacture of 2,4,6-trinitrotoluene (TNT) and is commonly found in ammunition wastewaters. Because of its toxic nature and large-scale use, 2,4-DNT is listed as a priority pollutant by EPA (1). Numerous studies have investigated the biodegradation of 2,4-dinitrotoluene (2-5) and these studies suggest that 2,4-DNT is inhibitory to biological treatment processes.

In this study, the cometabolic biodegradation process of 2,4-DNT with ethanol as the primary substrate under anaerobic conditions was investigated using an anaerobic respirometer. The inoculum for the respirometer was cultivated in a continuously stirred tank reactor (CSTR) fed with 2,4-DNT and ethanol. The pH and the temperature of the CSTR were kept at 7.2 and 35°C, respectively. The detention time in the reactor was 40 days and the influent 2,4-DNT concentration was 92 mg/L. The CSTR was operated until steady-state conditions were achieved and 2,4-DNT was completely degraded. Biological methane potential (BMP) tests with 2,4-DNT and ethanol as substrates were conducted in the anaerobic respirometer at the same pH and temperature as in the CSTR. The effects of the initial 2,4-DNT concentration on the anaerobic biodegradation of 2,4-DNT and on the pathway of the anaerobic biotransformation of ethanol were studied.

The degradation of 2,4-dinitrotoluene with different initial 2,4-DNT concentrations is shown in Figure 1. Higher initial concentrations of 2,4-DNT are more inhibitory.

The presence of 2,4-DNT also inhibits the acidogenesis of ethanol. The transformation of ethanol can be expressed as,

$$CH_3CH_2OH+H_2O=CH_3COO^-+H^++H_2 \qquad \Delta G^0=2.3kcal \tag{1}$$

Figure 2 shows that propionate and methane were formed in the reactors where ethanol was fed as the sole substrate or when low initial 2,4-DNT was present. These transformations are expressed as,

$$4H_2+HCO_3^-+H^+=CH_4+3H_2O \qquad \Delta G^0=-32.4kcal \tag{2}$$

$$3H_2+CH_3COO^-+H^++HCO_3^-=CH_3CH_2COO^-+3H_2O \qquad \Delta G^0=-18.3kcal \tag{3}$$

Almost no propionate was formed, however, with high initial 2,4-DNT concentrations (Figure 3).

Propionate was degraded to methane and carbon dioxide via acetate after all the previously formed acetate was completely transformed to methane and carbon dioxide.

$$CH_3CH_2COO^-+3H_2O=CH_3COO^-+3H_2+H^++HCO_3 \qquad \Delta G^0=18.3kcal \tag{4}$$

$$CH_3COO^-+H_2O=CH_4+HCO_3^- \qquad \Delta G^0=-7.4kcal \tag{5}$$

The BMP tests show that 2,4-dinitrotoluene with an initial concentration as high as 32 mg/L was completely degraded under anaerobic conditions. 2,4-DNT is self-inhibitory, however, and inhibits the acidogenesis of ethanol. The higher initial 2,4-DNT concentrations also are inhibitory to the formation of propionate.

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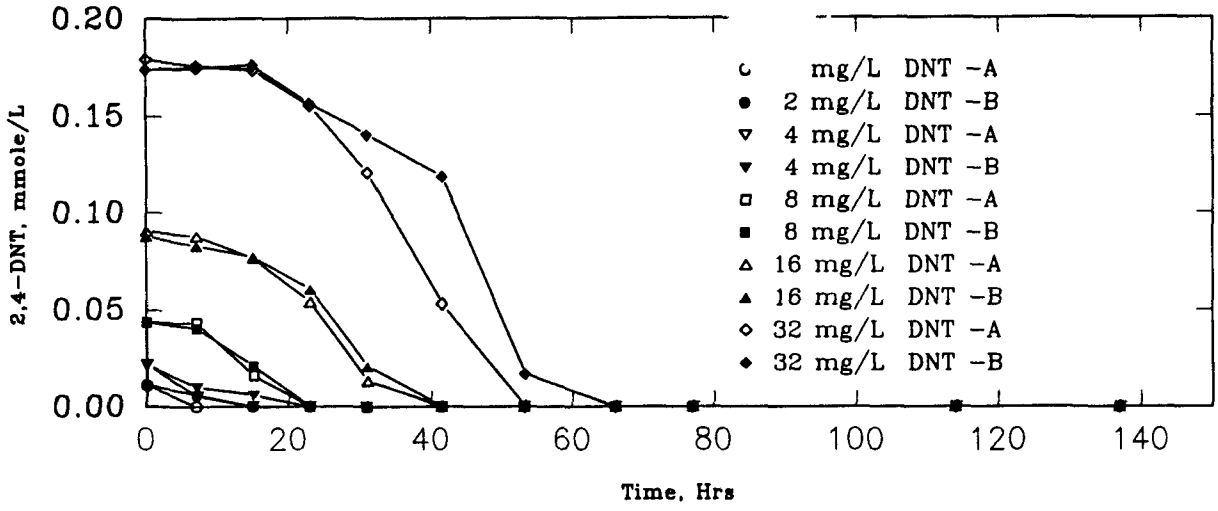

Figure 1. Anaerobic biodegradation of 2,4-dinitrotoluene.

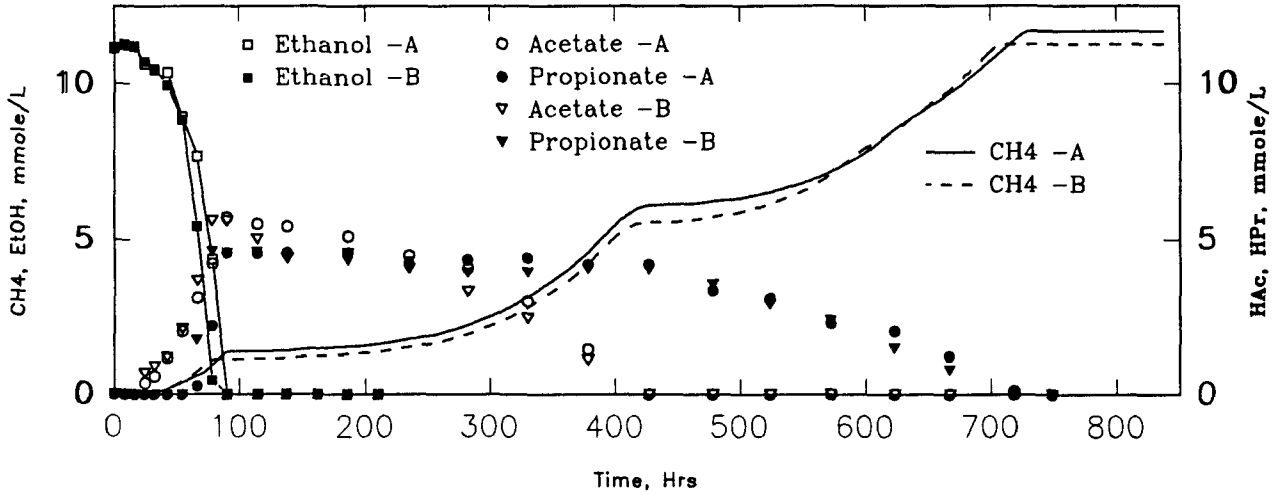

Figure 2. Ethanol biotransformation with initial concentration of 2,4-DNT of 2 mg/L.

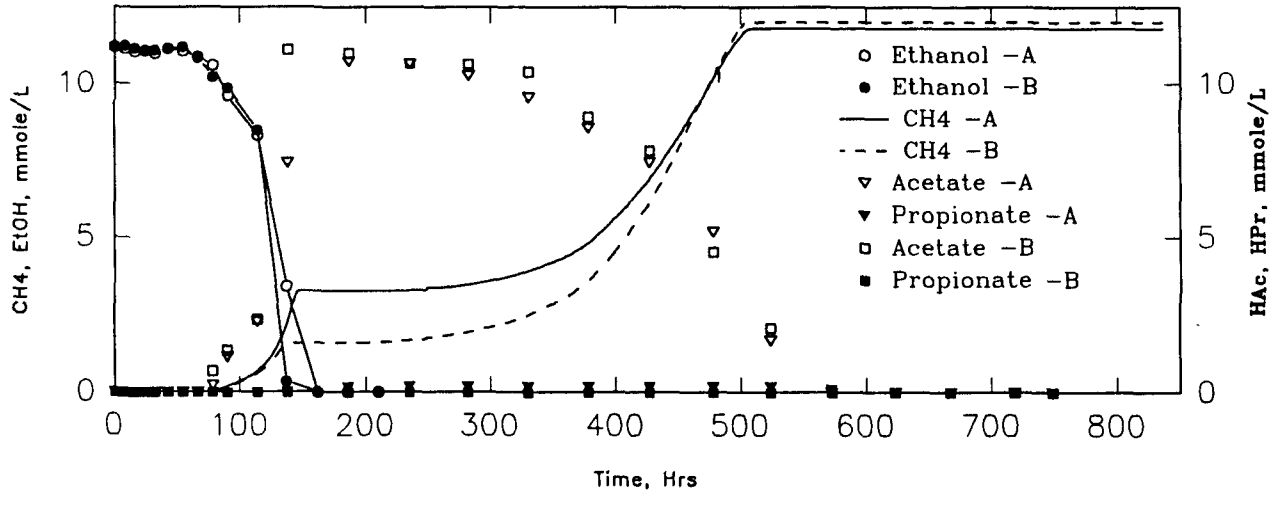

Figure 3. Ethanol biotransformation with initial concentration of 2,4-DNT of 16 mg/L.

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EFFECTS OF METALS ON ANAEROBIC TREATMENT PROCESSES

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INTRODUCTION

Recent interest in the use of bioremediation technology for the cleanup of contaminated soils and sediments has led to a greater understanding of the fate of a variety of toxic organic compounds in natural environments. Most of the research has focused on the biological transformation of a single organic contaminant in laboratory microcosms using naturally occurring microbial inocula. Yet, most polluted ecosystems and hazardous waste sites often are contaminated with a mixture of organic compounds as well as toxic inorganic wastes such as metals. Although the transport and transformation of organic compounds in natural environments such as soils and sediments has been a topic of considerable interest in recent years, little is known about the potential toxicity of metals to naturally occurring microorganisms and microbial processes of importance to the biotransformation of organic compounds.

Soils and sediments are quite varied in composition but generally consist of an array of mineral particles, organic matter, microbial cells and debris, and inorganic solutes, all of which are important in the formation of metal complexes. Further, recent studies have indicated the importance of microbial cell surfaces in the sorption and immobilization of metals in natural ecosystems.

RESULTS AND DISCUSSION

Ongoing studies at EPA's Environmental Research Laboratory in Athens, Georgia, are investigating the effects of heavy metals on anaerobic microbial processes on the biotransformation

of organic compounds. As a model system, the studies are focusing on the toxic effects of metals on the reductive dechlorination of chlorophenolic compounds in unadapted and chlorophenol-adapted microbial communities from freshwater sediments.

The onset, rate, and extent of biotransformation of several mono-, di-, and trichlorophenols were examined using unadapted and chlorophenol(CP)-adapted freshwater sediment slurries (pH 7.0) in the presence and absence of added metal solutions ($CuCl_2$, $CdCl_2$, $K_2Cr_2O_7$). A summary of the experimental results is presented in Table 1. The times required for unadapted control sediment slurries (no added metals) to dechlorinate the selected chlorophenols (10 mg/L) were 16 to 17 days for 2,4-DCP and 2,4,6-TCP; 21 days for 2,3-DCP; 31 days for 2-CP; 37 days for 2,4,5-TCP; and 30 days for 3-CP. Addition of a 20 mg/L concentration of the chloride salts of Cu(II) and Cd(II) had little or no effect on the onset, rate, or extent of dechlorination for most chlorophenols tested. Addition of Cr(VI) at 20 mg/L, however, increased the lag time before initiation of dechlorination for most chlorophenols tested. Addition of 100 mg/L (or greater) of Cr(VI), as $K_2Cr_2O_7$, caused total inhibition of dechlorination of all CPs tested with the exception of 2,4-DCP. Addition of 100 mg/L of Cr(VI) increased the lag time of 2,4-DCP dechlorination from 7.5 days (control) to 45 days. Higher concentrations of Cd(II) (100 to 200 mg/L) also caused complete inhibition of dechlorination for all chlorophenols tested with the exception of 2,4-DCP. The rate of dechlorination of 2,4-DCP was reduced by 70 percent and 100 percent at 100 and 200 mg/L Cd(II) concentrations, respectively. Addition of $CuCl_2$ at 100 mg/L increased the lag time before the onset of dechlorination for most chlorophenols tested, but dechlorination of 2,4-DCP was still evident at 200 mg/L of Cu(II). The distribution of the amended metals between the aqueous and complexed (sediment) phases of the experimental samples was determined by inductively coupled plasma (ICP) spectrometry (Table 2). Representative data for experiments amended with various metals and either 2,3- or 2,4-DCP are presented in Table 2. In most experiments, the aqueous phase concentrations of the added metals were low, varying from 0 to 2 mg/L for Cu(II), 0 to 30 mg/L for Cd(II), and 0 to 28 mg/L for Cr(VI) over the range of metals added (20 to 200 mg/L).

The effects of metal salts [20 to 200 mg/L of Cu(II), Cd(II)] on the reductive dechlorination of 3,4-DCP was compared to two distinct freshwater sediments of similar pH but of differing total organic carbon content. Control experiments (no added metals) for both sediment types differed slightly with regard to the onset of dechlorination, but rates of dechlorination were similar. In general, however, sediment cultures containing the higher organic carbon content (34 mg C/g) were more resistant to the inhibitory effects of the added metal salts. The onset and rate of dechlorination of 3,4-DCP were only slightly affected in the higher organic carbon sediment cultures amended with Cu and Cd salts at amended metal concentrations up to 150 mg/L. Reductive dechlorination in sediment cultures with a lower organic carbon content (17 mg C/g) was completely inhibited (total of 100 days incubation) in cultures amended with a total metal (Cu or Cd) concentration of 50 mg/L. Further, longer lag times (40 days compared to 14 days) were observed in those cultures with the lower organic carbon content at a lower (20 mg/L) amended metal concentration.

Results of recent experiments using 3,4-DCP-adapted sediment cultures of low organic carbon content indicated that dechlorination was more resistant to the inhibitory effects of the added metal salts than those reported above for the unadapted sediment cultures. Further, the effects of amended metals on the reductive dechlorination of mono- and di-CPs also was examined using mono- and di-CP-adapted sediment slurries. A summary of the result, plotted as the percentage of dechlorination activity (DAc) versus the amended metal concentration, is presented in Figures 1a-1c. The DAc is the ratio of the times required for a 50 percent reduction (T_{50}) in the initial concentration of the amended CP in metal-amended slurries and control slurries. Inhibition of

Table 1. Summary: Effects of Metal Amendment on the Onset and Rate of Dechlorination of Chlorophenols in Anoxic Freshwater Sediments

Subst. Rate		"Live" Rate	Metal Amendment[b]								
			Cu(II) [mg/L]			Cr(VI) [mg/L]			CD(II) [mg/L]		
			20	100	200	20	100	200	20	100	200
2-CP	Rate[a]	0.64	0.65	0.55	N	0.55	N	N	0.56	N	N
	Lag	12	10	24		15			10		
3-CP	Rate	0.62	0.56	N	N	0.45	N	N	0.46	N	N
	Lag (days)	59	58			65			58		
2-3-DCP	Rate	0.63	0.74	0.26	N	0.60	N	N	0.60	N	N
	Lag	10	7	37		10			7		
2,4-DCP	Rate	0.83	0.76	0.63	0.35	0.75	0.18	VS	0.87	0.10	N
	Lag	7.5	7	10	56	13	45		7	26	
2,4,6-TCP	Rate	0.60	0.89	0.76	N	0.51	ND	VS	0.56	N	N
	Lag	7	7	26		10	ND		14		

[a]Dechlorination rate is expressed as mg CP_{loss} $L^{-1}day^{-1}$.
[b]Final concentration of metal amended as $CuCl_2$, $CdCl_2$, or $K_2Cr_2O_7$.
N, negligible loss; VS, very slow; ND, not determined.

Table 2. Determination of Total and Aqueous Phase Metal Concentrations in Cherokee Pond Sediment Slurries (10% w/v) Amended with Various Metal Solutions and Specific Chlorophenols

	Substrate Amendment	Metal Amendment								
		20 mg/L			100 mg/L			200 mg/L		
		Cu	Cd	Cr	Cu	Cd	Cr	Cu	Cd	Cr
		Experimental Results								
Total[b] [mg/L]	2,4-DCP	25.1	16.0	19.7	106.4	78.4	115.8	202.7	178.2	222.0
	2,3-DCP	26.9	20.2	ND	113.4	90.8	111.4	192.0	182.2	ND
Dissolved[c] [mg/L]	2,4-DCP	<0.03	<0.03	<0.03	0.12	1.02	0.12	0.66	10.0	0.34
	2,3-CP	<0.03	<0.03	<0.03	0.12	1.07	0.12	0.6	9.24	0.42

[a]Values are the mean of duplicate determinations.
[b]Total metal concentrations after digestion with concentrated nitric acids.
[c]Metal concentrations of aqueous supernatant of sediment slurry after centrifugation at 10,000 xg and filtering.

ND, not determined.

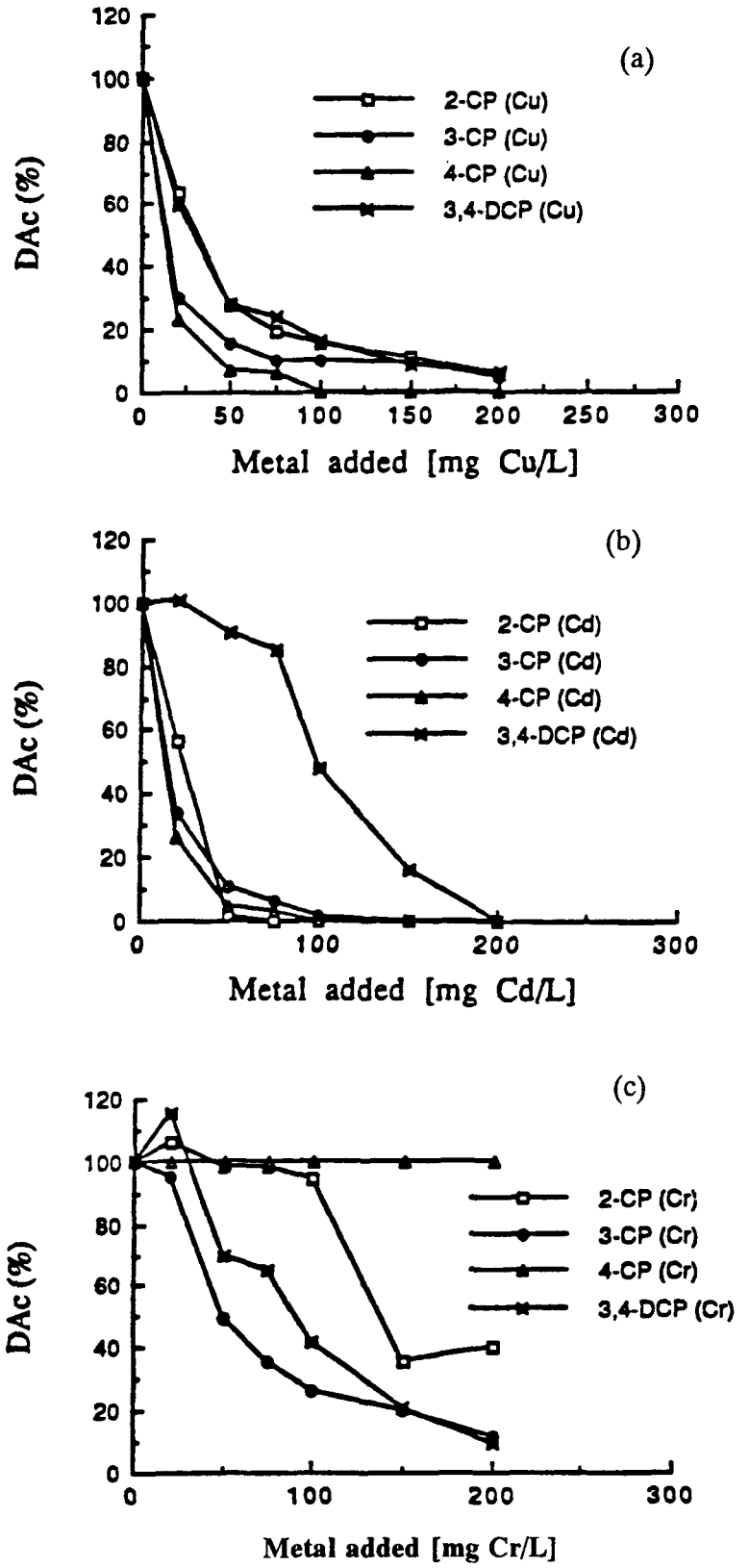

Figure 1. Reductive dechlorination activity (DAc) of chlorophenol-adapted sediments in the presence of (a) amended Cu, (b) amended Cd, and (c) amended Cr. The % DAc is the ratio of the T_{50} of the metal-amended slurry and the control slurry. Data are presented for dechlorination of 2-CP, 3-CP, 4-CP, and 3,4-DCP.

dechlorination was greatest in experiments amended with Cd and Cu; reductive dechlorination of 3,4-DCP was more resistant to the inhibitory effects of Cd compared to dechlorination of mono-CPs. Interestingly, amendment of Cr resulted in the least inhibition of reductive dechlorination for all CPs tested. In fact, reductive dechlorination of 4-CP was completely resistant to even the highest Cr concentration (200 mg/L) tested.

These preliminary data suggest that the metal type, aqueous metal concentration, and organic carbon content may affect the transformation of chlorophenols in anoxic sediments. Additional studies are in progress to determine the inhibitory nature of metals on the reductive dechlorination of higher chlorinated chloroaromatics, such as pentachlorophenol.

FATE OF HIGHLY CHLORINATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS IN ANAEROBIC SOILS AND SEDIMENTS

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INTRODUCTION

Chlorinated dibenzo-*p*-dioxins (PCDD) and dibenzofurans (PCDF) have been generated as unwanted by-products in many industrial and most incineration processes. Although their widespread distribution in different environmental compartments has been recognized, little is known about their fate in the ultimate environmental sinks, soils, and sediments. In particular, the susceptibility of PCDD and PCDF to biological oxidation and reduction reactions has only recently received attention, albeit limited.

Thus, the fate of selected highly chlorinated dibenzo-*p*-dioxins and dibenzofurans has been studied in PCB (polychlorinated biphenyl)-contaminated Hudson River sediments and creosote-contaminated aquifer samples. All samples were incubated anaerobically (i.e., in methanogenic conditions) and spiked with 144 ± 14 μg/kg of the following PCDD and PCDF congeners: 1,2,3,4,7,8-HxCDD; 1,2,4,6,8,9/1,2,4,6,7,9-HxCDD; 1,2,3,4,6,7,8-HpCDD; 1,2,4,6,8-PentaCDF; and 1,2,3,4,6,7,8-HpCDF.

BACKGROUND

Dehalogenation of highly chlorinated PCDD/PCDF on soil and particulate material has previously been demonstrated during physical-chemical processes, such as photolysis. During dehalogenation from the octa- to the tetra-chlorinated isomers, although the lateral chlorines (2, 3, 7, and 8 positions) were selectively retained in the case of dioxins, others were preferentially removed where dibenzofurans are concerned (1).

Reductive dehalogenation of PCBs with a high degree of chlorine substitution has been demonstrated to occur in previously contaminated Hudson River sediments and in methanogenic microcosms containing pristine or adapted sediments spiked with either Aroclor mixtures or individual PCB congeners (2-4). The PCB-molecule is preferentially dechlorinated at the *meta*- or *para*-position. Because of the structural similarity between dioxins or furans and PCBs, the potential for susceptibility of the PCDD/PCDF to reductive dehalogenation processes was investigated.

RESULTS AND DISCUSSION

The fate of the spiked PCDD/PCDF (except for 1,2,4,6,8,9/1,2,4,6,7,9-HxCDD) in anaerobic sediments and soils (expressed as C/Co) is shown in Figure 1. All time samples for the active microcosms represent an average of three replicates, while those for chemical (no inoculum) and autoclaved controls represent two replicates. In all cases, the losses over time in the chemical controls are negligible compared to the other treatments. The disappearance of PCDD/PCDF in the autoclaved controls, however, is in most cases nearly as extensive as that in the active microcosms, indicating the importance of sorption processes during the incubation period. The total decrease of PCDD/PCDF appears to be slightly less in the low organic carbon (0.02 percent) aquifer samples, when compared to the 7 to 8 percent organic carbon Hudson River sediment samples.

The first-order rate constants for disappearance—calculated from log (C/C_o) plots—are given in Table 1. Although it is difficult at this stage to differentiate between sorption alone and sorption combined with biodegradation, the net rates calculated indicate that the biological component increased the rate of PCDD/PCDF disappearance by 15 to 35 percent, dependent on the PCDD/PCDF isomer spiked and the inoculum used.

Previously up to 10 percent of the heptaCDD congener was dechlorinated to an unidentified hexaCDD after 2 months (5). This paper presents the analysis of heptaCDD-spiked microcosms inoculated with aquifer material. Two hexachlorinated isomers have been identified: 1,2,3,4,7,8- and 1,2,3,6,7,8-HxCDD, respectively. The 6-chlorine from the lesser chlorinated ring appeared to remove preferentially to the chlorine in the 4-position, as significantly more 1,2,3,4,7,8-HxCDD accumulated. The extensive removal of the peri-chlorines of the HpCDD congener, resulting in an enrichment in 2,3,7,8-chlorinated congeners is similar to what was found during photolysis (1).

Thus, a dechlorination pattern of 1,2,3,4,6,7,8-HpCDD is proposed to be consistent with the discrepant degree in accumulation of the two metabolites recovered (Figure 2). A pentaCDF isomer formed from dechlorination of 1,2,3,4,6,7,8-HpCDF has not been identified at this time.

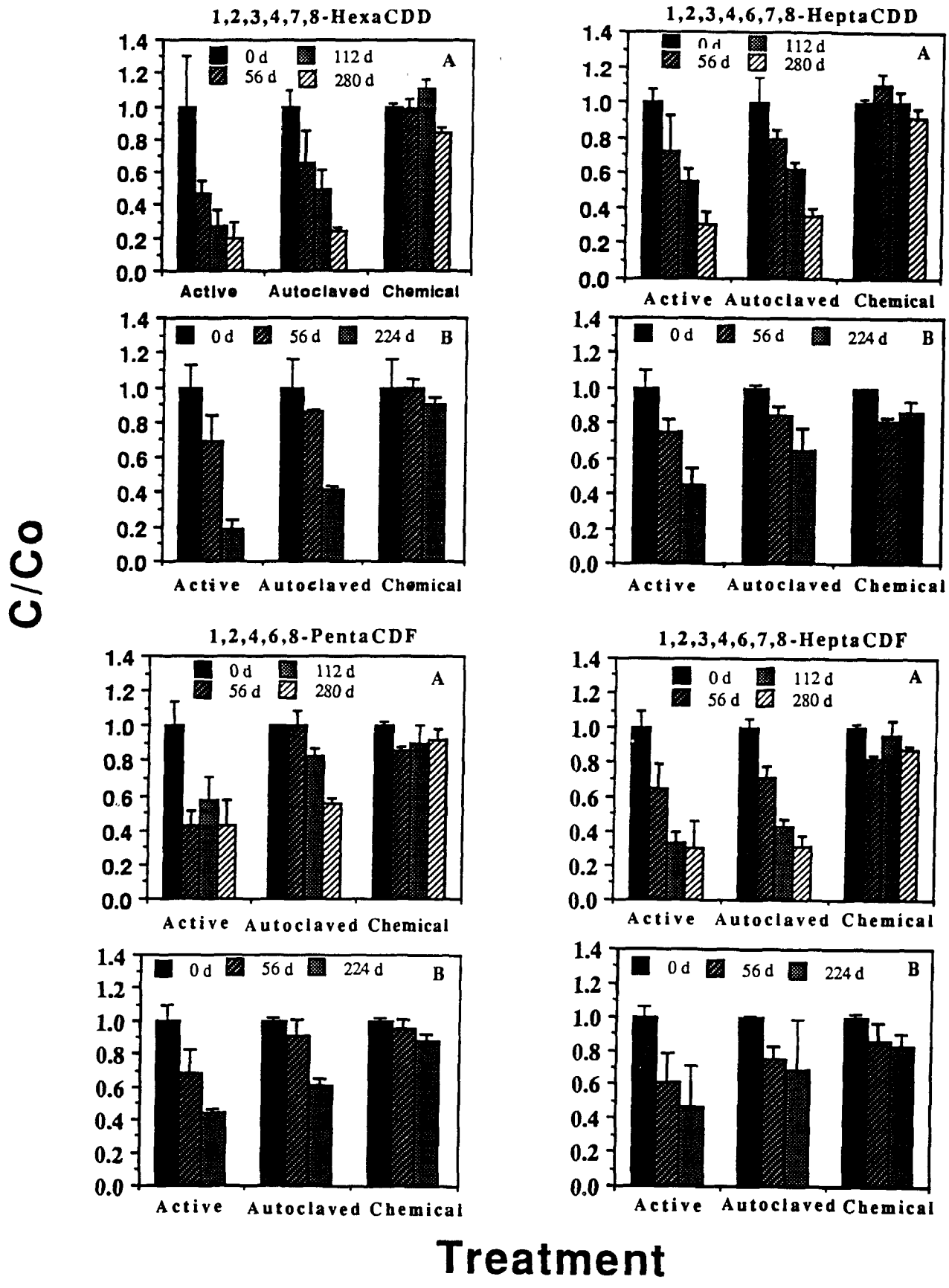

Figure 1. Fate of selected PCDD and PCDF in Hudson River sediments (A) and in Pensacola aquifer material (B).

Table 1. First-Order Disappearance Rates of PCDD/PCDF in Hudson River Sediment and Aquifer Material Inoculated Microcosms

PCDD/F Congener	Inoculum	Rate* (x 10-2 wk-1) Active	Autoclaved	Nettorate (%)
HpCDD	HR	2.81 ± 0.07	2.38 ± 0.14	0.43 ± 0.14 (15)
	PS	3.44 ± 0.10	2.19 ± 0.02	1.25 ± 0.10 (36)
HxCDD	HR	4.56 ± 0.30	3.19 ± 0.09	1.37 ± 0.30 (30)
	PS	2.48 ± 0.13	1.85 ± 0.16	0.63 ± 0.16 (25)
HxCDDi	HR	4.00 ± 0.16	4.13 ± 0.14	(-0.13 ± 0.16)
	PS	ND	ND	ND
HpCDF	HR	4.13 ± 0.10	3.56 ± 0.05	0.57 ± 0.10 (14)
	PS	4.88 ± 0.06	3.13 ± 0.01	1.75 ± 0.06 (36)
PentaCDF	HR**	2.69 ± 0.40	1.06 ± 0.02	1.63 ± 0.40 (60)
	PS	1.54 ± 0.10	1.23 ± 0.02	0.31 ± 0.10 (20)

* n = 6-12
** Not in 95 percent confidence interval.

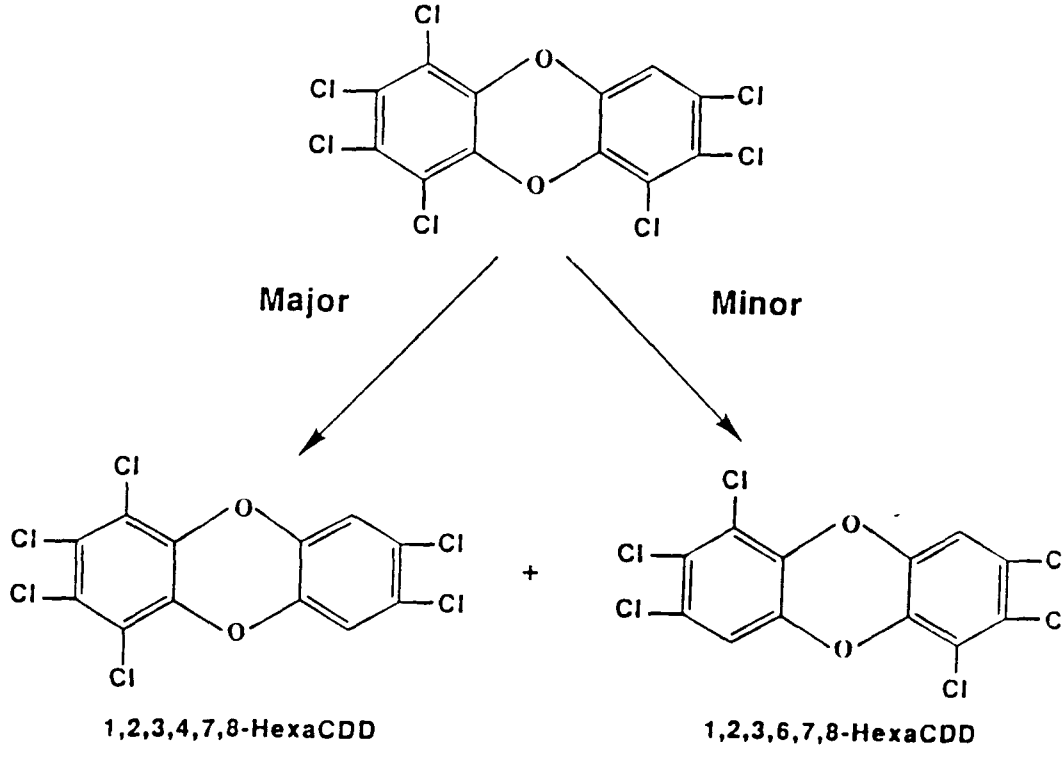

Figure 2. Proposed dechlorination of 1,2,3,4,6,7,8-heptaCDD in methanogenic aquifer microcosms.

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BIOREACTOR TREATMENT OF NITRATE CONTAMINATION IN GROUND WATER: STUDIES ON THE SULFUR-MEDIATED BIOLOGICAL DENITRIFICATION PROCESS

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INTRODUCTION

Nitrate contamination of ground water resources has become an increasingly serious problem in the United States and Europe (1,2). Water containing more than 45 ppm nitrate (10 ppm nitrate-nitrogen) is considered unfit for potable use. Consumption of water containing nitrate in excess of this level appears to be associated with adverse health effects, namely increased incidence of methemoglobulinemia in infants and gastrointestinal malignancies in adults (3).

The ground water basin underlying Orange County, California, serves a population of some 2.3 million people. Currently, over 250,000 acre feet of the basin's capacity is rendered unusable due to nitrate levels exceeding the federally mandated limit of 45 ppm.

At least six technological approaches to mitigation of the nitrate problem exist, including 1) recasing and/or redrilling wells to access uncontaminated zones of the aquifer, 2) blending contaminated water with low-nitrate water to achieve mandated standards, 3) demineralizing water with reverse osmosis, 4) demineralizing water with electrodialysis, 5) subjecting water to an ion-exchange process, and 6) subjecting water to biological denitrification (4). Orange County Water District has been exploring the use of biological denitrification as a means of producing potable-quality water from nitrate-contaminated ground water reserves.

BACKGROUND

The balance of existing research on biological denitrification relates to heterotrophic processes in which an organic compound, such as methanol, glucose, or glycerol, is added to the high-nitrate water (5,6). Such organics serve as a carbon and energy source for denitrification. While effective, the addition of organics to the water is undesirable since residuals might render the denitrified water toxic (i.e., if methanol is used). In addition, organics can give rise to trihalomethanes (THMs), if chlorine disinfection is used.

Autotrophic denitrification has been studied as a means to avoid these shortcomings. This paper deals with the use of elemental sulfur as an energy source for biological denitrification catalyzed by the autotrophic bacterium, *Thiobacillus denitrificans*, which in the absence of free oxygen reduces nitrate to dinitrogen gas by oxidizing sulfur to sulfate. All carbon required for biosynthesis is derived from carbon dioxide/bicarbonates present in the ground water. The denitrification process can be conducted in fluidized bed bioreactors, fixed (static) bed reactors, or in agitated (stirred) tank reactors.

EXPERIMENTAL REACTORS/METHODS

The denitrifying bacteria used in this study were obtained from an anaerobic enrichment culture consisting of basal mineral salts and sodium thiosulfate. Upon development, the culture was transferred to flasks containing basal salts and elemental sulfur. The culture consists of a consortium of bacteria: *Thiobacillus denitrificans* plus a variety of heterotrophic denitrifying bacteria.

The fluidized bed reactor was constructed from a 3.65 m section of 5.08 cm (internal) diameter transparent polyvinyl chloride (PVC) pipe closed at the ends with PVC unions. The reactor was mounted outdoors and fed with nitrate-amended well water, which was conveyed to the reactor using an adjustable peristaltic pump (Masterflex) capable of flows of up to 13.2 L/min. The reactor contained sulfur particles with an initial size range of -16/+30 mesh (U.S. Standard Screen Series). Depth of the sulfur bed under static (nonfluidized) conditions was 1.83 m. The reactor was inoculated with the denitrifying consortium.

The agitated (sulfur slurry) reactor system was constructed using a glass and stainless steel research fermenter (New Brunswick Scientific). The reactor (1.3-L liquid capacity) system provides the capacity for temperature control, variation of agitation rate, automatic pH control, and determination of dissolved oxygen tension. The reactor was charged with precipitated sulfur (100 percent - 80 mesh). Initial reactor runs employed a sulfur solids content of 1.5 percent (weight/volume). The reactor was fed (via a peristaltic pump) a synthetic salts solution formulated to simulate locally occurring high-nitrate ground water. The reactor system included a gravity separator unit to recover unutilized sulfur from the denitrified effluent. The separated sulfur was continuously returned to the main reactor vessel by means of a second peristaltic pump. The agitated slurry reactor was inoculated with the denitrifying consortium used in the fluidized bed reactor (see above).

During operation of both reactors, effluent samples were obtained on an hourly basis by means of an automatic sampling device (Isco). Samples were analyzed for nitrate, nitrite, sulfate, and pH. Studies were also conducted on the effluent to determine total autotrophic and heterotrophic bacterial counts.

RESULTS/DISCUSSION

Both reactor types were capable of maintaining prolonged periods of stable operation (effluent containing less than 0.3 ppm of either nitrate or nitrite from influent streams containing from 45 to 100 ppm nitrate). The autotrophic denitrification process resulted in a pH drop from approximately 8.2 (influent) to 7.0 (effluent). It was found that 1.64 mg of sulfate was added to the treated water for every 1.0 mg of nitrate removed. Typical nitrate removals for the fluidized bed

reactor and the sulfur slurry reactor are presented in Tables 1 and 2, respectively. Flow rate for the fluidized bed was 250 mL/min (reactor temperature at 18°C) while the flow to the sulfur slurry reactor was 5.0 mL/min (reactor temperature at 30°C).

SUMMARY/CONCLUSIONS

The operational characteristics of two sulfur-based biological reactor systems were investigated. Maximum hydraulic loading rates consistent with stable denitrification were determined. Efficient operation of the process in a field application might require supplementation of the high-nitrate ground water reactor feed with trace (1 to 2 ppm) amounts of ammonium and/or phosphate to supply bacterial biosynthetic requirements.

Both reactor types were successfully employed for the sulfur-mediated biological denitrification process. The fluidized bed reactor appears to offer several distinct advantages including 1) no complex solid-liquid separating unit (clarifier) is needed, 2) no multiple staging is required to prevent short-circuiting of water undergoing denitrification, 3) no costly mechanical agitator drives and reactor seals are required, 4) reactors can be constructed with large height-to-diameter ratios, thus minimizing space requirements, and 5) no recycle pumping of the sulfur substrate slurry is required.

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Table 1. Denitrification by Fluid Bed Reactor

RUN HOUR	INFLUENT NITRATE (ppm)	EFFLUENT NITRATE (ppm)
6600	46.7	0.7
6606	46.7	0.0
6612	46.7	0.0
6618	46.7	0.0
6624	47.0	0.0
6630	47.0	0.0
6636	47.0	0.0
6642	47.0	0.0
6648	42.5	0.0
6654	42.5	0.0
6660	42.5	0.0
6666	42.5	0.0

Table 2. Denitrification by Fluid Bed Reactor

RUN HOUR	INFLUENT NITRATE (ppm)	EFFLUENT NITRATE (ppm)
2303	57.2	0.0
2309	57.2	0.0
2315	57.2	0.1
2321	57.2	0.1
2327	59.8	0.0
2333	59.8	0.0
2339	59.8	0.0
2345	59.8	0.0
2351	55.7	0.0
2357	55.7	0.0
2363	55.7	0.0
2369	55.7	0.0

CHEMICAL INTERACTIONS AND pH PROFILES IN MICROBIAL BIOFILMS

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Substrate utilization kinetics within biofilms has been modeled by coupling Fickian diffusive transport with Monod-reaction kinetics (1,2). Substrate and product concentration profiles are effected in biological systems because of simultaneous diffusion and reaction. Compounds utilized or produced during biological transformations can have an impact on the rate of substrate utilization by affecting the local pH. For example, nitrification and the utilization of halogenated organic compounds produce acid equivalents and cause a decrease in pH, while denitrification consumes acid equivalents and causes an increase in pH. Production and utilization of total carbon dioxide also alters the chemical equilibrium. The rates of substrate utilization and growth of microorganisms are pH dependent and can vary significantly from the bulk solution to the attachment wall of biofilms because of the pH gradients influenced by diffusional resistance to mass transport. An approach was developed to analyze the effects of chemical interactions and pH changes within the biofilm on the overall rates of substrate removal in biofilms. This approach incorporates ionic mass transport effects accurately and accounts for the presence of background ions; charges on the surface or microorganisms; and corrections for the activity, electrophoresis, relaxation, and hydration of ions. Models have been developed for autotrophic and anaerobic biofilms. Results for an acetate-utilizing methanogenic biofilm are described.

The key features in this work are the incorporation of pH-dependent Monod kinetics and an attempt to account for the production and movement of gaseous products in methanogenic biofilms. Gaseous products accumulate within the biofilm as bubbles that periodically erupt to the surface, especially in systems under high organic loadings (3). Since a portion of the carbon dioxide that is produced does not remain in solution, the extent of pH change within the biofilm will be affected. In this study, the process of gas bubble formation and eruption is modeled by assuming that the advection of the gas generated from within the film to the bulk is described by a one-dimensional gas flow rate per unit area of the biofilm. Overall, the mass transfer of compounds in the biofilm consists of the diffusion of dissolved compounds in the liquid phase (including the biofilm) and the advection of gaseous products in the gas phase.

Assuming that the biofilm is at steady-state with respect to the consumption of substrate, a one-dimensional mass balance on total acetate (C_{TAc}), total inorganic carbon (C_{TIC}), and methane in the biofilm yields,

$$\frac{dJ_{HAc}}{dx} + \frac{dJ_{Ac^-}}{dx} = \frac{bX_f}{Y} - \frac{q_{max}X_f[HAc]}{K_s + [HAc]} f_{pH} \tag{1}$$

$$\frac{dJ_{CO_2}}{dx} + \frac{dJ_{HCO_3^-}}{dx} + \frac{dJ_{CO_3^{2-}}}{dx} + \frac{d}{dx}\left(\frac{q_g K_{h,CO_2}[CO_2]}{RT}\right) = \frac{q_{max}X_f[HAc]}{K_s + [HAc]} f_{pH} \tag{2}$$

$$\frac{dJ_{CH_4}}{dx} + \frac{d}{dx}\left(\frac{q_g K_{h,CH_4}[CH_4]}{RT}\right) = \frac{q_{max}X_f[HAc]}{K_s + [HAc]} f_{pH} \tag{3}$$

where:

J	=	the molar flux of the species
x	=	the spatial coordinate perpendicular to the biofilm surface
q_g	=	the gas volumetric flow rate per unit surface area of biofilm
K_h	=	Henry's constant
R	=	the universal gas constant
T	=	the absolute temperature
b	=	the respiration coefficient
Y	=	the cell yield per mass of acetate
X_f	=	the microorganism density in the biofilm
q_{max}	=	the maximum specific growth rate at the optimum pH
K_s	=	the Monod half-saturation constant
f_{pH}	=	the fractional decrease in the growth rate due to deviations from the optimum pH
		the brackets [ ] refer to the molar concentration of the species

The flux is given by Fick's law for neutral species and by the Nernst-Planck equation for ionic solutes (4). The fractional decrease in growth rate due to deviations from the optimum pH is represented by a Gaussian curve (5),

$$f_{pH} = e^{-\left(\frac{pH-7.02}{0.5}\right)^2} \tag{4}$$

Equations 1 to 4 were solved in conjunction with expressions for acid-base and gas-liquid equilibria and electroneutrality.

The profiles of (C_{TAc}) and pH for a biofilm depth of 1,000 μm, bulk pH=7.0, bulk $C_{TAc}=10^{-2}$ M, and various bulk partial pressures of carbon dioxide are shown in Figure 1. The profile of C_{TAc} predicted by using traditional biofilm models also is shown. Traditional biofilm models typically assume a constant pH in the biofilm. These models are expressed by a mass balance on total acetate similar to equation 1 but use Fick's law to describe the diffusion of both acetic acid and the acetate ion. The current model predicts that the pH increases in the biofilm. This increase is due to the utilization of a stronger acid (acetic acid) and the production of a weaker acid (carbon dioxide).

Depending on the partial pressure of carbon dioxide (P_{CO_2}), the pH at the wall of the biofilm can be as high as 7.49. This deviation from the optimum pH causes a drop in the rate of acetate utilization and increases the level of C_{TAc} within the biofilm. The lower the P_{CO_2}, the lower the buffer intensity, and the larger the increase in pH and C_{TAc} in the film. Since traditional models assume a constant pH, the concentration of C_{TAc} reaches very low levels in the biofilm.

The impact of the bulk pH on the flux of substrate into the biofilm is shown in Figure 2 for a bulk $C_{TAc}=10^{-2}$ M for various bulk C_{TIC} and a biofilm depth of 1,000 μm. The flux predicted by using the traditional model also is shown in the figure. Figure 2 shows that the traditional model predicts a maximum flux at a bulk pH of 7.0. This pH corresponds to the optimum pH of the microorganisms specified using equation 4. In contrast, the current model predicts an optimum pH less than 7.0 for acetate-utilizing biofilms. Since the pH increases in acetate-utilizing biofilms, operating at a lower pH causes a larger fraction of the biofilm to exist closer to the optimum pH. Decreasing the bulk C_{TIC} (or buffer intensity) causes a greater increase in the pH within the biofilm and shifts the optimum bulk pH to lower values. Overall, the approach developed can be coupled to a reactor model, and the operating conditions for a reactor (such as the buffering capacity of the influent feed, the influent pH, and the surface loading rates) can be specified to optimize reactor performance.

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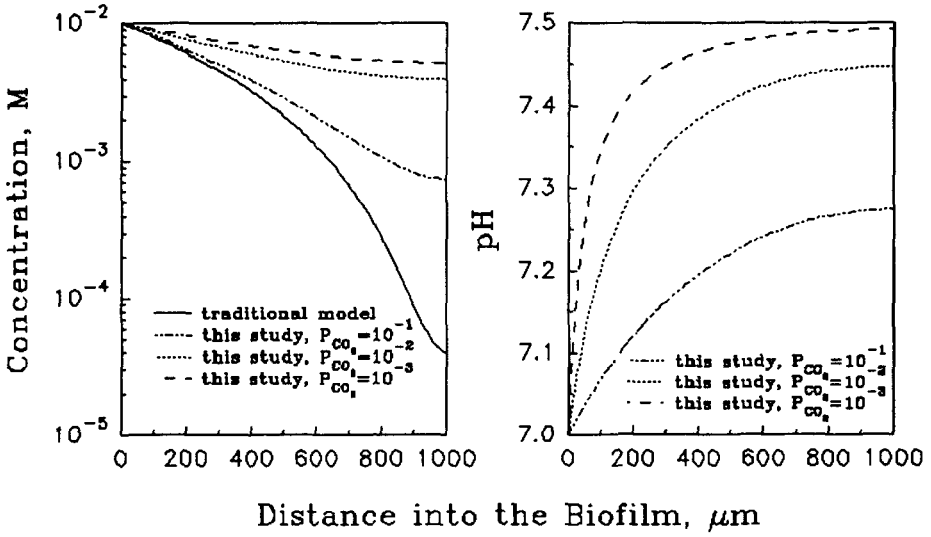

Figure 1. Profiles of total acetate (C_{TAc}) and pH for various bulk partial pressures of carbon dioxide, a biofilm depth of 1,000 μm, bulk pH=7.0, bulk $C_{TAc}=10^{-2}$ M, and $L_{CBL}=0$.

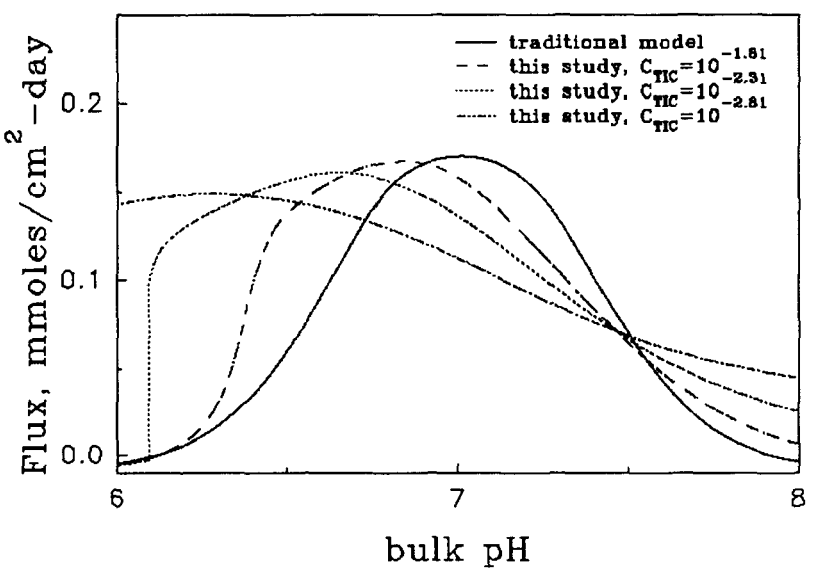

Figure 2. The flux of substrate into the biofilm as a function of bulk pH for a bulk $C_{TAc}=10^{-2}$ M for various bulk C_{TIC} and a biofilm depth of 1,000 μm.

CHARACTERIZATION OF BIOFILTER MICROBIAL POPULATIONS

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INTRODUCTION

Biofiltration, a promising, economical form of biological treatment, is becoming a more common means of removing environmental contaminants from waste gas (1). The conversion of target compounds to innocuous end products in the biofilter treatment process is mediated by a heterogeneous bacterial population that develops on the biofilter support material. Biofilter technology has been advancing rapidly in Europe since the late 1970s, but its application in the United States has been limited. Originally used at facilities such as animal rendering plants for odor abatement, biofilters are now being employed in the treatment of volatile organic compounds (VOCs) such as toluene, benzene, and ethylbenzene as well as more recalcitrant compounds such as methylene chloride and trichloroethylene (TCE) (2).

In addition to sound engineering design, improved biofilter efficiency as well as broadened VOC removal capabilities are ultimately dependent on the catabolic potential and activity of the biofilter microorganisms. Biofilter microbial communities are largely uncharacterized (3). Analysis of the structure and function of biofilter communities should provide useful information for biofilter performance optimization, detection and assessment of biofilter perturbation, and comparison of biofilters (e.g., between lab- and pilot-scale biofilters).

In this study, characterization of biofilter communities is being conducted by using both molecular biological techniques and standard microbiological protocols. Gene probes specific for aromatic hydrocarbon oxidation are being employed to determine which biochemical pathways are present in the biofilter, and phylogenetic probes are being used to determine what types of bacteria are predominant. In addition to these methods, DNA amplification fingerprinting (DAF) is being evaluated as a means of monitoring the microbial community structure of the biofilter.

BACKGROUND

Incorporation of some of the new DNA- based techniques in the study of biofilter microbial ecology has the potential to result in more precise and less time-consuming analyses that will directly contribute to improved biofilter performance. DAF technology has been successfully employed in a number of studies and has emerged as a powerful tool for rapid microbial identification (4,5,6). DAF also has been useful in the mapping of complex genomes (7). The utility of DAF in the analysis of mixed cultures is being evaluated as part of this study. Nucleic acids can be extracted directly from biofilter material and subjected to polymerase chain reaction (PCR) amplification in a relatively rapid timeframe. The ultimate goal is to determine whether the fingerprints generated can provide a means of comparing community structure over time or among different biofilters. Parallel studies using gene probes and standard culturing techniques are being conducted. The hope is that structural and functional assessments of biofilter communities can be made by combining these methods.

RESULTS

Initial experiments involved the generation of fingerprints for previously characterized VOC catabolic strains of *Pseudomonas cepacia* G4, *P. putida* F1, and *Pseudomonas* sp. JS150. These experiments served to evaluate the efficacy of various primers in the PCR reaction and provided fingerprints to compare with uncharacterized biofilter isolates.

Preliminary mixed-culture experiments were conducted using bench-scale culture microcosms inoculated with biofilter material and maintained on various VOCs. These cultures served as sources of biomass for the evaluation of lysis and DNA extraction methods. All PCR reactions were conducted using a single 8-base oligonucleotide as a primer. Microcosms, one with toluene as a sole carbon source and the second with toluene, ethylbenzene, and *p*-xylene, were maintained for a period of 5 months. The resultant fingerprints showed consistent profiles over a period of 3 consecutive days as well as some common bands between the microcosms (Figure 1). The patterns generated show distinct bands, not an indiscriminant smear as might have been predicted given the complexity of the sample.

Three operational VOC catabolizing biofilters, packed with peat and maintained in different configurations, were analyzed using DAF. These biofilters were maintained under different regimes: one with a pelletized medium, one maintained on channelized medium in a concurrent mode (gas stream and nutrient flow in the same direction), and one maintained on channelized medium in a countercurrent mode (gas stream and nutrient flow in opposing directions). The biofilters received toluene as a sole carbon source for 6 months prior to the initiation of sampling. Similar DAF profiles were obtained for the biofilters maintained on channelized support media, while the pelletized biofilter yielded a unique pattern. The individual organisms from these biofilters appear to constitute a relatively diverse population capable of growth on a number of VOCs including benzene and ethylbenzene. Fungi appear to be only a minor constituent of the population.

SUMMARY AND CONCLUSIONS

DAF has utility for generating rapid genetic profiles of biofilter microbial communities. Multiple samples can be easily processed, and culturing is not necessary. The significance of these

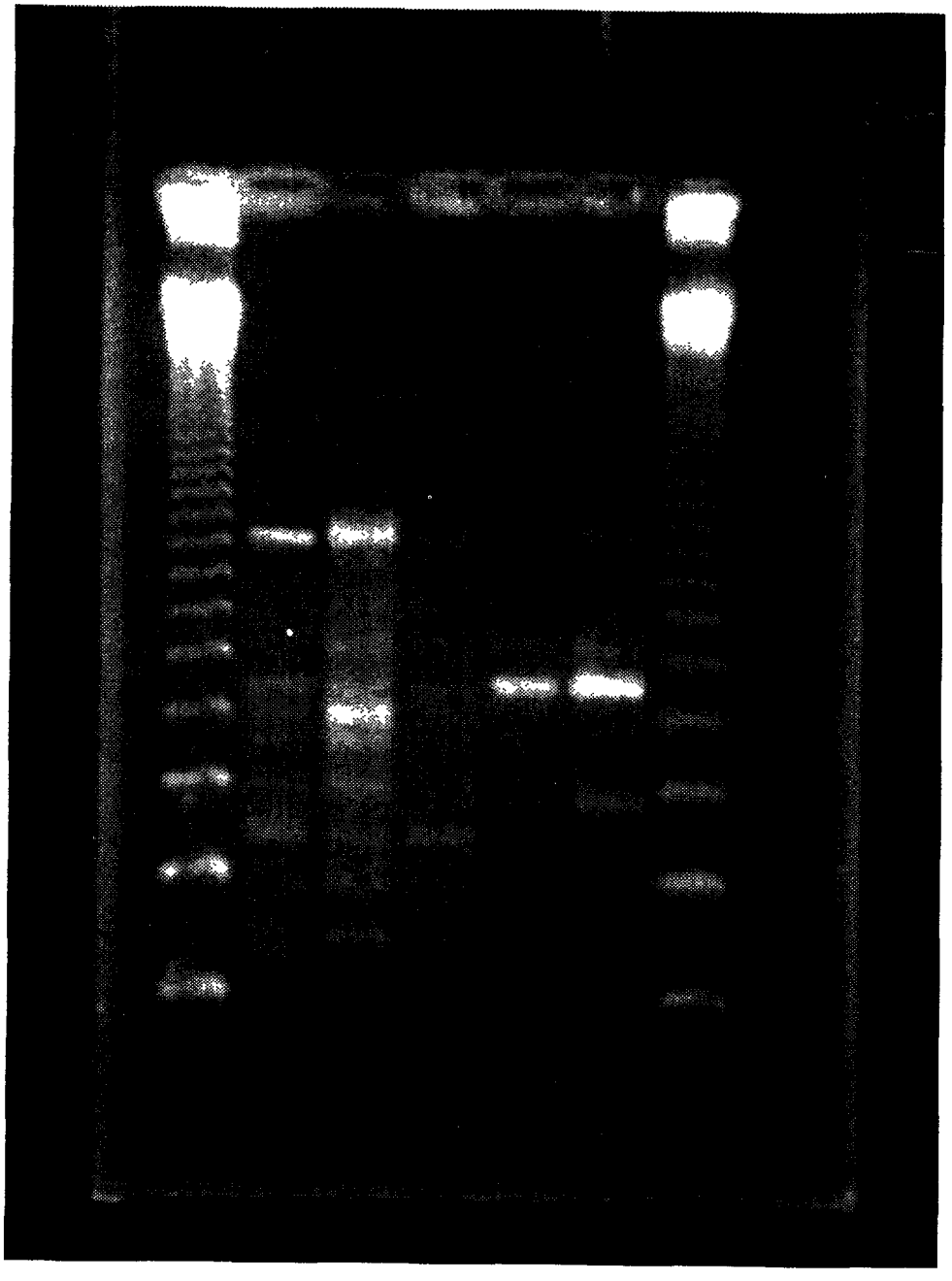

Figure 1. DAF analysis of mixed microbial populations. Patterns were generated from nucleic acid extracts from shake flasks inoculated with biofilter material and maintained on VOCs for five months. Lanes 1 and 7 are 123-base molecular weight standards. Lanes 2, 3, and 4 are patterns from the toluene-maintained culture over three consecutive days. Lanes 5 and 6 are patterns generated at 48-hour intervals from the flask maintained on toluene, ethylbenzene, and *p*-xylene.

profiles as means of monitoring biofilter performance is being evaluated by intensive sampling over time. DNA fingerprinting technology has potential as a rapid and economic tool for biofilter monitoring and for diagnostics. Ideally, a given DAF profile for a particular biofilter will prove to be diagnostic for particular modes of performance. Studies currently under way will involve gene probing to provide a functional component of the analysis.

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FUNDAMENTAL STUDIES IN THE DEVELOPMENT OF THE GAS PHASE BIOFILTER

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INTRODUCTION

The Superfund Amendments and Reauthorization Act (SARA) emission summary for petroleum and chemical manufacturing companies shows that the largest releases in air are volatile organic compounds (VOCs). Conventional technologies that have been used to control VOCs include 1) adsorption onto porous materials, such as activated carbon; 2) absorption into a liquid stream followed by stripping of the liquid; 3) combustion or incineration; and 4) pervaporation using membranes to selectively remove the harmful volatiles. These technologies present several problems, involving disposal of generated wastes, cost of equipment, and materials and energy required for operation.

A biofilter bed consists of a bed packed with a solid support with microorganisms in the form of a wet, biologically active layer, referred to as biofilm, immobilized on the surface of the support material. The solid support material can be either fine particulate material, such as soil, peat, compost, pellets of activated carbon, or ceramic media, or structured monoliths with passages of defined geometry. The microbial degradation of substrate is assumed to take place in the biofilm.

A wide range of VOCs can be removed by microbial degradation in biological filters. Air biofiltration is a well-established technology in Europe (1).

As compared to other options, biofiltration is cheap, reliable, and represents a more natural approach for control of VOCs. Moreover, it can handle chemically different compounds, and the compounds are completely degraded, unlike in some other technologies where they are simply transferred from one phase to other (2).

BACKGROUND

Biological filtration of VOCs implies the removal of compounds by contacting the contaminated air with microorganisms in a column or similar configuration. Three basic types of biological filters are available: bioscrubbers, bio-trickling filters, and biofilters (3). A biofilter has high specific surface area packing and water present as liquid holdup in the column. Either gas entering the biofilter has to be humidified or water has to be sprayed intermittently into the biofilter to prevent the drying of the column. Further, biofilters have to be washed with water during operation to prevent the accumulation of degradation products.

Biological methods have been employed for purification of waste gases since the early sixties. The concept of using biodegradation to control hydrogen sulfide from sewage works was first considered as early as 1923 (1). Soil beds have been used for controlling odors from sewage and wastewater plants. Bohn and Bohn (4) have discussed the theory and potential applications of soil beds for odor control. Such systems have recently been used for control of volatile aliphatic compound emissions (5).

Biofilters have been used extensively in Europe, especially in Germany and the Netherlands. Eitner (6) has presented data indicating that a significant reduction of hydrocarbon concentration is possible in approximately 1 week. Maximum removal rates were attained within 1 month of operation. He also has discussed the distribution of microorganisms in the biofilter. Ottengraf and van den Oever (7) have discussed the purification of waste gas containing toluene, butanol, ethyl acetate, and butyl acetate. The results show a zero-order dependence of degradation rate on concentration.

Don and Feenstra (8) have presented data comparing several alternative technologies for treatment of waste hydrocarbon gaseous streams and showed that biofiltration is the most cost-effective treatment method.

The soil bed biofilter systems mentioned above are inherently inefficient with respect to space utilization. One biofilter system, termed the BIKOVENT system, consists of prefabricated concrete parts that form an aeration plate to give uniform air distribution and drainage ducts. Developed by Drs. Hans Gethke and Detlef Eitner of Aachen, Germany, the BIKOVENT has been extensively used in Germany and Austria for odor control and for controlling VOCs in waste air streams. The technology was recently introduced to the United States by Biofiltration Inc., Gainesville, Florida.

A number of researchers have attempted to model biofilm processes since the 1970s. Few of the available models describe the situation of biofilm in three phase gas-liquid-solid systems where the compound undergoing degradation is present in the continuous gas phase. Further, none of the models are useful when the kinetic constants used in the rate expression are unknown.

This paper describes three experimental and theoretical studies conducted on the biodegradation of toluene, methylene chloride ($MeCl_2$), trichloroethylene (TCE), ethylbenzene, and chlorobenzene in air in aerobic biofilters.

MEASUREMENT OF BIOFILM KINETICS

A packed bed microbiofilter was set up to conduct kinetic experiments with immobilized biomass. The schematic of the experimental system is shown in Figure 1. A contaminant mixture in air was circulated through the packed bed microbiofilter (which was operated as a differential reactor) connected to the gas reservoir. Degradation of the substrate was monitored based on the substrate concentration in the gas reservoir. Mass balance equations were written for the system and the data obtained on the microbiofilter was analyzed. Figure 2 shows a typical experimental run for the system.

EXPERIMENTAL BIOFILTER STUDIES

The first study described the performance of a packed bed activated carbon biofilter (9). Complete degradation of toluene, methylene chloride, and TCE was demonstrated at the bench-scale at 2 minutes of gas retention time. After about 180 days of operation, the biofilter flooded due to excessive biomass accumulation and the carbon pellets had to be cleaned periodically. Figure 3 shows the removal efficiency of the biofilter as a function of time for the aerobic activated carbon biofilter.

The second study described the performance of a bench-scale packed bed biofilter with ceramic pellets (Celite, Manville Corporation, CA). Complete degradation of all compounds except TCE was achieved in our experiments. Figure 4 shows the removal efficiency of the compounds in the packed bed Celite biofilter.

The third study described the performance of a straight passages biofilter constructed from corrugated plates of Celite. The straight passages offer a means for excessive biomass to leave the biofilter bed, which does not occur in a packed bed system. Figure 5 shows the removal efficiency of the Celite straight passage biofilter for the compounds. The biomass loss from the straight passage biofilter is related to the flow rate of the nutrients, as shown in Figure 6.

Two models for a biofilter system have been developed that quantitate biofilm and plug flow regimes of biofilter operation. These models have been applied to the experimental data from the above three studies. Insights into biofilter design and operation gained from this analysis will be presented subsequently. That presentation will include a preliminary design procedure for biofilters and a comparison of costs with other technologies.

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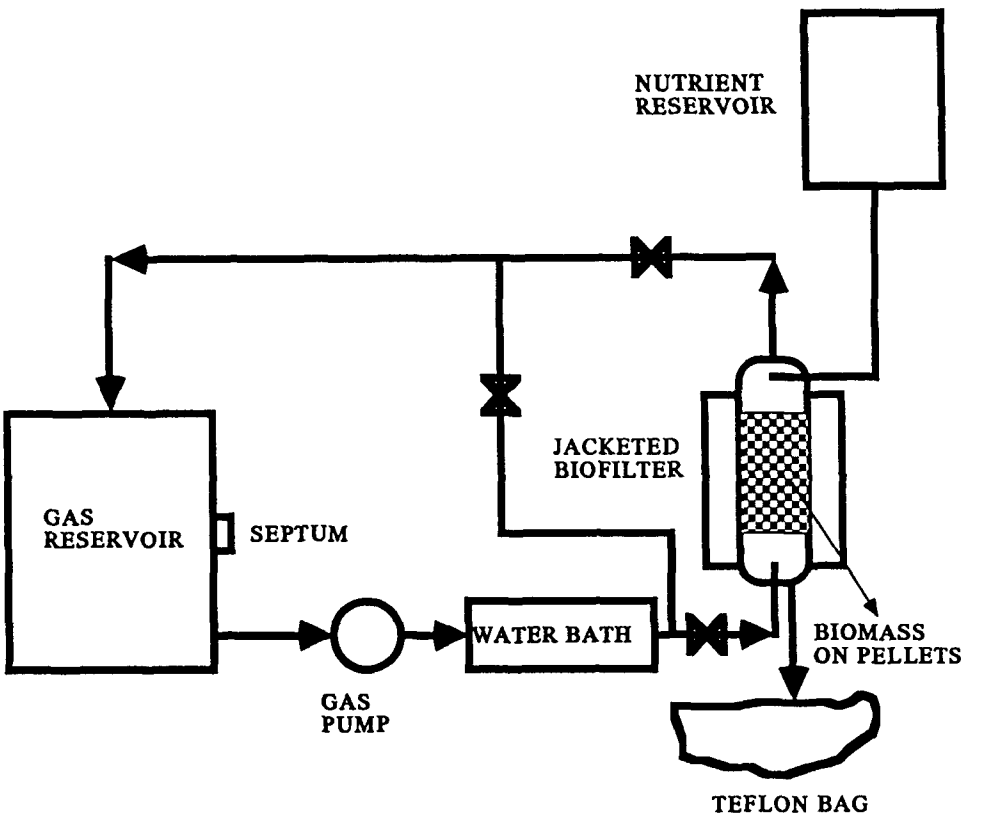

Figure 1. Schematic of the experimental apparatus for determining biofilm kinetics.

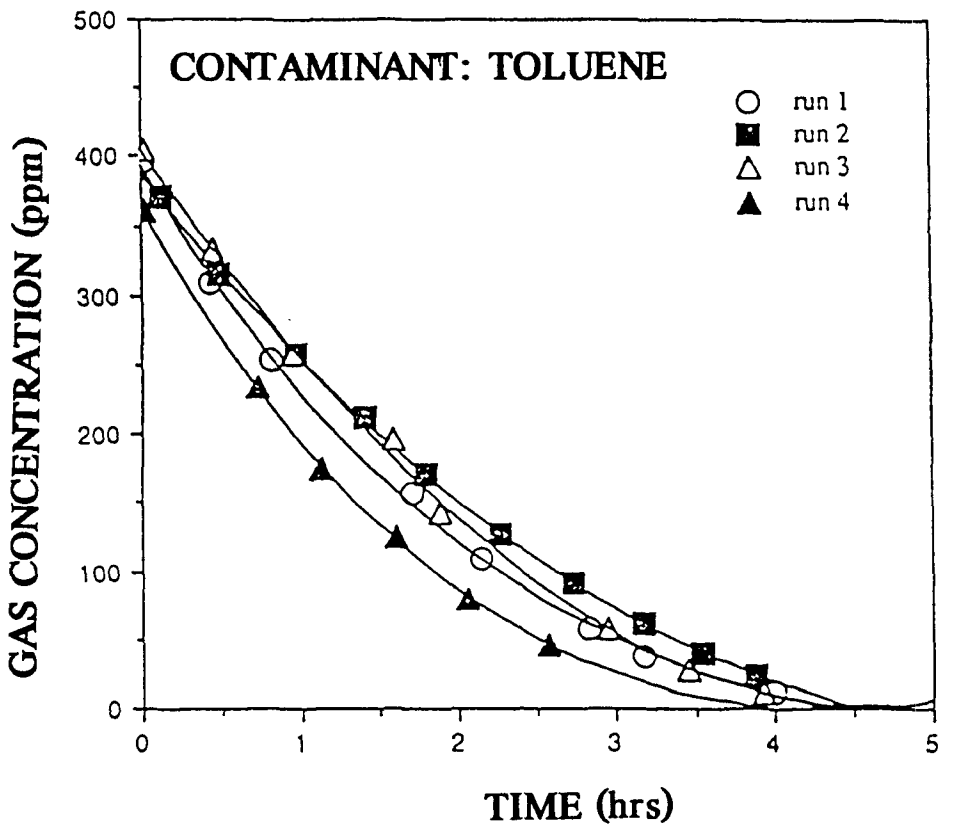

Figure 2. Experimental results on biofilm kinetics for toluene using the experimental apparatus shown in Figure 1.

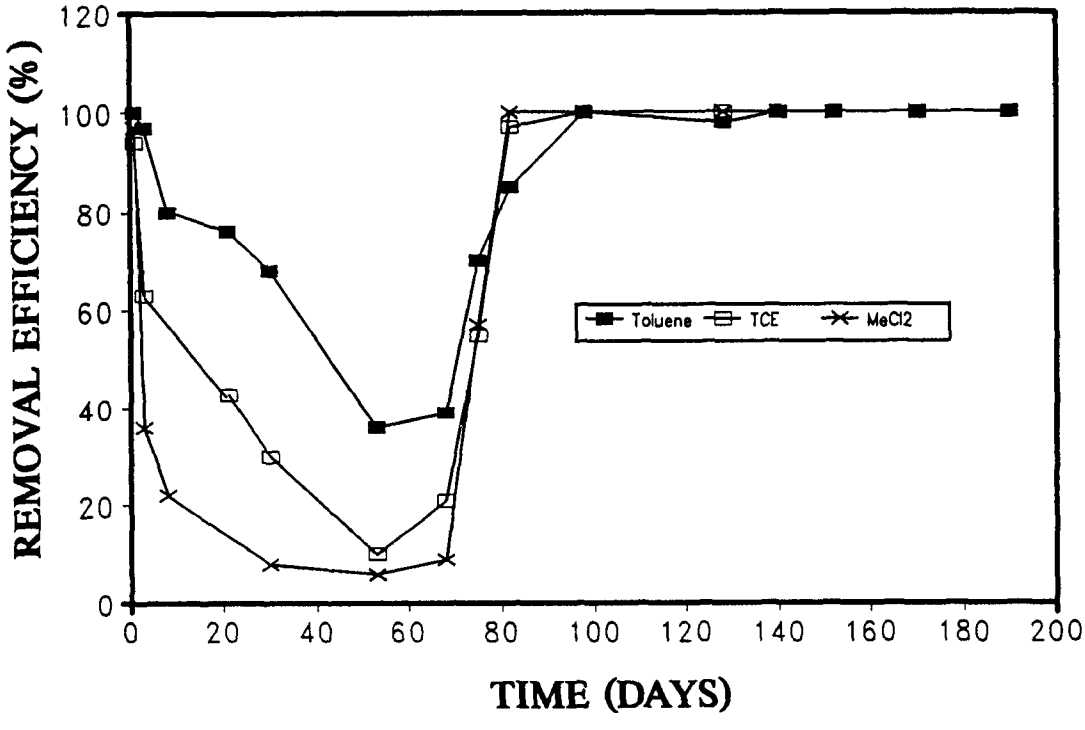

Figure 3. Experimental results on removal efficiency obtained for the activated carbon packed bed aerobic biofilter.

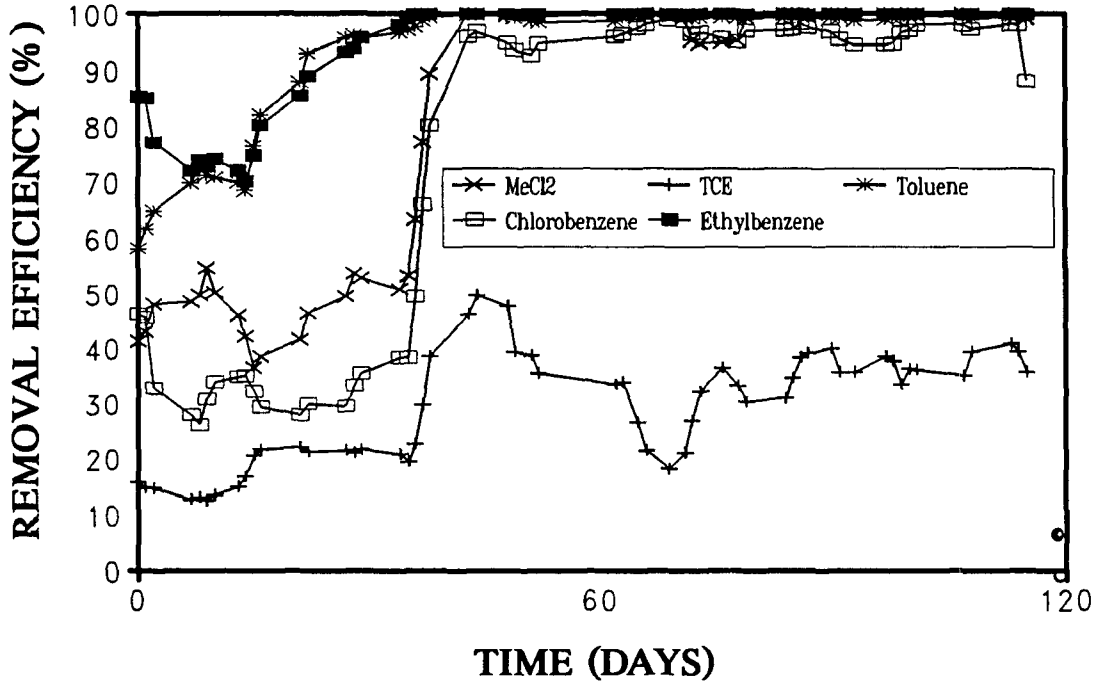

Figure 4. Experimental results on removal efficiency obtained for the Celite pellets packed bed aerobic biofilter.

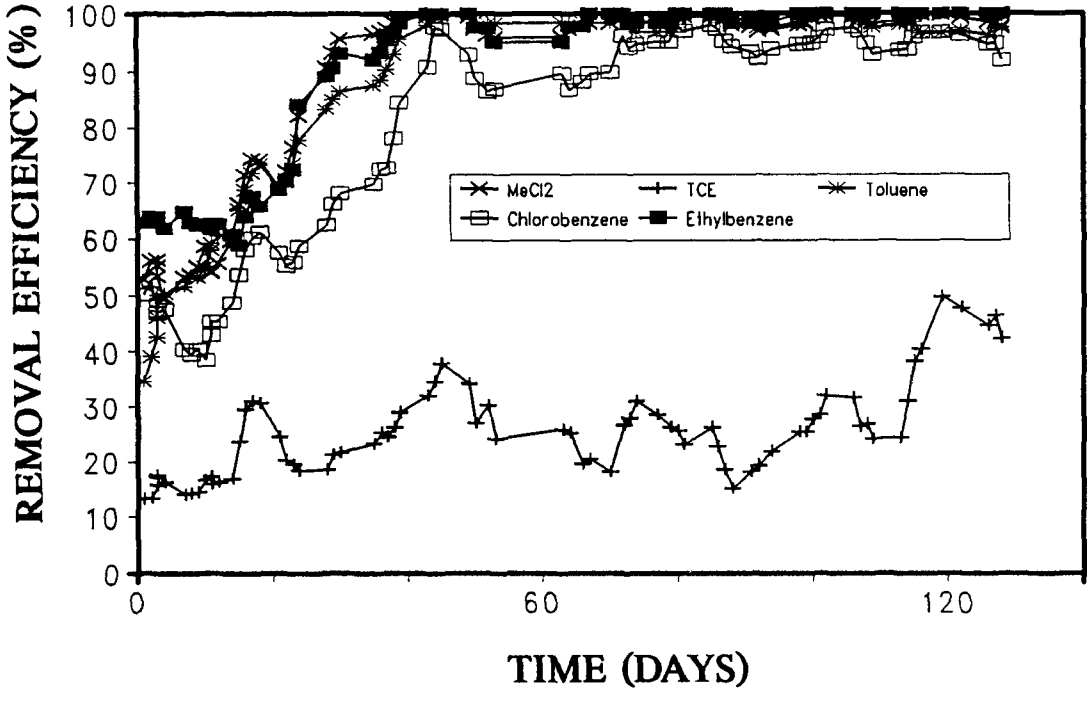

Figure 5. Experimental results on removal efficiency obtained for the Celite plates straight passages aerobic biofilter.

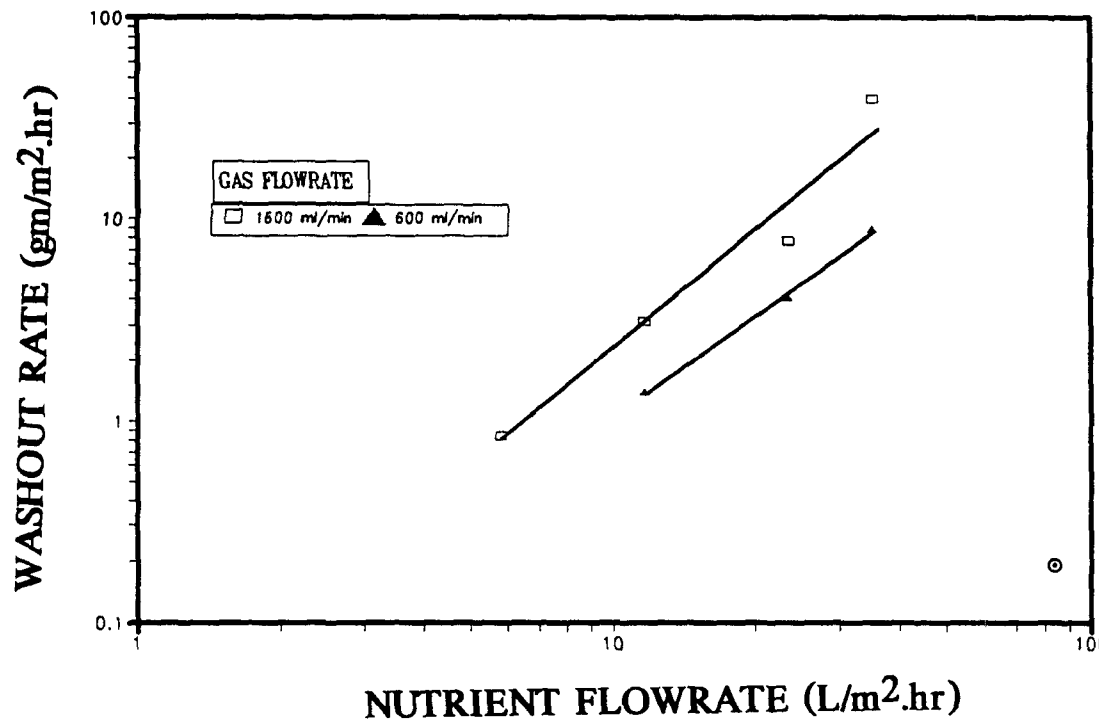

Figure 6. Experimental data on biomass slough off from the straining passages biofilter.

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SEQUENTIAL ANAEROBIC/AEROBIC TREATMENT OF CONTAMINATED SOILS AND SEDIMENTS

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INTRODUCTION

Every year many new Superfund sites are added to the National Priority List for extensive cleanup procedures. Feasible and low-cost technologies must be developed to expedite these procedures. One technology that meets these requirements is bioremediation (1), which uses naturally occurring microorganisms to convert hazardous substances into less-toxic or nontoxic substances.

The biodegradation rates of many highly chlorinated compounds can be accelerated by applying sequential anaerobic/aerobic treatment (2). 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. This scenario suggests that total degradation of the contaminants might occur when both anaerobic and aerobic treatments are sequentially applied.

The objective of this project is to conduct fundamental and applied research to aid in the development of sequential anaerobic-aerobic landfarming and composting technologies. These technologies will be used to biologically treat soils or sediments contaminated with highly chlorinated aromatic compounds and other low-solubility compounds that are susceptible to sequential treatment.

EXPERIMENTAL DESIGN

Methanogenic master culture reactors (MCRs) have been established to be used as a source of acclimated biomass for bench-scale biodegradation studies. The seed for the MCRs was anaerobically digested sewage sludge from a municipal wastewater treatment plant in the Cincinnati area. Ethanol is used as primary substrate to enrich methanogenic microorganisms. These reactors are kept under mesophilic conditions (35°C) in a controlled-temperature chamber. The reactors are fed semicontinuously with a mineral-salts medium and the contaminants of interest (Table 1). The ethanol loading rate is 1 g chemical oxygen demand/L/day. A computer-controlled respirometer (CES AER-200) is used to record daily gas production and track the performance of the MCRs.

Aerobic MCRs have been established by aerating a portion of the anaerobic MCRs at 20°C. These reactors are also fed semicontinuously using ethanol as the primary substrate. Anaerobic biodegradation products of the compounds listed in Table 1 will be used as co-substrates.

The rates and products of biodegradation will be measured in batch reactors under anaerobic and aerobic conditions using aqueous slurries of soils and sediments. The soil or sediment will be spiked with contaminant and transferred to serum bottles. Inoculum, co-substrate, and minimal salts media will be added, and substrate and product concentrations will be measured over time.

Table 1. Compounds of Interest

DDT
HEXACHLOROBENZENE
PENTACHLOROPHENOL
AROCLOR 1260 (PCBs)
NAPHTHALENE (PAHs)

RESULTS

Anaerobic MCRs that are acclimated to each of the compounds of interest are operating at steady-state. The accumulated gas production data for the DDT and hexachlorobenzene MCRs for a 6-month period are presented in Figures 1 and 2, respectively. The concentrations of the contaminants were increased until inhibition was experienced in the reactors as defined by loss of gas production capability. From these data, it was concluded that the optimal operating conditions for DDT and hexachlorobenzene were at a concentration of 2 mg/L.

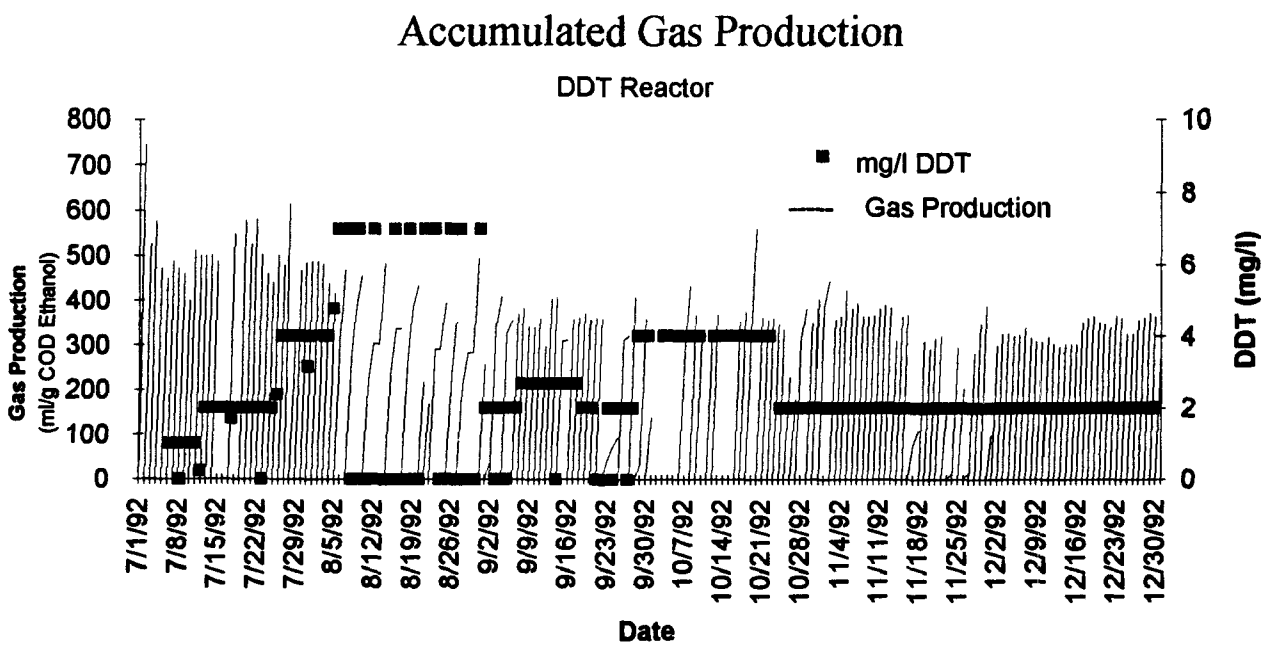

Figure 1. Gas production for anaerobic DDT reactor over a 6-month period.

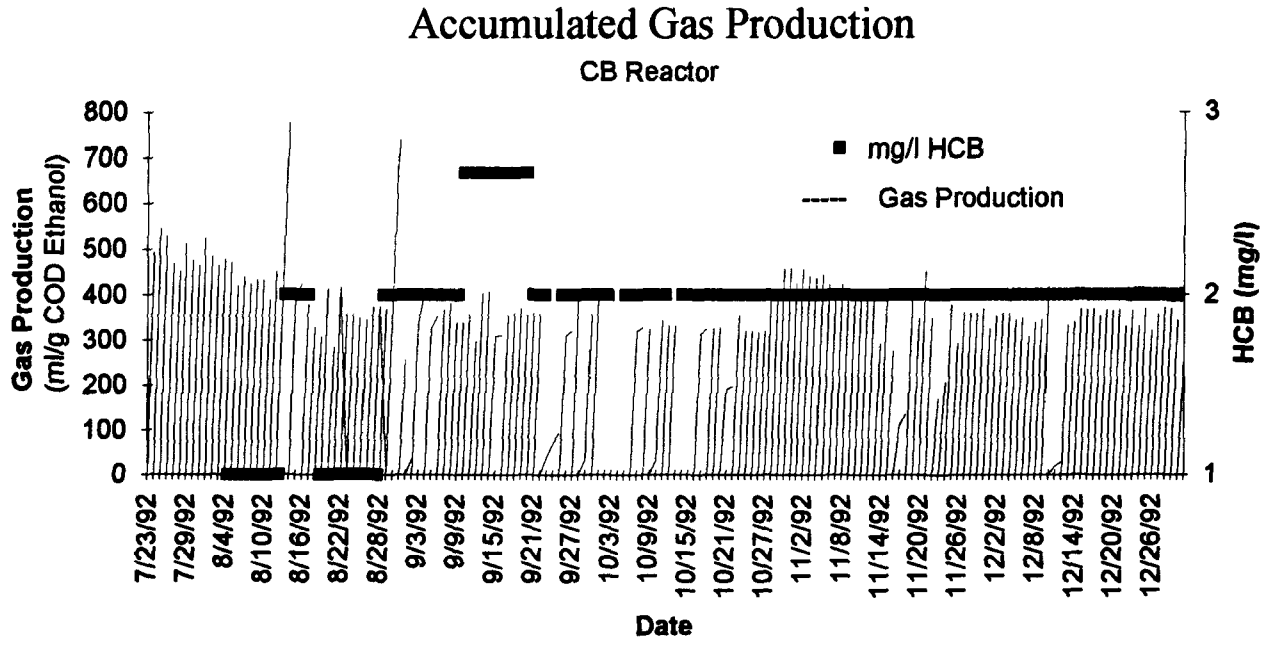

Figure 2. Gas production for anaerobic hexaclorobenzene reactor over a 6-month period.

CONCLUSION

The MCRs are operating at steady-state, and the biodegradation studies are in progress. These experiments will provide us with fundamental information on reaction kinetics that can be used to evaluate the feasibility of sequential anaerobic-aerobic treatment and to optimize its performance under field conditions. Further studies that will simulate field conditions more closely are planned.

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POSTER SESSION

APPROACHES TO THE DEVELOPMENT OF COMPARATIVE GENOTOXICITY RISK ASSESSMENT METHODS FOR EVALUATING HAZARDOUS WASTE CONTROL TECHNOLOGIES

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Environmental situations requiring remediation typically are present as mixtures of multiple toxicants and other pollutants. Most types of remediation efforts, including biological remediation endeavors, produce additional compounds that add to the complexity of evaluating the potential health effects of a contaminated site during and after remediation attempts. Likewise, when environmental applications of microorganisms are used for remediation purposes, the organisms and their products must be evaluated for safety.

Given the presence of multiple pollutants and the potential production of other pollutants by remediation processes, most of the actual toxicants within a typical remediation site are not identified. The Committee on Environmental Epidemiology of the National Research Council (1991) states, "There is evidence that NCP's [nonconventional pollutants] are a potentially important source of hazardous exposure. Some preliminary toxicologic studies suggest that NCP's have important biologic properties, environmental persistence, and mobility. . . . these unidentified substances represent risk of unknown magnitude." These NCP's, therefore, limit the ability to conduct risk characterizations of remediation sites when only analytical chemistry is used for exposure assessment studies.

By incorporating biological tests into assessment studies, it is possible to improve the estimations of potential human toxicant exposure before, during, and after remediation efforts. When appropriately coupled with analytical chemistry, bioassays also can be used to identify the major toxic pollutants. In addition, during the development of remediation methods, bioassays can be used for comparative assessments between differing technological approaches. Also, naturally occurring, mutant, and genetically engineered microorganisms have great potential for use in environmental remediation. Environmental releases, however, may result in direct human exposure to these organisms.

In the 1970s, using primarily *Escherichia coli*, researchers allayed public and scientific concerns that recombinant strains could survive and transfer genes for toxins to normal human microflora to a degree that would cause major health concerns. Because some microorganisms,

however, when present in large numbers can cause adverse health effects by other mechanisms, it is important to explore the potential health effects of environmentally released organisms (ERO). Recent efforts have examined competition and survival of EROs with normal human flora, their effect upon xenobiotic metabolism (environmentally and in vivo), and their effect on the competition and survival of pathogens. This poster presents the use of bioassays for comparative assessments of complex mixtures and introduces developing methods for the health assessment of microorganisms that are targeted for environmental release.

MODULATION EFFECTS OF BIOTECHNOLOGY MICROBIAL AGENTS FOLLOWING PULMONARY EXPOSURE OF MICE

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Endotoxin-resistant C3H/HeJ and endotoxin-sensitive CD-1 mice were challenged intranasally with environmental and clinical pseudomonads for evaluation of modulation effects (alteration of intestinal microbiota, translocation). At time intervals, the intestinal tract, spleen, liver, and mesenteric lymph nodes (MLN) were removed, homogenized, and plated for the enumeration of the dosed strain and/or resident microbiota. Environmental *Pseudomonas maltophilia* strain BC6, and *P. aeruginosa* strains BC16, BC18, and AC869, as well as clinical *P. aeruginosa* isolates DG1 and PAO1, were detectable in the intestinal tract 14 days following treatment. In CD-1 mice, *P. cepacia* strain AC1100 impacted the small intestinal anaerobic count, lactobacilli, and obligately anaerobic Gram-negative rods. Both strains AC869 and AC1100 modified the cecal obligately anaerobic Gram-negative rods and lactobacilli during the course of the experiment. Strain BC6 had an overall treatment effect on the cecal lactose negative enteric rods in C3H/HeJ mice. Strain BC18 increased the small intestinal lactose negative enteric rods, DG1 elevated the cecal lactose fermenting enteric rods, and BC16 increased the cecal anaerobic count. Strains BC16, AC869, and PAO1 translocated to the MLN, spleen, and liver during the experimental time. Strain BC18 was detectable in the liver at 3 hours following treatment, and strains BC17 and DG1 were recovered from the MLN and liver. No translocation was observed for strain BC6. Therefore, pulmonary treatment of mice with *P. aeruginosa*, *P. maltophilia*, or *P. cepacia* may alter the balance of the protective intestinal microbiota, which may cause further negative health effects such as multiplication of harbored pathogens, invasion by opportunistic pathogens, or translocation of bacteria to other organs. This abstract does not reflect EPA policy.

FIELD TREATMENT OF BTEX IN VADOSE SOILS USING HYDROFRACTURING, VACUUM EXTRACTION, AND BIOFILTERS

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INTRODUCTION

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

EPA's Risk Reduction Engineering Laboratory (RREL), in cooperation with the University of Cincinnati (UC), is developing engineering systems to bioremediate fuel-contaminated vadose soils. These systems include in situ bioventing of soil and consolidated soils, a system consisting of hydrofracturing and vacuum extraction to transfer volatile organic compounds (VOCs) from the soils to air, followed by air biofiltration to mineralize the extracted VOCs in the contaminated air.

BACKGROUND

This presentation describes a planned field demonstration of the hydrofracturing, vacuum extraction, and air biofiltration system. The hydrofracturing of consolidated vadose soil is achieved when fluid is pumped down a borehole until a critical pressure is reached that fractures the soil. Sand-laden slurry is then pumped into the fracture to create highly permeable pathways in the soil. The resulting horizontal sand lenses, at vertical separations as close as 15 cms apart, produce improved overall soil permeability for vacuum extraction of fuel hydrocarbons from the consolidated

soil. The extracted air contaminated with the fuel VOCs may then be treated in air biofilters to biodegrade the contaminant VOCs.

RREL and UC are developing improved biofilters to control VOC emissions for RREL's Superfund research program. The filter designs employ pellets or "straight-passages" support media, as shown in Figure 1. The VOC emissions in contaminated air passing through the biofilter are biodegraded by microorganisms attached on the surface of the media. A nutrient solution is recycled through the biofilter to support the microorganisms. Research on improved biofilters reveals efficient removal of biodegradable VOCs and a high degree of mineralization.

FIELD DEMONSTRATION

The site for the field demonstration has not yet been selected but is likely to be an Air Force base in Ohio. At the selected site, consolidated vadose soil contaminated with jet fuel VOCS will be hydrofractured by a RREL/UC team using the vacuum extraction equipment installed to permit air extraction of the VOCs. Hydrofracturing increases the overall soil permeability by nearly two orders of magnitude. Since a single horizonal sand lens, from fracturing with a radius of 7 m, typically provides approximately 0.1 m^3/min of extracted air, three horizontal sand lens (7-m radius) will be used to provide 0.28 m^3/min of contaminated air for biofilter treatment.

The field test will use two biofilters: one packed with approximately 1 meter (height) of porous ceramic pellets (6 mm avg. dia.), the other with approximately 1 m (height) of porous ceramic straight-passages media (127 passages/cm^2). Each biofilter will provide 2 min of empty bed contact time within the media at an air flow of 0.14 m^3/min. Steam and water will be used to raise the temperature of the extracted air to approximately 25°C and to prehumidify the air.

The biofilters will be constructed at EPA's Test and Evaluation (T&E) Facility in Cincinnati. The system will include continuous gas chromatography for automatic monitoring of appropriate air streams to characterize biofilter performance. The biofilm on the support media will be preacclimated to jet fuel hydrocarbons at the T&E Facility. The skid-mounted biofilters with acclimated biofilms will be transported to the site for connection to the hydrofracture/vacuum extraction systems. The performance of the integrated system will be characterized for approximately 3 months.

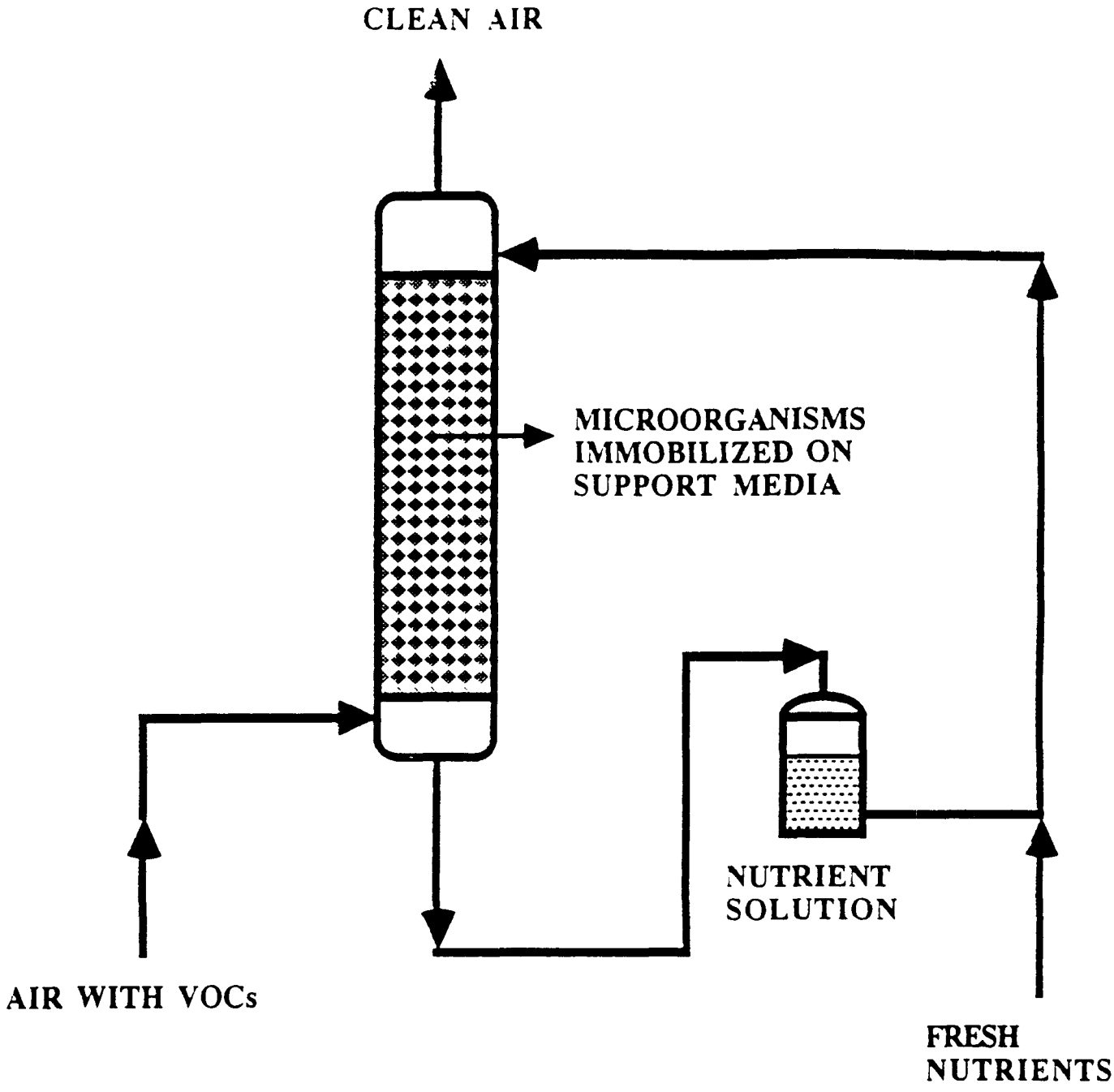

Figure 1. Schematic of the biofilter system.

PCB BIODEGRADATION DURING AEROBIC TREATMENT OF SLUDGE FROM THE FRENCH LIMITED NPL SITE

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INTRODUCTION

French Limited is a Superfund site near Houston, TX. The site is a sand pit that was used for disposal of chemical waste, primarily polycyclic aromatic hydrocarbons (PAHs), from API separator sludge, and polychlorinated biphenyls (PCBs), from discarded transformers. A technology vendor at the French Limited Superfund Site had claimed that PCBs codisposed with API separator sludge were degraded during aerobic biodegradation of the petroleum hydrocarbons. The concentration of PCBs in the sludge was reduced by a factor of 10, and the sludge volume was reduced by a factor of 10.

Aerobic biodegradation of the lower chlorinated PCBs in the presence of PAHs, in particular biphenyl, has been demonstrated, but the degradation of higher chlorinated species is more difficult (1,2,3). Therefore the confirmation of the observed PCB degradation in a controlled laboratory experiment was desirable. A laboratory experiment was proposed that would determine quantitatively the changes in the sludge under highly aerobic conditions. The purpose of this experiment was to determine if the vendor's claims of aerobic biodegradation of the PCBs under highly aerobic conditions were valid.

Although the aerobic conditions were maintained for 1 year and significant degradation of the organics in the aerobic reactor, measured as total petroleum hydrocarbons (Table 1), was observed, the total PCB concentration was not significantly affected (Table 2). Only certain of the lower chlorinated PCB congeners were significantly degraded under aerobic conditions, specifically BZ-7/9, BZ-6, and BZ-8/5 (Table 3). These congeners were dichlorobiphenyls. Figures 1 and 2

Table 1. Total Petroleum Hydrocarbon (TPH) Degradation

TPH (mg/L)	AVG.	SD (n = 3)
Start	18,919	5,944
Final		
Anaerobic	18,586	864
Aerobic	6,939	369

Table 2. Polychlorinated Biphenyl Depletion (mg/L of PCBs in Mixed Liquor; Average of Duplicate Samples)

	Aerobic	Anaerobic
Initial	18.5	15.9
Final	14.5	14.8
Depletion (%)	-21.9	-6.8

Table 3. Changes in Concentration of Polychlorinated Biphenyl Congeners during Incubation under Aerobic and Anaerobic Conditions

	Aerobic		Anaerobic		Reduction (%)	
	Before	After	Before	After	Aerobic	Anaerobic
BZ-10/4	2.80	3.01	2.94	2.45	-7.6	16.9
BZ-7/9	0.30	0.00	0.38	0.39	100.0	-2.9
BZ-6	0.48	0.14	0.65	0.65	70.7	0.1
BZ-8/5	2.81	0.49	3.67	3.70	82.4	-0.8
BZ-31	2.56	2.41	2.54	2.57	5.8	-1.2
BZ-28	3.26	3.37	3.28	3.36	-3.3	-2.5
BZ-33/53	4.22	3.35	4.21	4.26	20.5	-1.2
BZ-52	4.68	5.20	4.48	4.60	-11.2	-2.6
BZ-44	2.67	2.87	2.57	2.63	-7.5	-2.6

Note: Concentrations expressed as percent of total PCBs.

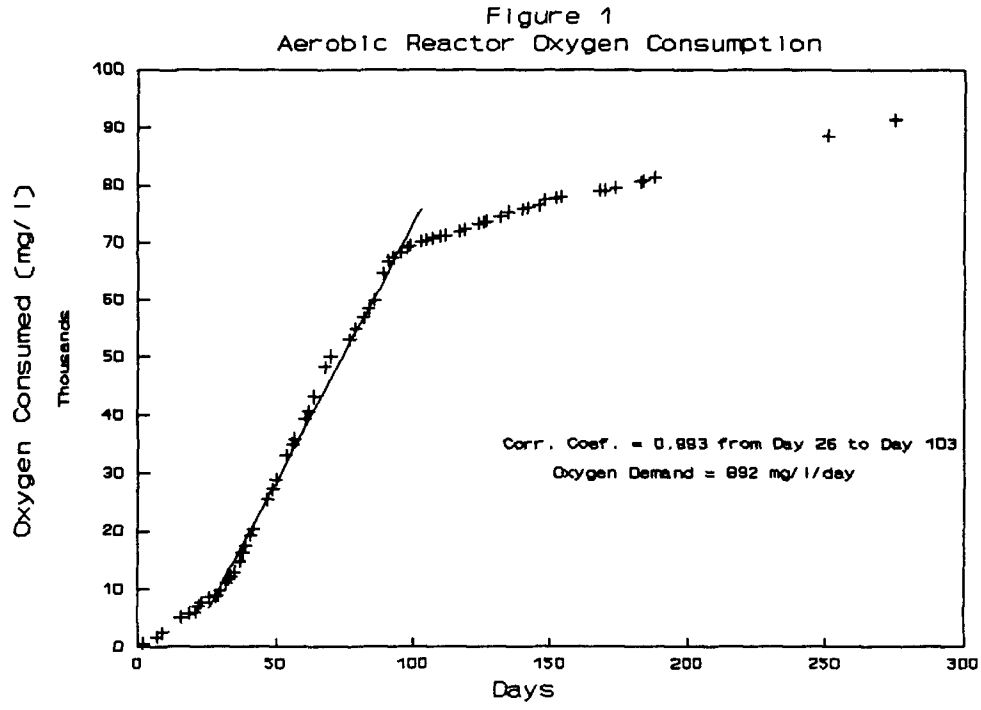

Figure 1. Aerobic reactor oxygen consumption.

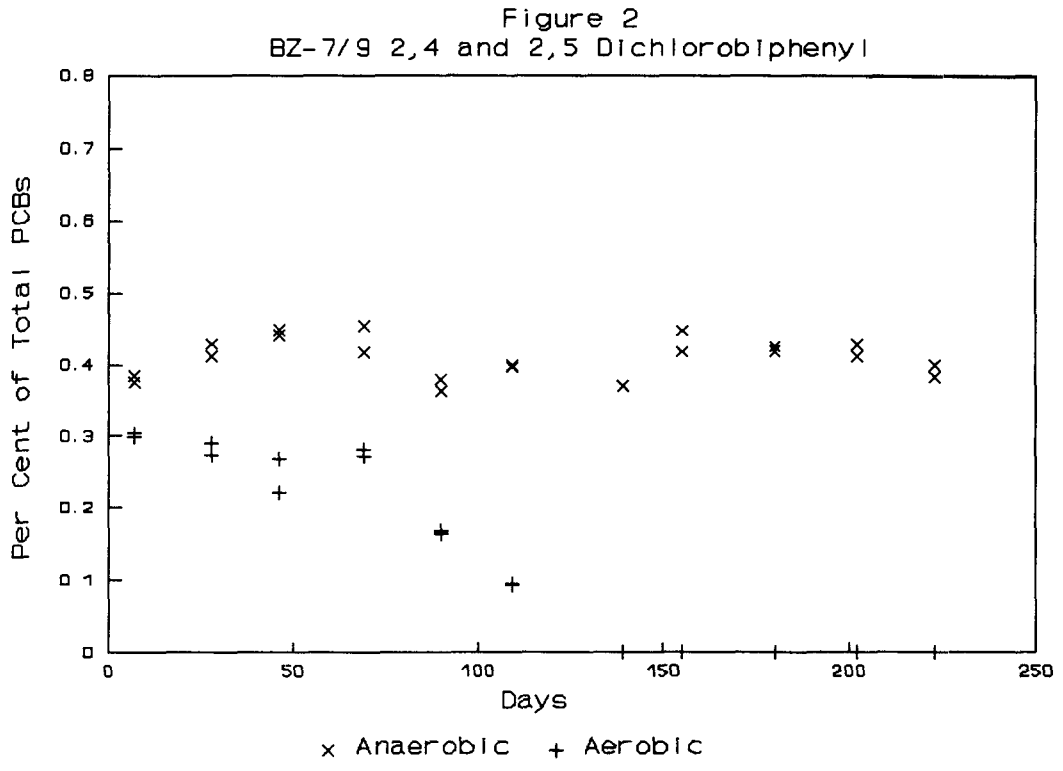

Figure 2. BZ-7/9 2,4- and 2,5-dichlorobiphenyl.

illustrate that the consumption of these PCB congeners occurred after biological activity had decreased. No congener was significantly degraded under anaerobic conditions (Table 3).

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DEVELOPMENT AND EVALUATION OF COMPOSTING TECHNIQUES FOR TREATMENT OF SOILS CONTAMINATED WITH HAZARDOUS WASTE

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This research investigates the potential for composting systems to remediate soils contaminated with organic pollutants. Significant progress in optimizing conditions and applying the power of biotechnology to large-scale soil compost systems will require a working understanding of the processes and mechanisms involved. Current commercial compost operations constitute black-box systems where optimization is approached on a hit-or-miss basis using scant process-related information. This research is designed to characterize physical and microbiological changes during composting, identify the stage of the composting process that yields the greatest destruction of contaminants, and determine mass balances of pollutant biotransformation.

The closed-vessel bench-top compost reactor design for this project was selected from many options to mimic a static compost pile. Considerable project design effort was invested in identification of materials-handling equipment and optimization of materials-handling operations. Since toxic soils will be handled during reactor loading, dust management procedures were carefully developed to eliminate spread of contaminants.

The composter consists of a modified 55-gal drum with five thermal wells extending 9 in. into the reactor core. Since heat production may be highly variable throughout the reactor, thermal wells are vertically spaced 5 in. apart to allow temperature measurements at various depths of the compost mixture. A screen located 4 in. above the reactor bottom supports the compost pile, and air forced through a 1-in. inlet port near the reactor bottom flows upward through the mixture. Increased air flow can be used to cool the reactor and help control compost temperature during thermogenesis. A top sample port enables collection of compost core samples for analysis. A draw is provided on the bottom to permit removal of excess liquid and collection of leachate samples for chemical analysis. The reactor is tipped on its side and rolled on a drum roller every 24 hours to mix the compost and rupture anaerobic pockets. Based on a prototype reactor investigation, a field of seven stainless steel reactors will be constructed for a treatability study using soil contaminated with creosote components.

Investigation of optimal composting conditions for destruction of soil contaminants involves determination of suitable soil types, moisture content, co-compost materials (e.g., wood chips,

sawdust, corn cobs), and nutritional adjustments (e.g., carbon/nitrogen ratio). The study also seeks to identify metabolically active microbial species and possible mechanisms of hazardous chemical transformation. Since it is difficult to trace all metabolites produced during the compost process, the end product will be bioassayed to assess the extent of detoxification by compost microorganisms. The finished compost soil will be evaluated for toxicity by Ames Salmonella mutagenicity, earth worm toxicity, and seed germination tests.

ENGINEERING OPTIMIZATION OF SLURRY BIOREACTORS FOR TREATING HAZARDOUS WASTES IN SOIL AND SEDIMENTS

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The operation and performance of soil slurry bioreactors are not well understood despite widespread use of these reactors in the field. This project's purpose is to enhance understanding of the factors controlling slurry bioreactor operation and performance using fundamental mechanical and treatability studies at bench and pilot scale. A systematic plan to evaluate slurry bioreactors and the required parameters for their optimal use is being developed by this research program. The information gained from these studies should be useful in improving treatment effectiveness in field applications.

Current research efforts are centered on developing and selecting suitable bench- and pilot-scale bioreactor designs and on preparing soil slurry. The bench-scale bioreactor selected is an original design, conical in shape with a round bottom. Three sample ports are built on one vertical side. The entire unit is airtight with four access ports configured on the lid to allow sealed entry of the air inlet and outlet tube and impeller shaft. The impeller is driven by a variable-speed motor. The fourth port on the lid is larger than the other ports and is used for slurry charging. Soil slurry preparation includes screening, preslurrification, soil particle size selection (by hydrocyclone), and treatment evaluation in either the bench- or pilot-scale bioreactors.

Bench-scale slurry reactors are being used to develop a more complete understanding of the physical, chemical, and biological factors controlling optimal operation. Bench- and pilot-scale reactors will yield information on solids loading methods, optimal residence times, optimal solids loading as a function of soil type, pollutant mass balances, and effectiveness of nutrient and microorganism additions. As bench-scale bioreactor performance information becomes available, it will be used to configure the larger pilot-scale bioreactors.

BIOTREATABILITY OF A VADOSE ZONE SOIL CONTAMINATED WITH DIOCTYL PHTHALATE

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Chemical spillage and waste disposal during 40 years of manufacturing vinyl wallcovering had contaminated portions of a field site with various organic chemicals, predominately xylene and dioctyl phthalate. Laboratory treatability studies were conducted on core samples obtained from the site. The objective was to determine whether natural biodegradation processes would remediate the site. Nine core samples were collected at three locations in 3-ft increments from site locations of low contamination, moderate contamination, and high contamination. Analysis of a nine-sample composite showed that 95 percent of organic compound contamination was dioctyl phthalate. All core samples contained sufficient nitrogen and phosphorus to support viable soil microbiological processes. Bacterial cell counts ranged from 7×10^8 to 53×10^8 cells/g for all cores, but were highest in moderately contaminated samples. The same trend was apparent with dehydrogenase activity. Dioctyl phthalate concentration means were 6, 101, and 333 mg/kg soil for low, moderate, and high contamination, respectively.

Equal amounts of core material on a 32 g dry-weight basis were contained in capped, air-tight 160-mL glass bottle microcosms. Consumption of headspace oxygen and carbon dioxide generation was measured during a 24-day, 22°C incubation period. Total mean oxygen consumption was 10, 77, and 66 percent of the 20.9 percent oxygen in headspace air for low, moderate, and high contamination, respectively. Headspace carbon dioxide for the same microcosms was 1.6, 12.3, and 13.1 percent. The rate of dioctyl phthalate biodegradation in laboratory microcosm bottles was approximately 0.1 g/kg soil/day for the contaminated cores. Extrapolating from laboratory data rates and assuming ideal field conditions, the predicted total time to naturally cleanse the site to less than 10 ppm phthalate would be 3 years and 10 years, respectively, for the moderate and high contaminated areas. Biodegradation of dioctyl phthalate was enhanced by the presence of xylene in a separate microcosm study.

INNOVATIVE BIOREMEDIATION STRATEGIES FOR CREOSOTE: GEOGRAPHIC DIVERSITY OF PAH DEGRADATION CAPABILITIES AT WOOD-TREATING SITES

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INTRODUCTION

The use of specially selected microorganisms to enhance bioremediation efforts has proved effective in a number of applications, especially when combined with bioreactor systems. The successful use of such isolates for the remediation of soil and water contaminated with organic wood preservatives (e.g., creosote and pentachlorophenol [PCP]) has resulted in the opportunity to employ these technologies at similarly contaminated sites throughout the world. Prior to worldwide dissemination of bioremediation strategies, however, concerns regarding the introduction of foreign biota had to be addressed. Therefore, a research program was initiated to ascertain: 1) whether microorganisms similar to those used in previous bioremediation strategies could be found in other soils, and 2) if so, whether the introduction of these isolates would offer any advantage to the bioremediation system.

MATERIALS AND METHODS

To address these issues, soils contaminated with polycyclic aromatic hydrocarbons (PAHs) were collected from creosote-contaminated sites in Norway, Germany, and the United States and screened for the presence of bacteria capable of utilizing phenanthrene (PHE) or fluoranthene (FLA) as a sole source of carbon and energy. Two soils from farmland in south-central Illinois, with no known history of PAH exposure, were also surveyed. Soil slurries (10 percent w/v) were prepared with 500 mg/L PHE or FLA in a sterile mineral salts medium (1) and incubated in the dark with shaking (150 rpm) at 30°C for 14 days. Following 14 days of aerobic incubation and enrichment,

cultures were diluted 1:5 (v/v) with fresh mineral salts medium containing PAHs. This transfer procedure was repeated two more times for a total of four enrichments over a 10-week period.

At selected time points, liquid samples were removed from each vessel and screened for the presence of bacteria capable of using PHE and FLA as primary growth substrates. Once individual colonies were purified, they were transferred to carbon-free mineral salts agar plates and complex agar plates, then overlaid with PHE or FLA. Plates were incubated for 14 days prior to scoring for individual colonies exhibiting zones of clearing of the PAH substrate. Colonies demonstrating PAH-clearing abilities were purified and transferred to 125 mL Erlenmeyer flasks containing 25 mL mineral salts broth plus 500 mg/L PHE or FLA. Cultures were incubated for 5 to 7 days with shaking (150 rpm) at 30°C. Bacterial growth at the expense of the PAH substrates was measured visually or by monitoring changes in absorbance at OD_{550}nm. As a control, growth in carbon-substrate free mineral salts broth was also monitored.

Once the PAH-degrading ability of purified cultures was validated, cultures were characterized by fatty acid profile analysis (GC-FAME) and substrate utilization patterns (Biolog Microplate System). The taxonomic relationships among these strains was analyzed by evaluating similarity measures from GC-FAME and substrate utilization patterns with principal component analysis (2). DNA/DNA hybridizations and 16S rRNA sequence comparisons also were performed to determine the phylogenetic relationships among the recovered isolates according to published methods (3-6).

In parallel studies, microbial respirometric responses (rate of liberation of carbon dioxide and the simultaneous consumption of oxygen) upon exposure to the following carbon sources were measured: 1) 500 mg/L naphthalene (NAH), PHE, or FLA; 2) 500 mg/L readily utilizable carbon (250 mg/L glucose + 250 mg/L glycerol); or 3) 500 mg/L specification creosote No. 450 (American Wood-Preserver's Association). Responses were recorded at 8-hr intervals over an 8-day incubation period (23°C, 100 rpm shaker speed) with a MicroOxymax™ respirometer (Columbus Instruments, Columbus, Ohio). These responses were compared to those observed upon treatment with no supplemental carbon and killed-cell controls (acidified to pH 2.0 with 1 N hydrogen chloride plus 3.7 percent formaldehyde) to discern the effect of nutrient amendment and aeration. At the end of each incubation period, slurries from nutrient-amendment-only, creosote-amended, and killed-cell (control) treatments were extracted and analyzed for the presence of creosote constituents as previously described (7). These values were compared with those determined at time zero with each soil.

DATA SUMMARY

Soils with previous PAH exposure possessed elevated numbers of PAH degraders that corresponded with increased respiratory activity and enhanced PAH biodegradation over uncontaminated soils. Following enrichment with PHE or FLA in soil slurries, only soils with known PAH contamination yielded bacteria that grew at the sole expense of these PAH substrates. Thus, all soils contaminated with PAHs harbored indigenous bacteria competent for PAH-degradation, but soils with no prior exposure to PAHs did not respond to the addition of these potential substrates.

The location of isolated strains and the corresponding PAH substrate are summarized in Table 1. Physiological and biochemical analyses of isolated bacteria showed that the PAH-degrading bacteria that recovered from PAH-contaminated soil via the specified techniques appeared to be related phylogenetically. For example, all soils with a history of PAH exposure harbored bacteria

Table 1. Bacteria Isolated from PAH-Contaminated Sites for Their Ability to Degrade Phenanthrene or Fluoranthene

Isolate Number	Culture Number	Source/ Reference	Enrichment Substrate	Soil of Origin
1	EPA505	(10)	fluoranthene	Pensacola, Florida
2	PJC 2282	GBERL	fluoranthene/pyrene	Pensacola, Florida
3	PJC 2283	GBERL	fluoranthene/pyrene	Live Oaks, Florida
4	PJC 2285	GBERL	fluoranthene	Live Oaks site, Florida
5	PJC 2286	GBERL	fluoranthene	Live Oaks site, Florida
6	PJC 2287	GBERL	fluoranthene	Pensacola, Florida
7	PJC 2288	GBERL	phenanthrene	Pensacola, Florida
8	PJC 2289	GBERL	phenanthrene	Live Oaks, Florida
9	PJC 2295	GBERL	phenanthrene	Pensacola, Florida
10	CRE7	(1)	phenanthrene	Pensacola, Florida
11	CRE11	(1)	phenanthrene	Pensacola, Florida
12	CRE12	(1)	phenanthrene	Pensacola, Florida
13	AK Phen6	(11)	phenanthrene	Prince William Sound, Alaska
14	N1F1	This Study	fluoranthene	Rade, midt pa bygg, Norway
15	N2P5	This Study	phenanthrene	Rade, ved kum, Norway
16	N2P6	This Study	phenanthrene	Rade, ved kum, Norway
17	N3P2	This Study	phenanthrene	Lillestrøm, karusell, Norway
18	N3P3	This Study	phenanthrene	Lillestrøm, karusell, Norway
19	N3F1	This Study	fluoranthene	Lillestrøm, karusell, Norway
20	N3F2	This Study	fluoranthene	Lillestrøm, karusell, Norway
21	N4F4	This Study	fluoranthene	Lillestrøm, prøvebrønn, Norway
22	N5F4	This Study	fluoranthene	Drammen, Norway
23	N6F4	This Study	fluoranthene	Hommelvik, ved tønner, Norway
24	G1F1	This Study	fluoranthene	Germany
25	G1F2	This Study	fluoranthene	Germany
26	G1P1	This Study	phenanthrene	Germany
27	G2P2	This Study	phenanthrene	Germany
28	FDA PYR-1	(12)	pyrene	Port Aransas, Texas
29	SR3	(13)	pentachlorophenol	northwest Florida

competent for PAH biodegradation, and principal component analysis of GC-FAME data showed relatedness between these strains (Figure 1). The strategy employed to enrich PAH-degrading bacteria thus appeared to select similar types of microorganisms that are indigenous to contaminated soils at each site. Hence, the export/import of these non-indigneous bacteria to augment bioremediation efforts would not seem to represent the introduction of exotic biota.

From a PAH biodegradation perspective, the strategy of relying exclusively on indigenous PAH degraders in bioremediation efforts needs to be closely evaluated for its ability to achieve site-specific cleanup standards in a timely manner. If the stimulatory effect of controlled nutriation, mixing, and aeration on the activity of the indigenous microflora results in acceptable rates and extents of biodegradation of targeted chemicals, then, on a site-specific basis, it may be possible to rely solely on the activity of such microorganisms to facilitate site remediation (8,9). Alternatively, utilization of non-indigenous microbes in optimized bioremediation systems could be advantageous for cost-efficient, effective bioremediation, while posing no discernible ecological risk.

ACKNOWLEDGMENTS

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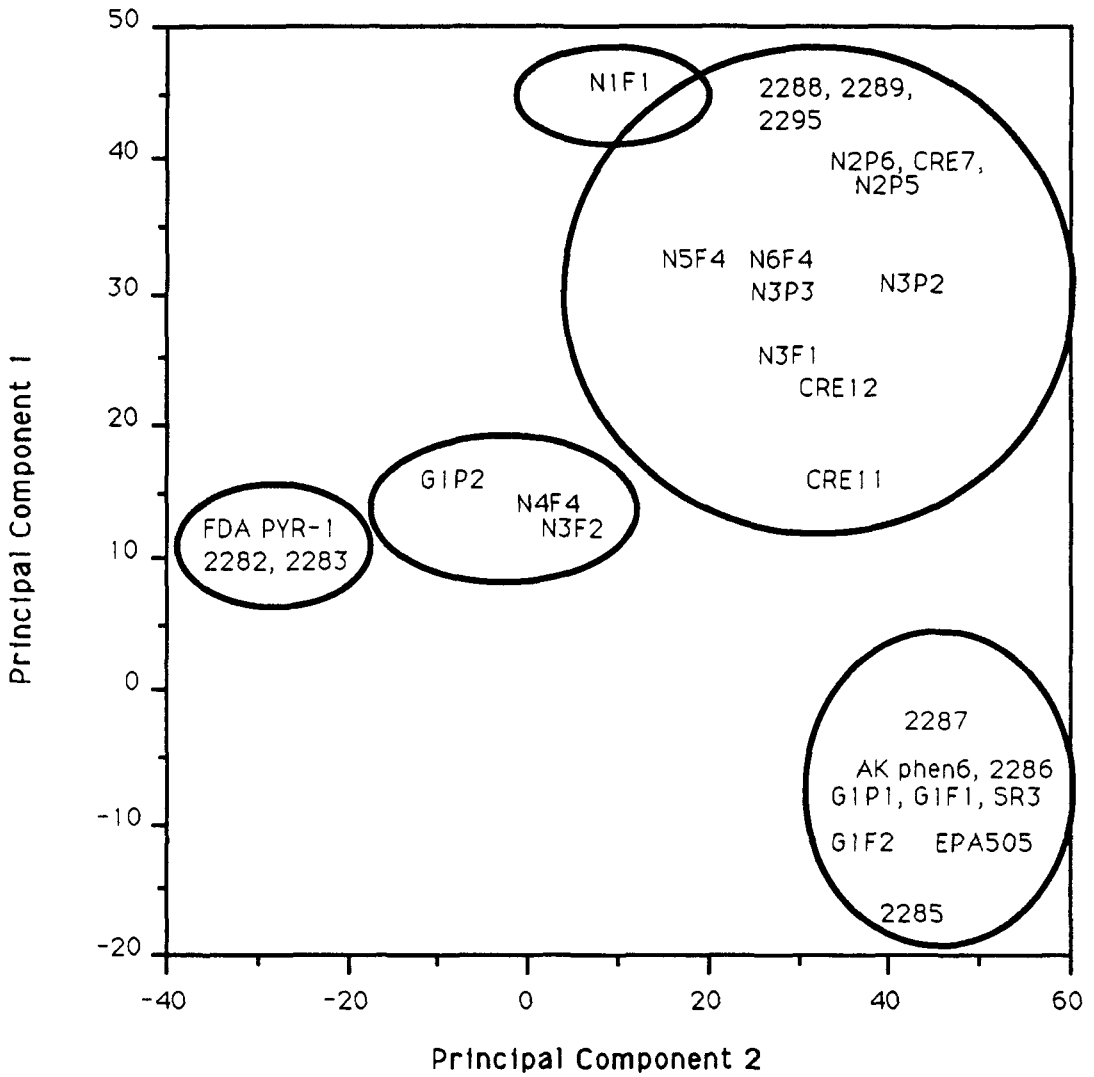

Bacterial similarity measurements using principal component analysis from GC-FAME data (see Table 1 for soil of origin and PAH enrichment substrates).

Figure 1. GC-FAME principal component analyses.

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DESIGN OF AN EXPERT SYSTEM TO SELECT AN APPROPRIATE BIOREMEDIATION TECHNIQUE

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INTRODUCTION

This project is developing a decision-making format that can be used by individuals interested in determining the appropriate bioremediation technologies to remediate soils contaminated by spills of petroleum products. The resulting information can be used for emergency response and remediation decisions. The project will integrate available information that the user can understand and utilize in a short time. The output will provide guidance to identify appropriate bioremediation action options for a specific situation. Thus, the project output will meet the following objectives: a) advance the understanding of applying bioremediation to specific problems, b) enhance the use of bioremediation through performance evaluations and technology transfer, c) evaluate the use of natural soil processes for treatment of hazardous wastes, and d) address informational impediments that constrain the use of new technologies.

The project will focus on in situ and ex situ bioremediation approaches that can be used for unsaturated soils. The technologies considered in this project are:

In Situ	Ex Situ
Bioventing	Slurry reactor treatment
Air sparging	Prepared bed treatment
Land treatment	Aerated pile treatment
Passive remediation	

As used in this project, petroleum spills are understood to consist of spills of gasoline, diesel, JP-4 fuel, processed and crude oils, and other raw, partially processed, and refined petroleum materials.

The anticipated users of the project output are expected to be a) emergency response team personnel, b) onsite coordinators, c) regional and state project managers, d) industry personnel, e) consulting engineers, and f) educational organizations interested in training students and others in need of the information.

BIOREMEDIATION

Bioremediation is the controlled application of the naturally occurring process of biodegradation. The process has been recognized as a relatively inexpensive and efficient method for removing organic chemicals from contaminated soils.

Despite the application of biological processes to many waste treatment situations, the wider use of bioremediation for contaminated soils continues to be limited by a general lack of understanding of the fundamentals that are involved and the processes that can be used. To date, each application has been, in many respects, an isolated occurrence that is unable to benefit from past experience and unable to provide guidance for future use. In recent years, however, both the relevant fundamentals for use with contaminated soils and the technical processes that can be applied in bioremediation have become more widely understood. Such knowledge will be used in this project.

Bioremediation processes have been used for soils that have contained a) petroleum hydrocarbons such as gasoline, diesel fuel, crude oil, and creosote; b) pesticides and their derivatives, c) chlorinated solvents such as methylene chloride, and d) hydrocarbons such as pentachlorophenol, naphthalene, and anthracene. An increasing number of sites are being remediated and closed using bio-processes, magnifying the need for an expert system.

PROGRAMMING LANGUAGE AND FORMAT

The expert system will be designed for a personal computer format. The system will be available for use by the audience discussed above.

The computer language used in the development of the expert system had to meet three criteria. First, it had to be in use throughout the country and not require a substantial financial investment or be difficult to set up. This requirement would ensure a wide user base. Second, the language had to be easy to upgrade and edit. This would allow for updating of the program to stay up to date with the changing field of bioremediation. Third, the language had to have superior graphics capabilities. This would be needed to effectively communicate the various bioremediation techniques and illustrations that would accompany text. The language that directly meets all three criteria is Hypercard, a language developed for information communication that is standard on all Macintosh systems.

The format of the program will contain three main branches:

Branch 1 Explanation of techniques
Branch 2 Description of illustrating methods, with examples
Branch 3 Analysis of the user's situation

The first branch will allow the user to become familiar with the various bioremediation techniques. It will include a written explanation of each process and will list the various soil or contamination criteria under which each can be effectively applied. This will provide a user who was only recently exposed to the bioremediation of hydrocarbons, for example, with a solid background; or will provide a comprehensive review of a technique that a person has not used in a while.

The second branch of the program will provide the user with case studies that illustrate decision processes. These studies will allow the user to match information with techniques. Then the expert system will either affirm the user's choice and provide supporting evidence, or correct the choice and provide the reasoning. This branch will allow the user to get a feel for the decision-making approach one should follow.

The final branch of the program will allow the user to input information pertaining to a specific situation. The expert system will then output the "best" choice or choices for this case and the logic behind the decision.

Note that the program is only an aid to the user. No program can adequately replace the logic of a user intimately involved with the particular situation. The program will only show the correct thought process and output the best possibilities. The user should then take these possibilities and thoroughly consider them, taking into account parameters the computer could not.

SUMMARY

An expert system is being developed to acquaint system users with the various bioremediation techniques applicable in the unsaturated zone and to indicate the conditions for which particular techniques are appropriate. The program will contain three main branches. The first will describe the bioremediation processes. The second will allow the user to view the decision process through case studies, and the final branch will allow the user to input specific information so that the program can output/suggestions on available techniques.

A DATA VISUALIZATION SYSTEM FOR BIOREMEDIATION ANALYSIS

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INTRODUCTION

Site characterization data play an essential role in determining the feasibility, design, and operation of a bioremediation system. These data represent a series of point measurements taken from wells, borings, and test pits of a highly heterogeneous, three-dimensional subsurface environment. The site engineer is faced with the task of interpreting these test-point measurements from a variety of perspectives. For example, the feasibility of a bioremediation strategy may depend on identifying a sufficient volume of contaminated media located within an acceptable soil stratum that is free of biological toxicants. Site characterization data should be displayed in a meaningful way so that they can be properly interpreted and used correctly when making remedial action decisions. The goal of this project is to develop a powerful yet simple-to-use data visualization tool for site engineers and managers that assists them in evaluating the feasibility of bioremediation at contaminated waste sites.

APPROACH

Our data visualization software, called ICASE (Integrated Computer Assisted Site Evaluation), takes measurements made at irregularly spaced locations at a site and uses a stratified interpolation process to estimate values within a uniform three-dimensional grid underneath the site. This procedure is carried out for any parameters of interest, which can include both soil properties and chemical species. It is then possible to display the geographical distribution of single parameters or pairs of parameters on a map of the site in a variety of formats that include:

- Shaded contour diagrams on a plan view of the site within any depth layer.
- Two-dimensional cross-sectional views across the site (in the form of single slices, fence diagrams, or an animated sequence of slices).
- Three-dimensional shaded volume views (similar to medical imaging) within the subsurface, with the ability to slice open the volume along any plane to get a cross-sectional view.

In addition to the data visualization methods, ICASE is being equipped with a bioremediation knowledge base. This is a collection of rules and conditions (perhaps even a scoring system) that will infer the feasibility of a particular form of bioremediation technology based on conditions measured at the site. These include soil and aquifer geotechnical properties along with contaminant properties and nutrient levels. At the user's command, ICASE will let the knowledge base reason over the site's characteristics and graphically display areas where a particular type of bioremediation technology may (or may not) be technically feasible.

CURRENT STATUS

The first year of the project has focused on developing map display and data visualization features. Base map display, ad hoc data queries, contouring, and two-dimensional cross-sectioning have all been completed. The three-dimensional visualization methods are currently being enhanced. The major effort in the second year will be the development of the bioremediation knowledge base, its incorporation into ICASE, and the testing of the completed system on a suite of site remediation case studies.

POSTER PRESENTATIONS
SUPPORTED BY
EPA'S HAZARDOUS SUBSTANCE RESEARCH CENTER PROGRAM

The following research is being carried out under the auspices of the EPA's Hazardous Substance Research Center (HSRC) program. EPA established this program in response to provisions in the 1986 amendments to the Comprehensive Environmental Response, Compensation, and Liability Act. These provisions authorized EPA to establish HSRCs with a mission to study all aspects of the "manufacture, use, transportation, disposal, and management of hazardous substances" and made the Agency responsible for the "publication and dissemination of the results of such research." The program is managed by the director of EPA's Office of Exploratory Research (OER) in the Office of Research and Development (ORD).

EPA has established five research consortia, with each serving two adjacent federal regions. These include:

Northeast Hazardous Substance Research Center—Region-Pair 1 and 2, which includes the New England states, New York, New Jersey, and the territories of Puerto Rico and the U.S. Virgin Islands. The lead institution is the New Jersey Institute of Technology, and the center's director is Dr. Richard Magee. Other consortium partners include the Massachusetts Institute of Technology, Tufts University, Rutgers University, Stevens Institute of Technology, Princeton University, and the University of Medicine and Dentistry of New Jersey.

Great Lakes and Mid-Atlantic Hazardous Substance Research Center—Region-Pair 3 and 5, which comprises the Great Lakes states and the mid-Atlantic states of Virginia, West Virginia, Maryland, Pennsylvania, and Delaware. This three-university consortium is headed by Dr. Walter Weber of the University of Michigan; Michigan State University and Harvard University are partner institutions.

South/Southwest Hazardous Substance Research Center—Region-Pair 4 and 6, which is made up of Gulf Coast and southern states. Louisiana State University heads this center, in partnership with Georgia Institute of Technology and Rice University. The center's director is Dr. Louis Thibodeaux of Louisiana State University.

Great Plains and Rocky Mountain Hazardous Substance Research Center—Region-Pair 7 and 8, which includes the states on the eastern side of the Great Basin along with the Great Plains states. This large consortium is run by Dr. Larry Erickson of Kansas State University. The other six participating institutions are Montana State University and the Universities of Iowa, Missouri, Montana, Nebraska, and Utah.

Western Region Hazardous Substance Research Center—Region-Pair 9 and 10, which includes the West Coast states along with Alaska, Arizona, Hawaii, and Idaho. Stanford University and Oregon State University make up this consortium. Dr. Perry McCarty of Stanford University is the center's director.

DEVELOPMENT OF A KNOWLEDGE-BASED BIOREMEDIATION ADVISER

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INTRODUCTION

Bioremediation is an increasingly promising technology for cleaning up hazardous waste sites, but because of a lack of data and knowledge to make informed decisions, owners and consultants often do not think of it as a viable treatment alternative. Yet bioremediation is establishing itself with a credible track record as a cost-effective technology for certain types of contaminated sites. A limitation, however, is that bioremediation processes are not universally applicable. It is thus important to determine whether bioremediation can be applied on any specific site, before resorting to other treatments.

This study aims at gathering and classifying disparate pieces of knowledge on bioremediation processes in such a way that they can be used in an interactive knowledge-based advisory system, called the Bioremediation Adviser. Such an advisory system would be useful to field practitioners in determining the applicability of bioremediation and enable novices to gain insight in the factors affecting the technical feasibility and success of bioremediation.

BACKGROUND

Whether bioremediation is feasible for cleaning a contaminated site depends on many factors including the characteristics of the spill and the site conditions, and knowledge of the degradability of the contaminant. These factors, in turn, depend on knowledge of the contaminants (e.g., physical and chemical properties), and of the microorganisms present (e.g., enzyme-producing capabilities) in the contaminated environment.

The literature contains considerable information on laboratory experiments on the degradation of pollutants, albeit studies with pure microbial cultures or undefined mixed cultures. While such information will be contained within the Bioremediation Advisor, some of it will be precluded from application in the field. Alternatively, some laboratory studies have provided direct evidence for the potential of either indigenous or added microbes in bioremediation schemes.

The Bioremediation Advisor will help bridge the gaps among practitioners' collection of traditional data, theoretical knowledge about bioremediation treatment processes and characteristics of pollutant-degrading microbes, and biological characteristics of the existing environment (Figure 1). Ultimately, this information will be used in conjunction with a rule-based system to guide field data collection and to match field data with bioremediation technologies to suggest the feasibility of bioremediation as a treatment for a given contamination and recommend possible bioremediation technologies.

RESULTS AND CONCLUSIONS

To date, a literature survey has been performed, including a review of papers and textbooks, pertaining to the biological degradation as well as the chemical properties of over 70 organic contaminants. The characteristic chemical properties and observed biodegradation of 20 relevant contaminants of concern, ranging from halogenated aromatic and aliphatic compounds to pesticides, have been classified in Hypercard stacks. Environmentally relevant chemical properties include redox potential, aqueous solubility, organic partition coefficient, and molecular structure. When values were not available in the literature, they were estimated based on established methods. The compiled biodegradation parameters include the nature of the degradation mechanism (i.e., oxidation or reduction, and mineralization or transformation), and the environmental redox potential under which degradation may occur.

Since both the redox of the environment and that of the compound determine to a great extent what type of (and whether) biodegradation will occur, correlations are currently being developed using these parameters for a range of organic pollutants. The pollutants themselves have been grouped according to a system reflecting their relative redox potential, taking into account the level of carbon atom substitution (scaled from 1 to 4), the number of halogen atoms and hetero-atoms, and presence or absence of aromaticity.

Even though data collection from the literature constitutes the bulk of the work reported here, the Hypercard format is continuously being developed to suit the eventual implementation in the Bioremediation Adviser's architecture.

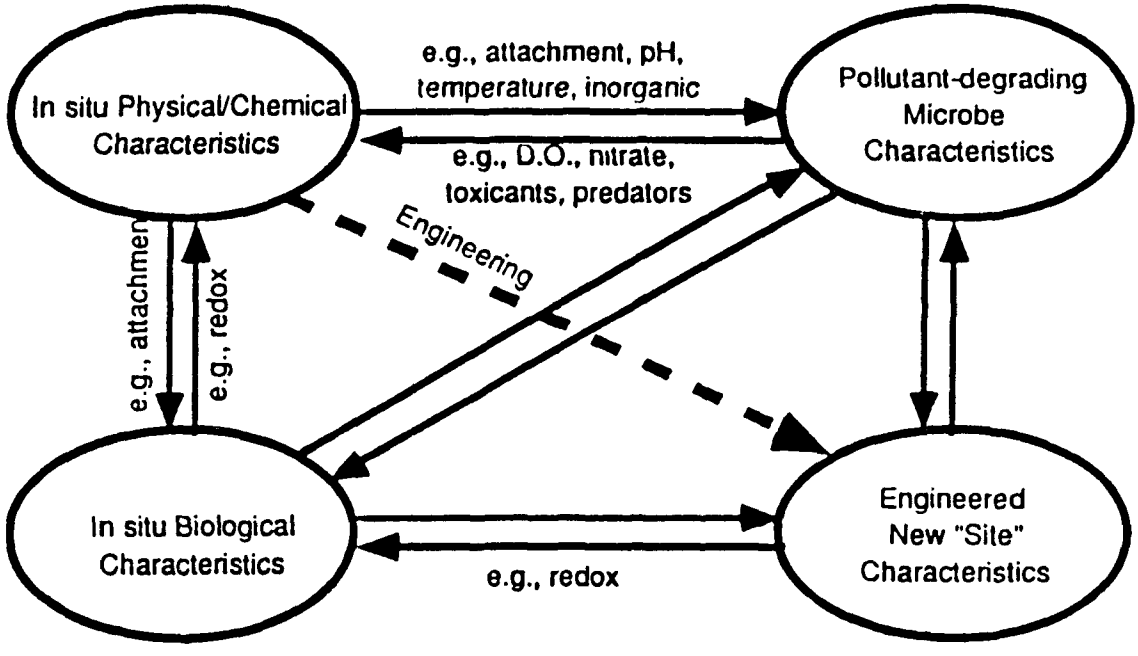

Figure 1. Proposed expert system scope.

USE OF COMPOSTING TECHNOLOGIES TO TREAT A TNT-CONTAMINATED SOIL

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Trinitrotoluene (TNT), an aromatic hydrocarbon known to be carcinogenic and mutagenic, has been extensively used by the military as an explosive for decades. As a result of its uses and disposal techniques, the U.S. government now owns approximately 1 million tons of soils contaminated with TNT and other explosive compounds.

Previous laboratory and field research has shown that TNT can be microbially transformed. The transformation products are the result of the reduction of the nitro groups and include diaminonitrotoluene and aminodinitrotoluene isomers. Many of these compounds appear to be toxic. The result is that method of transformation of TNT may not reduce risks to known populations.

The aim of the current project is the mineralization of TNT. The approach is to pretreat a TNT-contaminated soil using a chemical oxidant to yield trinitrobenzoic acid (TNBA) followed by microbial transformation of TNBA using composting technology. Many methods have been developed to oxidize the methyl ($-CH_3$) group(s) of a benzene ring to a carboxyl (-COOH) group. Many, however, require a very harsh environment (i.e., high-temperature, high-pressure, or severe acidic or basic conditions). The most benign and field-applicable oxidant and the one chosen for this project is ozone (with a radical initiator). Initial efforts using an aqueous system have shown that as the number of nitro groups increased (i.e., methylbenzene to TNT), the oxidizing efficiency of ozone decreased (80 percent to less than 50 percent). The use of ozone in combination with hydrogen peroxide or ultraviolet light is currently under investigation.

The disappearance of TNBA was investigated under thermophilic (55°C) conditions. Ninety-eight percent of the TNBA was reduced in a period of 20 days in a soil matrix contaminated at 10 mg/kg. The high pressure liquid chromatography indicated the presence of two new peaks, suggesting the appearance of metabolites. The identification of the metabolites and ^{14}C-TNBA studies are under way.

EFFECT OF PORE-SCALE HYDRODYNAMICS ON BULK REACTION RATES

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The objective of this research is to determine the effective first-order volumetric reaction rate of a biologically reactive solute. The effective reaction rate is the rate that would be measured in a column experiment, and that would subsequently be used in a transport model commensurate with the scale of the Darcian velocity. The fundamental question addressed is how does the effective reaction rate depend on the cumulative effects of pore-scale geometry and mechanisms. A model is developed that includes geometrical structural features such as pore shapes and surface roughness, spatially variable transport mechanisms such as diffusion and convective mass transfer, and spatially variable reaction rates within a biofilm or microcolony.

The microbiological processes by which subsurface microorganisms transform pollutants are controlled by the detailed pore-scale physical mechanisms that affect the organisms' functioning. The understanding of the mechanisms and processes controlling in situ biorestoration are best at the pore scale. For the purposes of ground water and soil remediation, however, it is desirable to make measurements, predictions, and assessments at a much larger scale. It is not feasible to bridge this gap in scales by simply having a computational domain that spans both. First, there is not enough data to specify the spatial structure with such detail; second, current computational resources are inadequate to handle such large problems, even if the data did exist. A method for upscaling from the pore to field scale is required.

This study starts with a detailed description of the mechanisms and processes thought to be significant in the biodegradation of a reactive contaminant. The detailed model includes 1) the pore water velocity field through a channel with variable surface roughness and tortuosity; 2) spatially variable diffusion in the pore water, biofilm, and solid matrix; and 3) spatially variable reaction rates within the biofilm. The approach adopted in this work is to average spatially the equations governing the pore-scale transport of the reactive contaminant over an appropriately large volume. The upscaling methodology is based on a moment method approach. The upscaling procedure yields the parameters that describe the transport of the less-detailed, spatially averaged solute concentration, and in particular the effective first-order volumetric reaction rate.

As a first step, a simple geometric model of a porous medium was used to examine the effects of the subgrain-size structural features and processes on the measurable laboratory-scale

reactivity of a biologically reactive solute. In addition to providing some rough insight into the problem at hand, it will provide a means for verifying the subsequent, more complicated models. The geometry of the problem consists of two parallel plates a distance 2a apart. The surfaces of the parallel plates are assumed to be coated with a shallow uniform biofilm of a reactivity that is approximated with a first-order surface reaction rate coefficient, λ. At the column scale, the measurable quantity of interest is the global scale solute concentration (C^*), which is defined as the point concentration averaged over the distance between the plates. An analytical solution for the temporal behavior of C^* is developed for the case of a solute undergoing diffusion D_m and a first-order surface reaction λ. For a very wide range of parameter values, including those values associated with enhanced subsurface biological activity, it is found that 1) for times satisfying $a^2/D_m<t$, C^* decays according to a first-order volumetric rate γ^*; 2) the magnitude of the global-scale reactivity coefficient γ^* is determined by the Damkohler group II number defined as $Da=\lambda a/D_m$; 3) for the case of a small Damkohler number (weak reaction), valid for $Da < 0.2$, the limiting value of the rate coefficient is $\gamma^*=\lambda/a$; 4) for the case of a large Damkohler number (strong reaction), valid for $20<Da$, the limiting value of the rate coefficient is $\gamma^*=\pi^2D_m/4a^2$; and 5) for intermediate values of the Damkohler number, the function $\gamma^*a/\lambda=0.70-0.47 \log(Da)$ is approximately valid for $0.2 < Da < 20$.

For the more general case of interest in this research, the equation governing the effective first-order reaction rate is solved. The behavior of the global reaction rate γ^* is investigated as a function of different pore geometrys, Damkohler numbers, and spatial distributions of the microorganisms.

IN SITU BIOREMEDIATION USING A RECIRCULATION WELL

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Presenter: Perry L. McCarty, Stanford University

INTRODUCTION

A promising microbial system for in situ bioremediation is aerobic cometabolic degradation by methanotrophic bacteria. Methanotrophic bacteria produce the enzyme methane monooxygenase (MMO), which can degrade many hazardous volatile organic compounds. During cometabolic degradation, the target contaminant is fortuitously degraded by the MMO that is produced in order for the microorganisms to utilize the supplied electron donor (methane). Studies at the Moffett Naval Air Station (3,4,5,6) have demonstrated successful removal of several volatile organics, including trichloroethylene (TCE) and vinyl chloride (VC), by promoting the growth of methanotrophic bacteria in the subsurface.

Several investigators (7,8,9) have examined the flow characteristics of vertical recirculation wells. This poster investigates the use of a vertical recirculation well to promote cometabolic transformation of VC using methanotrophic bacteria. A vertical recirculation well contains both an injection and extraction screen with a pump between the two screens to force recirculation. Ground water enters the well through the upper screen and is pumped in the downward direction. Oxygen and methane are added to the water below the pump, and the enriched water is injected through the lower screen. The screens have a 10-m separation. The target contaminants are recirculated, which increases their contact time with the biologically active zone, allowing a greater extent of contaminant removal. In previous in situ bioremediation applications (3), the target contaminant passed through the biologically active zone only once, and any remaining contaminant required further treatment above ground. The simulations presented here evaluate different oxygen and methane delivery schemes for a recirculation well operated to promote in situ bioremediation.

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CHLORINATED ALIPHATIC HYDROCARBON BIODEGRADATION BY METHANOTROPHIC BACTERIA

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Methanotrophic bacteria, which oxidize methane for energy, have been found capable of oxidizing chlorinated aliphatic hydrocarbons (CAHs) such as trichloroethylene (TCE) by cometabolism. A two-reactor treatment system for TCE destruction by methanotrophic bacteria is being evaluated. The system consists of a growth reactor (completely mixed) that continuously produces the active culture for addition to a TCE-contaminated waste stream in a separate transformation reactor (plug flow). This reactor configuration eliminates TCE transformation product toxicity during cell growth, benefits from the controlled conditions in the growth reactor, and gives enhanced TCE removal through the absence of competitive inhibition and more favorable plug flow kinetics in the transformation reactor.

Methane/TCE Interactions. The TCE transformation rate by a methanotrophic mixed culture in batch studies was found to be enhanced by the presence of a high concentration of methane (5 to 6 mg/L) at high TCE concentration (5 to 9 mg/L). This result was contrary to the expectation that the presence of methane would cause a reduction in the TCE transformation rate due to competitive inhibition. At a lower TCE concentration (0.9 mg/L), the TCE transformation rate was found to be lower in the presence of methane, indicating that competitive inhibition does occur. It appears that at the higher TCE concentration, energy depletion and cell inactivation attributable to TCE transformation product toxicity controlled the TCE transformation rates, while competitive inhibition controlled at the lower TCE concentration. These results indicate that methane addition to the transformation reactor in the proposed treatment system may be beneficial in some cases.

Mixed-Culture Growth Conditions. Two aspects of growth reactor performance were studied, reactor solids retention time (SRT) and nutrient nitrogen concentration (nitrate nitrogen, mg N/L). Three laboratory growth reactors were operated at various combinations of 2-day and 8-day SRT and 82 mg N/L and 165 mg N/L influent concentrations. The results showed that similar TCE transformation capacities (T_c, g TCE/g cells) could be obtained at both SRTs and that the higher growth yield of the 2-day SRT led to a higher TCE transformation yield (T_y, g TCE/g methane).

Nitrogen starvation did not affect the T_c at the 2-day SRT, but led to higher growth yields and higher T_y.

Poly-beta-hydroxybutyrate (PHB). The T_c of resting (non-fed) methanotrophic cells has been suggested to be a function of the cells' PHB content, which increases under nutrient deficiency. PHB serves as an internal source of reducing power when the energy substrate (methane) is absent. When the cultures of two laboratory growth reactors were compared, T_c increased with PHB content.

The activity of the soluble methane monooxygenase (MMO_s, the enzyme that performs TCE oxidation) was determined by measuring naphthalene oxidation rate. The naphthalene oxidation rate was directly proportional to PHB content in samples taken from the 8-day SRT growth reactor, which had PHB contents of 2 to 8 percent. When the samples were amended with 20 mM formate, a readily available electron donor, the specific naphthalene oxidation rate was constant and higher than in unamended samples. These results indicate that in resting cells MMO_s activity is limited by the availability of a suitable source of reducing power.

ANAEROBIC BIODEGRADATION OF BTEX COMPOUNDS AT SEAL BEACH, CALIFORNIA

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Orange County Water District built a facility at the Seal Beach site in California where there was a significant gasoline spill resulting in contamination of the ground water aquifer with hydrocarbons including benzene, toluene, ethylbenzene, and *m*-, *p*-, and *o*-xylene (BTEX). The facility was designed for the operation of in situ bioreactors wherein strategies for bioremediation of the contaminated soil were tested. The bioreactors consisted of aquifer sediment-filled stainless steel cylindrical vessels with near complete capability to control and monitor both hydrodynamic flow and influent/effluent composition. The operation of two anaerobic/anoxic bioreactors focused on developing the native microbial populations under natural and induced denitrifying conditions. The ground water at the site had a high naturally occurring background concentration of sulfate (85 mg/L). The influents to the "natural background" control and denitrifying reactors were contaminated ground water from the site and contaminated ground water amended with nitrate (13 mg/L as nitrogen), respectively. Although some biological removal of toluene and *m,p*-xylene was observed in the nitrate-amended bioreactor, toluene and *m,p*-xylene also were removed in the unamended control reactor, though to a lesser degree.

In the laboratory, batch bottle microcosms with sediment and ground water from Seal Beach then were used to verify the field results and evaluate other conditions under which in situ biotransformation could be enhanced at the Seal Beach field site. Both toluene and *m,p*-xylene were removed in the unamended microcosms, just as observed in the field. Corresponding loss of sulfate in the sample bottle suggested that the observed aromatic removal was probably due to sulfate-reducing bacteria. Addition of nitrate to the samples stimulated denitrificaytion, which resulted in faster disappearance of toluene, complete loss of ethylbenzene, but less complete removal of *m,p*-xylene. Addition of a nonindigenous hydrocarbon-degrading consortium to the microcosms resulted in some enhancement of *o*-xylene transformation. In several nitrate-amended microcosms in which nitrate was not replenished after it was completely utilized, sulfate reduction commenced. Thereafter, both nitrate and sulfate were utilized in the microcosms. Biotransformation of benzene in several microcosms was tied to active sulfate reduction. In those microcosms that had both active denitrification and sulfate reduction, there was complete loss of the full range of BTEX aromatic compounds. The results of this experiment indicate that utilization of a combination of the electron acceptors nitrate and sulfate may provide benefits in restoration of BTEX-contaminated sites.

BIOTRANSFORMATION OF INDOLE AND QUINOLINE UNDER DENITRIFYING CONDITIONS

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The anaerobic conditions prevalent in many contaminated ground water plumes provide an impetus to investigate the anaerobic biodegradation of petroleum hydrocarbons. In this study, sediment from an oil refinery waste pond that had been found to be biologically active against single-ring homocyclic aromatic compounds under denitrifying conditions was used as inoculum in nitrate-containing enrichment cultures. The cultures were prepared according to a protocol of rigorous oxygen exclusion; they contained 2 mm of nitrate and 100 mm of an aromatic substrate, individually, in a mineral medium; and they were incubated within an anaerobic chamber. The aromatic substrates were indene, naphthalene, indole, quinoline, furan, benzofuran, thiophene, and benzothiophene. Two live and two autoclaved controls were prepared per substrate.

Within 20 days, cultures exhibited transformation of indole and quinoline as determined by high pressure liquid chromatography (HPLC). The other aromatic substrates have not been transformed after 7 months of incubation. At 9 days, the concentration of indole had decreased and an as yet unidentified more polar transformation product had been produced. By 37 days, both indole and the transformation product were no longer detectable. Quinoline was decreased in its cultures by 20 days and a transformation product that HPLC retention times indicated was 2-quinolinol had appeared. By 39 days, quinoline was undetectable and 2-quinolinol had accumulated. By 54 days, 2-quinolinol was undetectable. Ion chromatography revealed that nitrate was consumed in all live cultures and that nitrate consumption occurred simultaneously with indole and quinoline transformation. Gas partitioner analysis of headspace samples failed to detect methane or hydrogen sulfide. Examination of the quinoline-fed culture with phase-contrast microscopy revealed a microbial community of diverse morphology. Neither nitrate nor any of the aromatic substrates were consumed in autoclaved controls.

Sediment-free subcultures retained the ability to completely transform indole and quinoline. These subcultures were dominated by motile, Gram-negative rods growing singly and in dense and macroscopically visible flocs. Subcultures exposed to hydrogen consumed nitrate without degrading the nitrogen heterocycles. The addition of 0.3 percent mercury chloride prevented consumption of any substrates.

COMETABOLISM OF TCE BY NITRIFYING BACTERIA

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The soil nitrifying bacterium *Nitrosomonas europaea* is a lithoautotroph that obtains all of its energy for growth from the oxidation of ammonia (NH_3) to nitrite (NO_2^-). Ammonia oxidation is catalyzed by ammonia monooxygenase (AMO), which converts NH_3 to hydroxylamine (NH_2OH). The oxidation of NH_2OH to NO_2^- provides both the reductant required for AMO and the sole source of electrons for adenosine triphosphate (ATP) synthesis. AMO can also oxidize alternate substrates including alkanes, alkenes, aromatics, and many C_1 to C_3 halogenated aliphatics, including trichloroethylene (TCE). The products of these oxidations are not assimilated and the oxidation process requires simultaneous NH_3 oxidation as a source of reductant. These oxidation reactions are therefore regarded as cometabolic.

TCE cometabolism by autotrophic nitrifying bacteria has several attractive features. First, AMO is a constitutive enzyme and requires no induction. Second, NH_3, is inexpensive, and very water soluble. Third, nitrifying bacteria are widely distributed in soils. In situ processes could rely on indigenous nitrifier populations and avoid the use of genetically engineered and potentially less-competitive species.

Despite these features, *N. europaea*, like all other aerobic TCE-degading bacteria, suffers a toxicity from TCE oxidation resulting from the formation of a reactive TCE-epoxide intermediate. Much of this TCE toxicity involves inactivation of AMO. This research uses *N. europaea* as a model system to study the physiological consequences of TCE toxicity on the cometabolic process. The objective is to determine the factors that allow for the maximal sustainable rate of TCE degradation. Physiological studies are being used to provide operating parameters for concurrent studies using reactors capable of sustainable TCE degradation.

In short-term (10 min) experiments correlating TCE degradation (measured as Cl^- ion release) and the extent of inactivation of several enzyme activities, *N. europaea* has been found to oxidize 60 nmol of TCE/mg protein before complete inactivation of AMO. This value ranges from 30 to 100 nmol TCE/mg protein, depending on the specific activity of batch-grown cells. TCE toxicity

is irreversible without protein synthesis and affects both AMO and the enzymes and/or proteins associated with NH_2OH oxidation. Since sustainable TCE oxidation will require the ability of cells to constantly resynthesize these inactivated proteins, these data suggest that the maximal sustainable rate of TCE degradation will occur at considerably lower concentrations of TCE than those that support the maximal initial rate of degradation. This is supported by the observation that the growth of *N. europaea* in the presence of TCE can only occur at very low TCE concentrations (<5 nmol/mL) where the extent of enzyme inactivation is sufficiently low that net growth can occur despite the need to resynthesize inactivated components. To quantify a maximal tolerable level of TCE inactivation, the kinetics of recovery of AMO activity in cells after partial and complete inactivation of AMO by TCE have been examined. These kinetics have been compared with cells in which AMO has been specifically inactivated by light. These results indicate that a level of TCE inactivation of greater than 20 percent adversely affects the rate of recovery of AMO activity.

Reactor-based studies investigating TCE-degradation over longer periods (>1 hr) have been initiated and have confirmed that sustainable TCE-degradation can be maintained but only at low TCE concentrations (< 250 ppb). An interesting consideration in these studies is whether cells respond to partial TCE inactivation by the *de novo* synthesis of AMO (and other proteins) and what contribution newly synthesized enzymes make to the kinetics of TCE degradation. Initial studies following the kinetics of *de novo* protein synthesis using $^{14}CO_2$ have been conducted, and these studies are continuing.

A third approach to the question of TCE toxicity involves studies of TCE-degrading, alkene-oxidizing bacteria. These species contain epoxide-metabolizing systems and may be able to circumvent TCE-mediated toxicity by consuming the epoxide intermediate. Initial studies indicate that a substantial increase in tolerance to TCE toxicity compared to *N. europaea* is not realized in this system. There is some evidence that TCE-epoxide may in fact inhibit or inactivate the epoxide metabolism of these bacteria.

ASSESSING THE EFFECT OF ENVIRONMENTAL CONDITIONS ON CHLOROPHENOL REDUCTIVE DECHLORINATION PATHWAYS AND KINETICS

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INTRODUCTION

A knowledge of biotransformation pathways and kinetics is essential to assess risks at contaminated sites, implement biological treatment processes, or design effective bioremediation strategies. In addition to the microbial consortium, environmental conditions such as pH, oxidation/reduction potential, or the presence of toxicants may alter biodegradation pathways and kinetics from those observed in the laboratory.

Pentachlorophenol (PCP) was selected for study because it is toxic to a wide variety of organisms (1), it is widely distributed in the environment, and the anaerobic biotransformation pathways for chlorophenols have been well studied. PCP has been used extensively as a wood preservative and pesticide. Hundreds of sites in the United States are contaminated with PCP as a result of wood-treating activities. Many of these sites are on the National Priority List for cleanup under the Superfund Program.

The goal of this study is to better understand the effects of certain environmental conditions on the rate of anaerobic biotransformations of chlorophenols. Preliminary studies were conducted to characterize the pentachlorophenol degradation pathway for an acclimated culture. This consortium will be used for all experiments, and the kinetics of these biotransformation reactions will be measured.

A computer-interfaced reactor system has been developed to allow measurement of biodegradation rate constants under constant conditions of biomass, pH, sulfate, sulfide, and acetate concentrations. The reactor is being used to develop progress curves under varying, but constant, environmental conditions.

RESULTS

Pentachlorophenol Biotransformation Pathway by the Acclimated Consortium. Pentachlorophenol biotransformation pathways were determined for a methanogenic consortia fed 5,300 mg/L acetate, 3.4 μm (0.9 mg/L) pentachlorophenol, and nutrients for 9 months. Within 1.2 days, 4.38 μM PCP was biotransformed at 99.7 percent efficiency (Figure 1). Degradation of PCP was accompanied by the production of all three tetrachlorophenols, as well as 3,4,5-, 2,4,5-, and 2,3,5-trichlorophenol. The "sum" identified in Figure 1 represents the sum of the concentrations of PCP and all tetra- and tri-chlorophenols identified in the reactor. After 0.6 days, the mass balance appears to fall off due to the production of dichlorophenols, which were not included in the "sum."

2,3,4,5-Tetrachlorophenol (2,3,4,5-TeCP) reached a maximum concentration of 0.37 μm after 0.5 days and was not detected in the reactor after 1 day. 2,3,5,6-TeCP accumulated rapidly in the reactor to a maximum concentration of 3.1 μM or approximately 70 percent of the initial PCP concentration. The accumulation of 2,3,5,6-TeCP ceased after 0.88 days when PCP was removed from the reactor. 2,3,4,6-TeCP, the meta dechlorination product, was observed at very low levels (less than 0.07 μM) during the experiment.

The experiment demonstrates that the consortium acquired the ability to dechlorinate PCP at all three chlorine positions after exposure to PCP for six months. Although dechlorination at the ortho position is preferred by unacclimated consortia (1,2,3,4), removal of para and meta chlorines from PCP to produce 2,3,5,6-TeCP and 2,3,4,6-TeCP is observed following acclimation. The development of a split degradation pathway has generally not been observed in previous laboratory experiments, although it has been documented in rice paddy soils exposed to PCP (5,6). Additionally, each of the tetrachlorophenols observed in this study were also reported in studies performed examining PCP biodegradation by sludges individually acclimated to either 2-CP, 3-CP, or 4-CP (1).

Similar progress curves were developed for the three tetrachlorophenols and their resulting metabolic products. The overall pathway appears in Figure 2.

Development of a Reactor System to Control Environmental Conditions. A computer-interfaced reactor system has been constructed and is now being refined. It provides control of pH, sulfide concentrations, acetate concentrations, the apparent oxidation/reduction potential, and the biomass concentration (there is little growth compared to the initial biomass in the reactor). Acetate and sulfate are added with computer-controlled dispensers to change or maintain the concentrations of these species.

The reactor system provides constant monitoring of the following parameters using an analog-to-digital conversion board:

- pH with an Orion Ross glass electrode

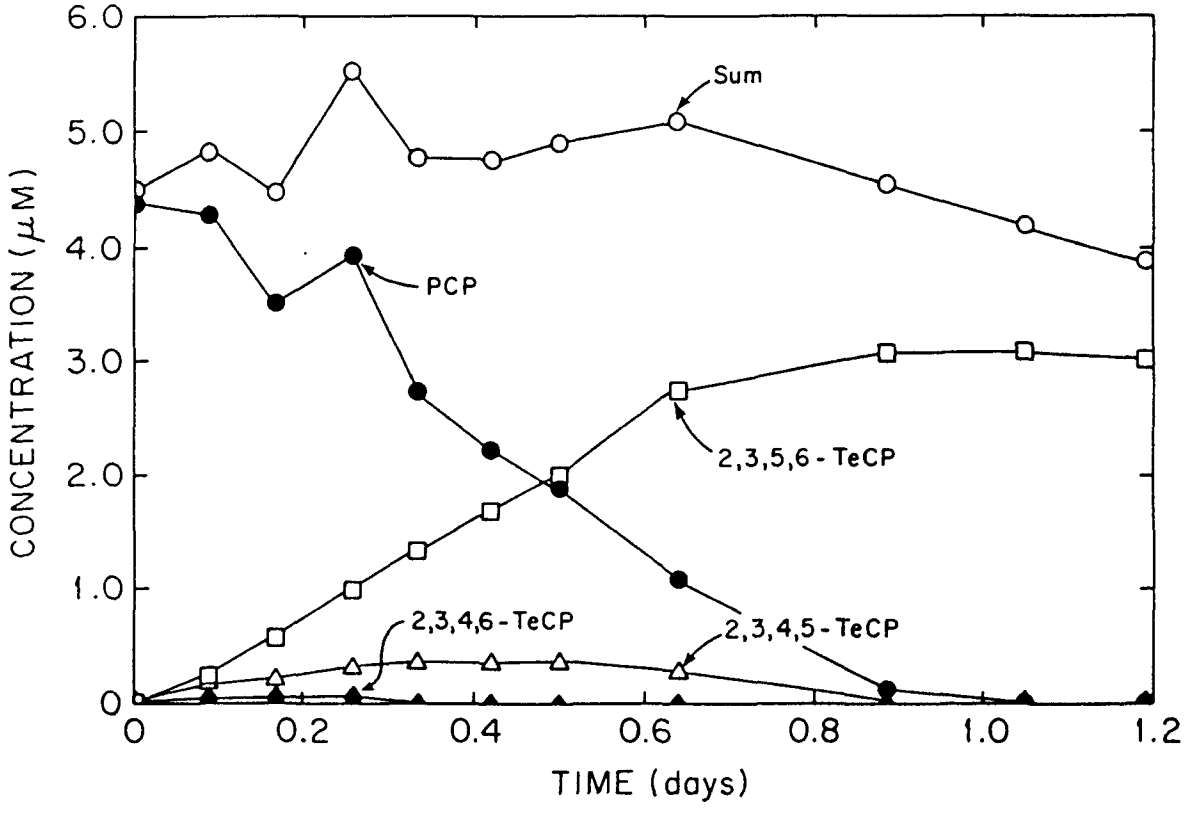

Figure 1. Progress curve for pentachlorophenol reductive dechlorination by a pentachlorophenol-acclimated methanogenic consortium.

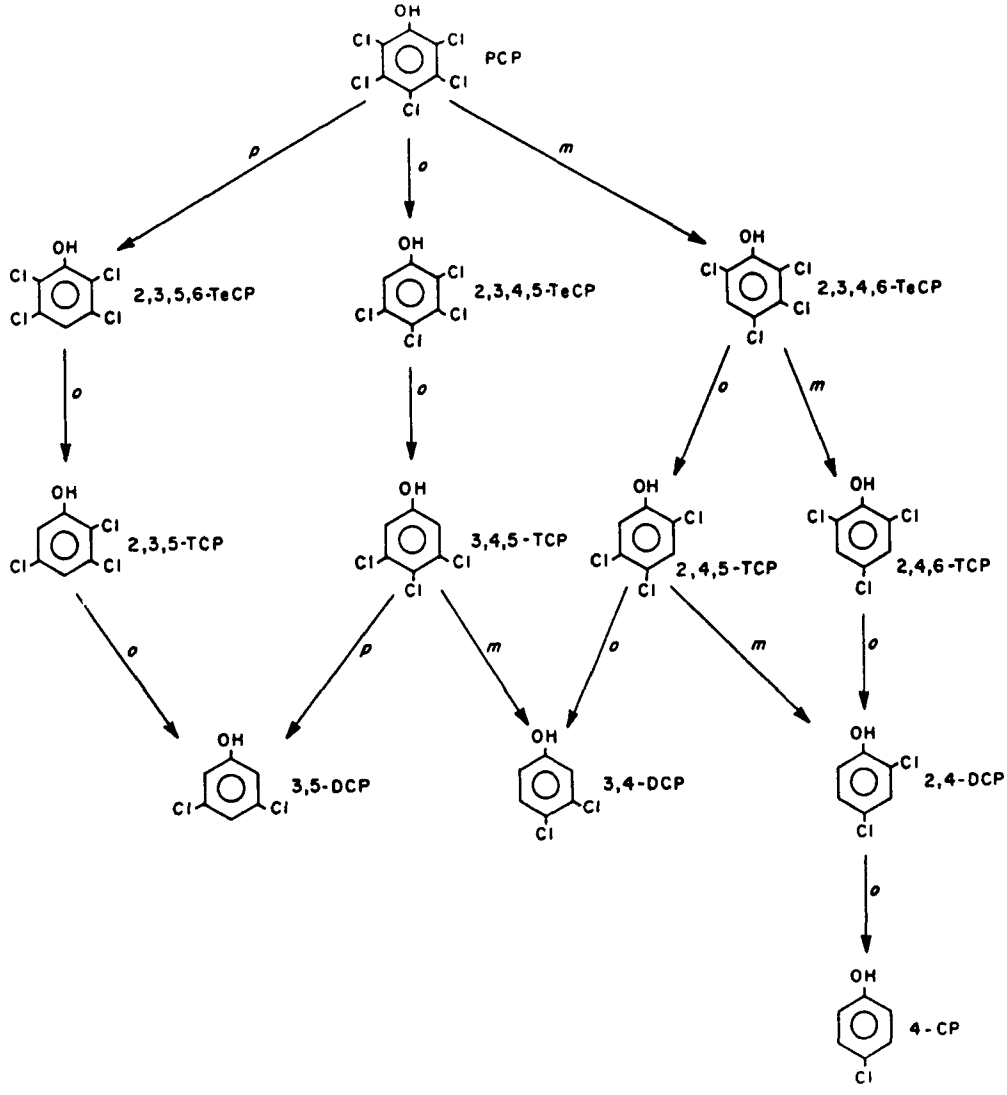

Figure 2. Summary of the observed reductive dechlorination pathway for pentachlorophenol and its metabolites by a pentachlorophenol-acclimated methanogenic consortium.

- Apparent E_H at a platinum electrode
- Potential at a silver/sulfide ion selective electrode

The same Orion double junction Ag/AgCl electrode is used as the reference electrode for all the above indicator electrodes to eliminate problems that can arise when two or more reference electrodes are present in the same solution. The sulfide electrode is calibrated by multiple standard additions of Na_2S at the end of data collection. Since there is no on-line sensor for acetate or sulfate, samples are periodically withdrawn from the reactor to determine their concentrations by ion chromatography.

The E_H, sulfide electrode, and pH measurements for an experimental run are shown in Figures 3 and 4. The apparent oxidation/reduction potential mirrors the sulfide concentration and remained at a fairly constant level of -530 mV for a period of nearly 60 hours. During this period, the reactor's ability to maintain constant acetate concentrations was evaluated by the programmed addition of acetic acid. Slug additions of acetic acid or sodium acetate were made three times to bring the acetate concentration to a desired level. The pH changed after slug additions of acetate acid or sodium acetate. Once adjusted, the pH was maintained at a fairly constant value by automated addition of acetic acid to compensate for acetic acid consumption.

SUMMARY AND CONCLUSIONS

Based on the results of preliminary experiments, the complete biotransformation pathway observed for the reductive dechlorination of PCP by this acclimated consortium is shown in Figure 2. PCP was dechlorinated at three positions to produce 2,3,4,5-TeCP, 2,3,4,6-TeCP, and 2,3,5,6-TeCP. 2,3,4,5-TeCP was dechlorinated at the ortho position to form 3,4,5-trichlorophenol (3,4,5-TCP), which then gave 3,5-dichlorophenol (3,5-DCP) and lesser concentrations of 3,4-DCP as persistent products. 2,3,4,6-TeCP produced both 2,4,6-TCP and 2,4,5-TCP. 2,4,6-TCP was dechlorinated sequentially at the ortho positions to produce 2,4-DCP and 4-CP; and 2,4,5-TCP was dechlorinated to produce 3,4-DCP and 2,4-DCP. Sequential ortho dechlorination of 2,3,5,6-TeCP yielded 2,3,5-TCP and 3,5-DCP.

Ortho dechlorination was observed most frequently. Every possible metabolite due to ortho dechlorination was observed except for the production of 2,3,4-TCP from 2,3,4,6-TeCP. Therefore, eight of the nine possible ortho dechlorination products were observed. In contrast, only two para dechlorination products (of seven possible products) and four meta dechlorination products (of nine possible products) were observed. Of the possible polychlorinated phenolic congeners, only five are not included in the pathway: 2,3-DCP, 2,5-DCP, 2,6-DCP, 2,3,4-TCP, and 2,3,6-TCP. It is important to note that these five undetected di- and tri- chlorophenol congeners possess at least one ortho chlorine, and would not be expected to be observed because of the consortium's preferential removal of ortho chlorines.

A reactor system has been developed to allow measurement of reductive dechlorination kinetics while maintaining relatively constant biomass concentration, pH, sulfide concentration, and apparent oxidation/reduction potential. Once the control of acetate and sulfate concentrations have been refined, reductive dechlorination kinetics will be measured for varying, but constant, environmental conditions.

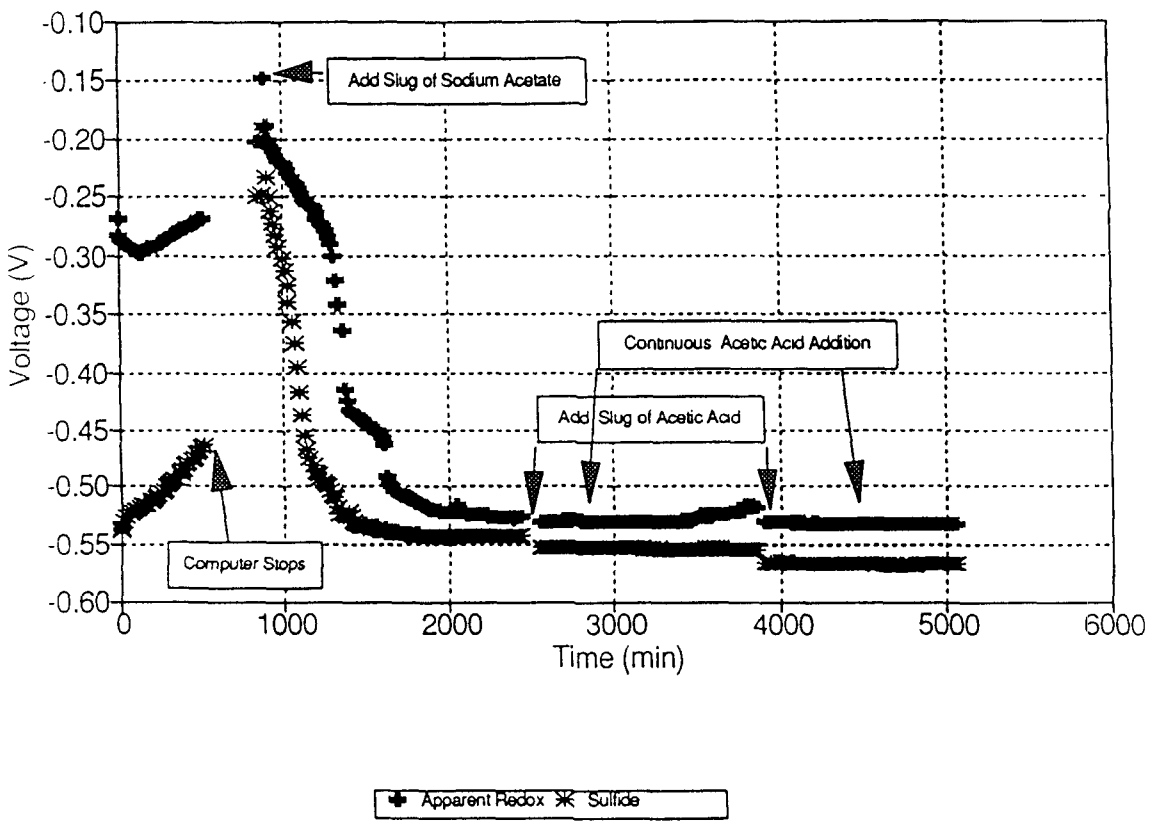

Figure 3. Apparent E_H and sulfide concentrations.

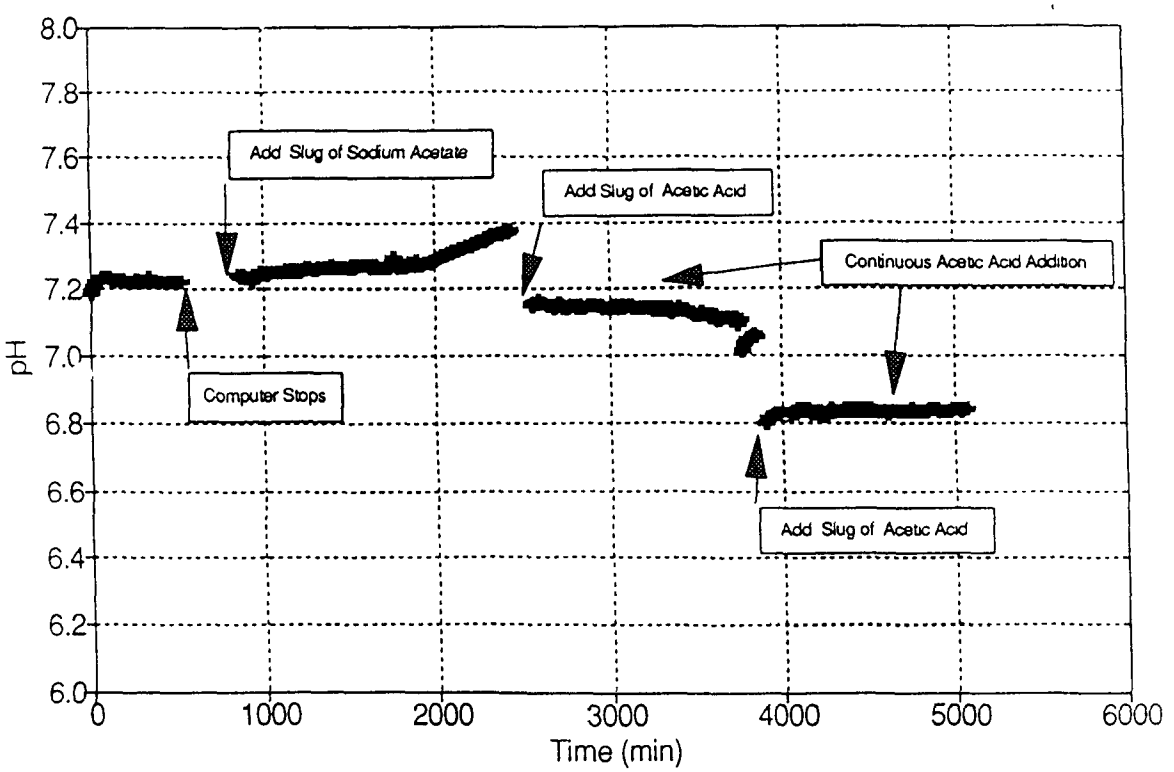

Figure 4. pH measurements.

ACKNOWLEDGEMENTS

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SPATIAL DISTRIBUTION OF NONAQUEOUS PHASE LIQUID IN SAND CORES USING X-RAY COMPUTED TOMOGRAPHY

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The small-scale spatial distribution of entrapped nonaqueous phase liquid in porous media is important in controlling dissolution kinetics, bioremediation performance, and the effectiveness of pump-and-treat schemes. This distribution is difficult to measure, however, which limits experimental understanding of the effect of entrapment processes on remediation. This study investigated the use of x-ray computed tomography (CT) to measure nondestructively the spatial distribution of trichloroethane (TCA) concentration inside constructed sand cores.

The 76-mm-diameter and 52-mm-long plexiglass cores were uniformly packed to a bulk density of 1.57 g/cm^3 with sand having a d_{50} of 0.49 mm, producing a mean porosity of 0.41. The cores were slowly saturated with water under vacuum pressure. Then a core was placed in a Siemens Somatom DRH CT unit such that the longitudinal axis of the core was horizontal and normal to the CT scan plane. The core was scanned prior to the introduction of TCA using a sweep of 21 adjacent scans with a slice thickness of 2 mm and pixel size of 0.32 x 0.32 mm. Then an injection of 1.0 mL of TCA was made at the center of the core using a needle and syringe placed through a septum in the core wall. Immediately after injection, an additional sweep of 21 adjacent scans was made.

Each scan produced a 256 x 256 matrix of x-ray attenuation coefficients (μ). A theoretical equation was developed to relate μ to mass TCA in each pixel. The equation requires an estimate of the effective energy (E) of photons passing inside the core. The mean E was computed for this particular core geometry and elemental composition of sand using the known photon energy spectrum emitted by the x-ray tube. The equation was applied to compute the mass in each pixel, and images of mass distribution were produced. For these mass calculations, μ values were averaged over groups of 6 x 6 pixels, producing computed masses for volume elements of 2 x 2 x 2 mm. Using a mean E inside the core of 75 kev, the total computed mass was equal to or greater than 95 percent

of the injected mass. Subsequent sweeps of scans measured the change in mass in each pixel and movement of the plume with time. This study demonstrates the use of CT for studying issues related to small-scale, three-dimensional, nonaqueous phase liquid movement and entrapment in porous media.

SCALE-UP IMPLICATIONS OF RESPIROMETRICALLY DETERMINED MICROBIAL KINETIC PARAMETERS

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Great Plains and Rocky Mountain Hazardous Substance Research Center

Recent attention to bioremediation of contaminated surface water and aquifer systems has necessitated the development of methods to estimate in situ rates of biodegradation. Specifically, biodegradation kinetic parameters must be determined with sufficient accuracy to justify their use in predictive models for field degradation rates. Respirometry has been used for many years to determine the oxygen demand and stoichiometry of the degradation of organics in wastewater. More recently, researchers have adapted the standard biochemical oxygen demand (BOD) respirometry apparatus to determine the kinetics of microbial degradation within the batch BOD vessel. Methods used to date have relied on a thorough knowledge of initial and final conditions within the respirometer for accurate determinations of kinetic parameters. The method used in this research employs a curve fitting model that uses a single Monod kinetic expression with cell decay to approximate the accumulated oxygen demand curve over time. The curve-fitting routine uses initial substrate concentration and the actual oxygen demand curve as inputs, and iteratively calculates a best fit solution for μ_{max}, K_s, biomass yield, initial biomass concentration, and cell decay. Mean values and 95 percent confidence intervals (CIs) were determined for these kinetic parameters. The kinetic parameters were then used in a bioprocess model to estimate the extent of biotransformation activity at a field site. To assess the effects of kinetic parameter variation on the size of the area biotransformed, the values of two important parameters (μ_{max} and K_s) were varied through their CIs. Results indicate that the area biotransformed in a 4,800-day model run varies only slightly from the least favorable (low μ_{max}, high K_s) to the most favorable (high μ_{max}, low K_s) microbial kinetic condition.

DISSIPATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE RHIZOSPHERE

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Vegetation may play an important role in the biodegradation of toxic organic chemicals in soil. The beneficial effects of vegetation may include uptake, metabolism, or volatilization of hazardous organics by the plants, and/or increased biodegradation by the rhizosphere microflora. The objective of this research was to evaluate the contribution of plants and associated microflora to the biodegradation of recalcitrant organic compounds in soil. The degradation of selected polycyclic aromatic hydrocarbons (PAH) in the rhizosphere of four plant species was investigated in a greenhouse experiment. Soil was contaminated with 100 mg/kg of PAH and planted with one of the following: alfalfa (*Meticago sativa*), fescue (*Festuca arundinacea*), big bluestem (*Andropogon gerardii*), or sudan grass (*Sorgham vulgare sudanese*). Plant roots, plant shoots, and soils were analyzed for PAH content after 4, 8, 16, and 24 weeks. Contaminants were detected in some of the plant samples, and PAH concentrations were smaller in rhizosphere than in unvegetated soil. Target compounds were not detected in soil leachates. Microbial numbers were greater in the contaminated rhizosphere of all plant species when compared to unvegetated soil. These results indicate that the interaction between plants and rhizosphere microflora enhances remediation of soils contaminated with hazardous organics.

EFFECT OF IRREVERSIBLE SORPTION ON BIOAVAILABILITY

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Biodegradation of organic pollutants in the environment depends largely on the bioavailability of the adsorbed pollutants. Steinberg et al. (1) has shown that ethylene dibromide (EDB) from a contaminated soil sample is not biodegradable by indigenous microbes after 38 days, whereas added (^{14}C) EDB is rapidly mineralized. Similarly, the sediment bound polycyclic aromatic hydrocarbons (PAHs) from Tamar Estuary, U.K., retained remarkable compositional uniformity to that of parent compounds (2). The bound PAH is not available for leaching, microbial breakdown, or photodegradation.

The present experiment was conducted to determine the reversibility of naphthalene adsorption/desorption on soil. Naphthalene readily adsorbed onto the soil and reached equilibrium within 1 day. The adsorption isotherm yielded an organic carbon-based partition coefficient (K_{oc}) of 793 cm_3/g, which was comparable to either the literature or estimated values (3).

Desorption experiments were conducted by replacing successive fractions of the aqueous phase with clean water. The supernatant fluid was separated from the soil by centrifugation at approximately 1,500 g. The hysteresis phenomenon was observed for naphthalene adsorption/ desorption (i.e., the adsorbed naphthalene was not readily desorbed from soil). Only about 11 to 23 percent desorbed in the first five successive desorption steps, whereas 97 percent of the adsorbed naphthalene should desorb if assuming reversible desorption. The desorption appears to be controlled by two rate-limiting mass transfer steps. When first-order kinetic reactions were assumed to approximate the desorption process, the reaction half-life was 6 days for the fast desorption and 451 days for the slow desorption. The rate constants were one to three orders of magnitude slower than the literature or estimated values (4,5).

The results of this study show that naphthalene or other structurally similar compounds can be adsorbed to the soil/sediment and rendered less available for extraction, chemical, and biological transformation.

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THE EFFECT OF POPULATION AND SUBSTRATE INTERACTIONS ON SBR DESIGN OPTIMIZATION

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Sequencing batch reactors (SBRs) operate in a cyclic mode, with each cycle usually consisting of five distinct periods: fill, react, settle, draw, and idle. Such reactors are known to offer a number of advantages over conventional activated sludge systems, including greater flexibility in meeting changes in feed conditions, effective control of the process and the quality of the discharge, and higher volumetric efficiency. Also, a separate clarifier is not required. The optimal design of SBRs for treatment of hazardous and toxic chemicals requires development of process models for making predictive and scale-up calculations.

In this project, 5-L SBRs are being used to investigate two categories of issues: the first deals with the effect of population interactions (i.e., competition) on the performance of the unit; the second involves identifying the interactions among pollutants at the kinetic level and then determining the effects on SBR design.

Phenol was used as the model compound in studying the effect of microbial competition. Two species were employed: *Pseudomonas putida* (ATCC 17514) and *Pseudomonas resinovorans* (ATCC 14235). Substrate concentrations were monitored using high pressure liquid chromatography (HPLC) measurements. Total biomass concentration measurements were based on optical density, while biomass concentrations of individual species were monitored through colony counts on a nutrient agar and a citrate-containing agar on which only *P. resinovorans* could grow. A relatively slow fill phase with aeration was found to permit biodegradation to start and to lead to better results. Depending on the phenol concentration in the untreated waste, the residence time, the fraction of the cycle time allocated to the fill phase, and the ratio of minimum to maximum volume of the reactor contents in a cycle, the biomass composition was found to change substantially with time. Unless the conditions are properly selected, a mixed culture cannot be maintained in the long run. A detailed model has been derived and used in extensive numerical studies. These studies determined the conditions leading to stable operation of the unit. The model predicts and the experiments verify that under certain conditions of operation multiple types of reactor behavior are possible, as determined by conditions at start-up of the unit.

Phenol and 4-chlorophenol (4CP) are being used as model compounds for studying the effects of substrate interactions. Two cultures have been tested. One was not capable of degrading 4CP unless phenol was present; this cometabolic process led to complete mineralization of 4CP. The second culture was capable of completely mineralizing both phenol and 4CP, but each compound affects the degradation of the other. These cross-inhibitory kinetics have been mathematically described, and the SBR model predicts the best values for the operating parameters. Experiments are under way to verify the model's predictions.

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