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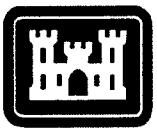

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Federal Integrated Biotreatment Research Consortium (FIBRC): Flask to Field Initiative

Rakesh Bajpai, Deborah R. Felt, Catherine C. Nestler, Altaf Wani, Jim C. Spain, Joanne Jones-Meehan, Guy W. Sewell, James M. Tiedje, Lance D. Hansen

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Federal Integrated Biotreatment Research Consortium (FIBRC): Flask to Field Initiative

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Under Project CU-720

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Preface

This report summarizes results of research conducted by the Federal Integrated Biotreatment Research Consortium (FIBRC) during 1994-2001. The Strategic Environmental Research and Development Program (SERDP) sponsored this project under its Cleanup Thrust Area Project CU-720. The U.S. Army Engineer Research and Development Center (ERDC) directed the FIBRC research program, which was entitled "Biotreatment: Flask to Field Initiative." Active membership of the FIBRC consisted of the following organizations:

ERDC Environmental Laboratory (EL), Vicksburg, MS

U.S. Army Natick Research, Development and Engineering Center, Natick, MA

U.S. Army Engineer District, Baltimore, Baltimore, MD

U.S. Air Force Research Laboratory, Tyndall Air Force Base, Panama City, FL

U.S. Naval Research Laboratory, Washington, DC

U.S. Naval Command, Control and Ocean Surveillance Center Research, Development, Test and Evaluation Division (NRaD), San Diego, CA

U.S. Environmental Protection Agency (USEPA) Environmental Research Laboratory, Athens, GA

USEPA Robert S. Kerr Laboratory, Ada, OK

Great Lakes and Mid-Atlantic Hazardous Substance Research Center (GLMAC), Ann Arbor, MI

In addition, the following organizations also participated in the FIBRC in an advisory capacity:

ERDC Cold Regions Research Engineering Laboratory (CRREL), Hanover, NH

ERDC Construction Engineering Research Laboratory (CERL), Champaign, IL

U.S. Army Environmental Center, Aberdeen Proving Ground, MD

U.S. Department of Energy (DOE) Argonne National Laboratory, Argonne, IL

The FIBRC Director during the preparation of this report was Dr. Jeffrey W. Talley, ERDC, Vicksburg, MS. Lead authors were Dr. Jim Spain, Air Force Research Laboratory; Dr. Jim Tiedje, Michigan State University, East Lansing, MI; Dr. Hap Prichard, Naval Research Laboratory; and Dr. Guy Sewell, Robert S. Kerr Research Laboratory. Executive Assistant for the FIBRC was Ms. Deborah R. Felt, Applied Research Associates (ARA), Vicksburg, MS.

During the time of writing of this report, Ms. Cathy Vogel served as SERDP's Program Manager for the Cleanup Thrust Area. Program managers included Dr. Femi Ayorinde, Joint UXO Coordination Office, Fort Belvoir. SERDP Directors were Mr. Bradley Smith, SERDP Program Office, and Dr. John Harrison, EL.

Dr. Rakesh Bajpai, Professor, University of Missouri-Columbia, wrote the main body of this report based largely on the key aspects of the appendices written by the thrust area leaders. Mr. Richard Conway, Union Carbide Corporation Senior Fellow (retired), SERDP Project Shepherd, Dr. Talley, and Mr. Daniel E. Averett, Chief, Environmental Engineering Branch, Environmental Processing and Engineering Division, (EPED), EL, reviewed and edited the main report, as well as the appendices. Ms. Felt and Ms. Catherine C. Nestler, ARA, assisted in the preparation, technical review and editing of the manuscript. Other authors of this report were Dr. Altaf Wani, ARA; Dr. Spain; Dr. Joanne Jones-Meehan, Naval Research Laboratory; Dr. Sewell; Dr. Tiedje; and Mr. Lance D. Hansen, EL. Authors of individual appendices are Dr. Spain, Dr. Tiedje, Dr. Prichard, and Dr. Sewell.

This study was conducted under the direct supervision of Mr. Averett and under the general supervision of Dr. Richard E. Price, Chief, EPED, and Dr. Edwin A. Theriot, Director, EL.

At the time of publication of this report, Dr. James R. Houston was Director of ERDC, and COL John W. Morris III, EN, was Commander and Executive Director.

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Summary

The Federal Integrated Biotreatment Research Consortium (Flask to Field) represented a 7-year Strategic Environmental Research and Development Program funded effort by several research laboratories to develop bioremediation technologies for contaminated U.S. Department of Defense (DoD) sites. The project was proposed as a new 6.2-level research program to enhance, but not duplicate, the existing funded efforts in the DoD. It also complemented both the U.S. Environmental Protection Agency and U.S. Department of Energy short- and long-term research strategies. The primary objective of this project was to develop the most promising biotreatment processes at the bench, intermediate, and pilot scale in each of four thrust areas: polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, energetics and explosives, and polychlorinated biphenyls (PCBs).

The consortium structure consisted of a lead principal investigator or director and four thrust area coordinators. Lead researchers at various government installations and academic institutions carried out the individual projects in each thrust area. Engineering groups worked closely with scientists in evaluating the potential of the resulting technologies and in the transfer of technologies from bench scale to field. A technical advisory committee consisting of leading biotechnology specialists in academia, industry, consulting, and government assisted and advised the director and reviewed individual projects for technical merit.

Many bioremediation projects were performed within the four thrust areas during Project CU-720. This report presents a complete list of projects and the lead researchers associated with them. The following are highlights of the accomplishments from each thrust area:

a. Energetics and Explosives.

(1) Bacteria able to utilize 2-nitrotoluene (2NT), 4-nitrotoluene (4NT), 2,4-dinitrotoluene (2,4DNT), and 2,6-dinitrotoluene (2,6DNT) as sole carbon and nitrogen sources were isolated, and the degradative pathways have been identified.

(2) Most (>98 percent) of the nitrotoluenes (NTs and DNTs) in contaminated groundwater from Volunteer Army Ammunition Plant, Chattanooga, TN, were removed at a retention time of 3 hours using a fluidized-bed reactor (FBR).

(3) A strain of *Pseudomonas* was isolated and found to rapidly transform 2,4,6-trinitrotoluene into previously unknown metabolites using a novel pathway. Two biodegradation pathways that model nitrobenzene remediation were also elucidated.

(4) In a 5-month field study at Badger Army Ammunition Plant, Baraboo, WI, the concentration of 2,4DNT in recirculating groundwater dropped from 16,000 to 130 µg/L and the ratio of 2,4- to 2,6DNT changed from 50:1 to 1:5.

(5) In regard to trinitrotoluene (TNT), some success in degradation was achieved with a FBR charged with granulated activated carbon. Other laboratory and field tests have not indicated that TNT is removed effectively during aerobic degradation of DNT. However, understanding the initial oxidative reactions of DNT-degrading bacteria could provide the basis for metabolic engineering to develop a new biochemical strategy for the initial attack on TNT.

b. Polycyclic Aromatic Hydrocarbons (PAHs).

(1) A bacteria strain capable of degrading high-molecular-weight PAHs in a laboratory setting and a surfactant-producing bacterium were isolated and characterized.

(2) Researchers developed an inoculum carrier technology using vermiculite for bioaugmentation of contaminated soil.

(3) Results from pilot-scale field tests in land farm treatment units (LTU) indicated PAH concentrations had decreased by 69 percent starting from an initial value of 13,000 mg/kg. PAH reduction in a highly contaminated soil was 86 percent and 87 percent using biostimulation and bioaugmention, respectively.

(4) While tilling appears to enhance the biodegradation of PAHs initially, landfarming of PAHs does not appear to be driven by soil mixing.

c. Chlorinated Solvents.

(1) Molecular probes were developed to monitor microbial potential of reductive dehalogenation of chlorinated solvents at contaminated sites.

(2) Post-extraction reductive dehalogenation of dense, nonaqueous phase liquid contamination was implemented and evaluated at a solvent extraction field site.

(3) Comparison of core material collected prior to and following the cosolvent-flushing test indicated removal effectiveness of polychlorinated ethenes at 65 percent.

(4) A design guidance document for implementing and monitoring Solvent Extraction Residual Bioremediation was written.

d. Polychlorinated Biphenyls (PCBs).

(1) Aerobic PCB metabolism by biphenyl-degrading organisms was characterized.

(2) Recombinant variants that metabolize PCBs were constructed.

(3) Polymerase chain reaction techniques were developed and tested for tracking genetically engineered microcorganisms in situ.

(4) Pilot scale activities are underway to evaluate the ability of PCB-degraders to reduce PCB concentrations in contaminated soil under controlled conditions.

Besides accomplishments, discussions of the utility of each thrust in remediation practice, transitional research, and technology transfer are presented.

1 Introduction

The Strategic Environmental Research and Development Program (SERDP)[1] Cleanup Project CU-720 was a multiagency project titled "Flask-to-Field Initiative" spearheaded by the Environmental Engineering Branch, Environmental Processes and Engineering Division, of the Environmental Laboratory, Vicksburg, MS, of the U.S. Army Engineer Research and Development Center (ERDC) (formerly the U.S. Army Engineer Waterways Experiment Station (WES)). This multiyear project was conducted between Fiscal Year (FY) 1994 and 2001 under the auspices of the Federal Integrated Biotreatment Research Consortium (FIBRC). The overall goal of the project was to develop field-implementable, cost-effective bioremediation technologies for contaminants of interest to the DoD. The active FIBRC membership consisted of the following agencies:

ERDC
U.S. Army-Natick Research Development and Environment Center, Natick, MA
U.S. Army Engineer District, Baltimore, Baltimore, MD
U.S. Air Force Research Laboratory, Tyndall Air Force Base, Panama City, FL
U.S. Naval Research Laboratory, Washington, DC
U.S. Naval Command, Control and Ocean Surveillance Center Research, Development, Test and Evaluation Division (NRaD), San Diego, CA
EPA Environmental Research Laboratory, Athens, GA
EPA Robert S. Kerr Laboratory, Ada, OK
Great Lakes and Mid-Atlantic Hazardous Substance Research Center (GLMAC), Ann Arbor, MI

Work was conducted at these institutions during the project period. Lead researchers associated with the project at each institution are listed in Table 1.

A number of these projects were possible because of the sustained funding of research and collaborations of different scientists, engineers, practitioners, regulators, and site managers associated with Project CU-720. The Consortium

[1] SERDP is the U.S. Department of Defense's (DoD) corporate environmental research and development (R&D) program, planned and executed in full partnership with the U.S. Department of Energy (DOE) and the U.S. Environmental Protection Agency (EPA), with participation by numerous other Federal and non-Federal organizations.

Table 1
Lead Researchers Associated with FIBRC Projects Between FY 1994-FY 2001

Government Agency	Lead Researcher
U.S. Air Force Research Laboratory Tyndall Air Force Base, Panama City, FL	Dr. Jim Spain
U.S. Army Engineer District, Baltimore Baltimore, MD	Ms. Pam Sleeper
ERDC Vicksburg, MS	Dr. Herbert Fredrickson Dr. Douglas Gunnison Mr. Lance Hansen Mr. Jerry Miller Ms. Catherine Nestler Dr. Kurt Preston Dr. Mohammad Qasim Dr. Jeffrey W. Talley Dr. Altaf Wani Dr. Mark E. Zappi
U.S. Army Natick Research and Development Center Natick, MA	Dr. Charlene Mello
EPA Ada, OK	Dr. Guy Sewell
EPA Athens, GA	Dr. Steven C. McCutcheon Dr. Lee Wolfe
NRaD San Diego, CA	Dr. Sapine Aptiz
U.S. Naval Research Laboratory Washington, DC	Dr. P. Hap Pritchard
Howard University Washington, DC	Dr. Jim Johnson
Georgia Institute of Technology Atlanta	Dr. Kurt Pennell
Michigan State University East Lansing	Dr. James M. Tiedje Dr. John F. Quensen III
University of Michigan Ann Arbor	Dr. Walter Weber

provided the scientists with access to field samples for research and to the practical aspects of developing new technology. The practicing engineers and site management personnel had an opportunity to work with the scientists developing the technology, and the interactions resulted in bidirectional flow of information between several projects.

In addition, the following organizations also participated in the consortium in an advisory capacity:

U.S. Army Engineer Cold Regions Research and Engineering Laboratory, Hanover, NH
U.S. Army Engineer Construction Engineering Research Laboratory, Champaign, IL
U.S. Army Environmental Center, Aberdeen Proving Ground, MD
DOE Argonne National Laboratory, Argonne, IL

Between FY1994-2001, the Consortium (FIBRC) was headed by several directors who supervised the Project CU-720:

Dr. Mark Zappi	FY 1994 - 1995
Dr. Kurt Preston	FY 1995 - 1996
Dr. Jeffrey Talley	FY 1996 - 1997
Dr. Rakesh Bajpai	FY 1997 - 1999
Dr. Jeffrey Talley	FY 1999 - 2001

2 Project Background

It has been estimated that the DoD has over 21,000 contaminated sites containing hazardous organic chemicals requiring some form of remediation; the total cost of their cleanup has been estimated to be $30 billion (Table 2). The hazardous organic chemicals on these sites can be classified in the following categories:

a. Low-molecular-weight fuels (hydrocarbons), aldehydes, ketones, alcohols, phenols.

b. Polycyclic aromatic hydrocarbons (PAHs).

c. Explosives and energetics (nitrotoluenes, nitroazides, perchlorates).

d. Chlorinated solvents (chloroethenes and chloroethanes).

e. Polychlorinated biphenyls (PCBs).

Table 2
Typical Costs for Remediation of Soils and Groundwater

Matrix	Treatment Technology	Cost
Soil	Chemical extraction	$110-440 / m ton
	Base catalyzed decomposition	$220-550 / m ton
	Composting	$190-290 / m ton
	Excavation and landfill disposal	$300-510 / m ton
	Incineration	$470-590 / m ton
	Thermal desorption	$45-330 / m ton
Water	Carbon adsorption	$0.32-1.70 / 1000 L
	UV/Oxidation	$0.03-3.00 / 1000 L

Reference: Federal Remediation Technologies Roundtable Web site (updated in 2001), http://www.frtr.gov

All hazardous chemicals have the potential to impact the environment, but those under categories *b* through *e* are particularly problematic due to both their potentially toxic, mutagenic, teratogenic, and/or carcinogenic effect on the exposed receptors and their recalcitrant nature (resistance to degradation).

Environmental restoration technologies consist of nondestructive and destructive techniques. The nondestructive technologies (air stripping, soil washing, thermal desorption, adsorption, extraction, landfilling, containment, stabilization) generally use physical processes either to contain the contaminant or to transfer the contaminant from one phase or media to another, thereby at times postponing the task of contaminant destruction. For the sake of many site restorations, the nondestructive technologies represent the best available

technology (BAT) at present. While these are accepted today, technologies that destroy or recover/reuse the chemicals are more desirable and may even be mandated in the future. As a result, the government funding and regulatory agencies (DoD, DOE, EPA, U.S. Department of Agriculture (USDA)) have been encouraging development of innovative destructive technologies.

The destructive technologies include thermal, chemical, and biological transformations. Thermal and chemical technologies usually require excavation of the contaminated material and additional material handling. These activities result in high energy costs and potential release of the contaminant to the environment and exposure to personnel. Bioremediation (the biological technology for transformation/mineralization of contaminants and site clean-up/restoration) has been successfully commercialized for treatment of low-molecular-weight total petroleum hydrocarbons (TPH) and other easy-to-degrade compounds; it offers potential for recalcitrant compounds, but needs further advances for widespread commercial application.

Bioremediation takes advantage of diversity of microbial metabolism to transform/degrade the contaminants of interest. Biological treatment methods offer the advantage of transformation under mild conditions, environmental friendliness, potentially in situ operation, conversion of organics to less toxic or innocuous compounds (carbon dioxide and water in the limit), and potentially lower costs. It can also be more toxic, e.g., generation of vinyl chloride from degradation of polychlorinated ethenes (PCE), trichlorothylene (TCE), and dichloroethylene (DCE). The basic premise of this technology is that the microbial capability to metabolize the hazardous chemicals (whether anthropogenic or natural) either already exists in nature or can be developed by the currently available tools of molecular biology. While this is generally true, widespread commercial application of bioremediation technology is hampered by the following considerations:

a. Design, operation, and evaluation of bioremediation technologies require sophisticated knowledge of microbial transformation pathways, process control, and analysis of large number of samples. A good understanding of the microbial metabolism of the specific hazardous compound(s) is essential for the success of the technology. The metabolic pathway(s) used to transform a compound, the extent of transformation, and the degree of its completion depend on the type of microorganisms as well as the environmental conditions present in a medium. One cannot take for granted that the microbial transformation would always result in formation of innocuous compounds, as previously indicated. Failure to realize this fact may result in an inability to achieve the desired level of cleanup, in rare cases may even exacerbate the problem, and thus inappropriately brand the biotreatment technology inappropriately as "unsuccessful" in spite of its potential. One example is the formation of vinyl chloride (a known carcinogen) as an intermediate during anaerobic microbial transformation of trichloroethylene (a suspected carcinogen). Accumulation of vinyl chloride must be avoided by appropriate control of the bioremediation operation.

b. Given the heterogeneity and diversity in the nature of hazardous chemicals, the microbial population, and the subsurface geochemistry,

one cannot make an a priori assumption that microbial capability to degrade a given hazardous chemical will be present at a specific site. Even in the presence of sufficient microbial capability in an environment, physical considerations (availability of the hazardous chemical to the microbial community and mass transfer) and/or chemical considerations (nutrients, electron acceptors and donors) may hinder the requisite transformation. It is well documented that some naturally occurring microorganisms require a separate energy source (phenomenon of cometabolism) in order to transform trinitrotoluene (TNT), high-molecular-weight (HMW or hPAH) PAHs, polychlorinated aliphatics, and PCBs. Sound knowledge of microbial ecology and physiology, and interactions of nutrients and electron donors and acceptors with the environmental matrices is necessary for successful application of bioremediation.

c. The technologies and processes developed by scientists and engineers at bench scale still must overcome the issues of field-scale heterogeneity, uncertainty, implementation, economics, robustness, and regulators' and vendors' caution toward an unproven technology. This is one of the key roadblocks to widespread use of bioremediation in site restoration.

The expertise to address these considerations encompasses many diverse disciplines (basic sciences, engineering, program and project management, economics, public affairs). Development of bioremediation technology for the restoration of contaminated sites requires a multidisciplinary team approach that must comprehensively address the issues related to microbial ecology, metabolism, physiology, metabolic engineering, reactor analysis and development, process scale-up, economic analysis, field implementation, and technology promotion. Such a coordinated action has generally not been possible under the normal framework of single institutional research and development. Sporadic and often sparse funding, inaccessibility to actual contaminated sites, poor communication between research and development groups, inadequate design and application engineering input into research plans, and poor technology transfer to applied technology user groups have severely hindered bioremediation process development.

SERDP Cleanup Project CU-720, "Flask to Field Initiative," was initiated in FY 1994 to address these concerns for recalcitrant compounds. It was a multi-year and multidisciplinary team effort. Multidisciplinary teams involving several eminent laboratories and engineering organizations of DoD, EPA, and academia were formed under the umbrella of a consortium led by ERDC, Vicksburg, MS. These laboratories and remediation specialists were selected based on their expertise in one or more of the disciplines mentioned previously, history of cooperation, and their ability to contribute to a complete solution for specified problems.

The project was proposed as a new 6.2-level research program to enhance, but not duplicate, the existing funded efforts in the DoD. It also complemented both the EPA and DOE short- and long-term research strategies. In planning this project the following 1993 DoD user requirements were considered and later prioritized:

- Processes to remediate groundwater contaminated with hydrocarbon fuels.
- Treatment systems for water contaminated with organic contaminants.
- Treatment systems for water contaminated with chlorinated and defense hydrocarbons.
- Treatment systems for water contaminated with mixtures of chlorinated solvents.
- Treatment of Navy-relevant contaminants in salt/brackish/groundwater matrices.
- Dredged sediments area decontamination and reclamation.
- Improved marine sediment remediation technologies for metals, organics, and PCBs.
- Contamination under buildings and roads.
- Technologies for isolation and decontamination of sludges.
- Decontamination of soils containing energetic materials.
- Remedial treatment technology for soils contaminated with chlorinated and nonchlorinated organics.
- Isolation and treatment technology for contaminated surface water impoundments.
- Improved shore and open ocean hazardous materials cleanup/restoration.
- Improved fate, effects, and transport models for groundwater.
- Enzyme and bacterial treatment technology.
- Improved cleanup procedures at locations where underground storage tanks (UST) have leaked.

These user problems and technology requirements pertained to a large list of remediation technologies identified by the DoD installation environmental officers and U.S. Army Corps of Engineers Districts (users) as needing futher investigation by DoD researchers based on the existing problems facing the users in their efforts to remediate federal lands. These problems also represented those experienced by environmental engineers involved with Comprehesive Environmental Response, Compensation, and Liability Act (Superfund) (CERCLA) and Resource Conservation and Recovery Act (RCRA) activities.

3 Goals and Objectives

The overall goal of the project was to develop field-implementable, cost-effective bioremediation technologies for contaminants of interest to the DoD. The specific objectives were to increase understanding of bioremediation (metabolism, process mechanisms, and limitations) and to bring at least one process in each problem contaminant class to field status. Priority contaminants of interest were energetics and explosives, PAHs, chlorinated solvents, and PCBs. The matrices of concern were soil and groundwater. Specific objectives for each contaminant class were as follows:

a. Energetics and Explosives.

- Discover microorganisms able to destroy nitroaromatic contaminants.
- Characterize pathways, enzymes, and reactions.
- Adopt these systems for remediation of environmental contamination in bioreactors and scale-up.
- Demonstrate bioremediation potential at pilot and field scale.
- Develop technologies for in situ bioremediation of nitroaromatic compounds.
- Gain an understanding of the control and organization of degradation pathways for nitroaromatic compounds so that organisms with an expanded catabolic range can be metabolically engineered to degrade more recalcitrant nitroaromatic compounds.

b. Polycyclic Aromatic Hydrocarbons.

- Isolate and characterize bacteria capable of degrading HMW PAHs.
- Evaluate biosurfactants for enhancing bioavailability of sorbed PAHs and isolate/characterize bacteria producing the biosurfactants.
- Develop techniques for bioaugmentation of contaminated soil.
- Implement and test bioremediation of HMW PAHs in pilot-scale field tests.

c. Chlorinated Solvents.

- Develop tools to monitor microbial potential of reductive dehalogenation of chlorinated solvents at contaminated sites.
- Implement and evaluate post-extraction reductive dehalogenation at a solvent extraction field site based on pre-established flask-level potential for reductive dehalogenation at a given site.

- Prepare a design guidance document for implementation and monitoring of Solvent Extraction Residual Bioremediation.

d. Polychlorinated Biphenyls.

- Develop genetically engineered organisms capable of growing on PCBs.
- Evaluate additives (surfactants and other chemicals) to enhance PCB dechlorination.
- Implement and test PCB bioremediation in pilot-scale field tests.

4 Technical Approach

The organizational structure of the multiteam project is shown in Figure 1, with the Consortium Director as project leader responsible for overall project coordination and management, and reporting to the SERDP directorate and its Science Advisory Board (SAB). The task of biotreatment process development was organized into four thrust areas, one for each priority contaminant class. An eminent scientist/engineer was assigned the task of thrust area leadership. The thrust area leader was responsible for coordinating the activities of the different research and development teams related to the contaminant class, as well as for reporting the activities to the Consortium Director. The membership of the thrust areas was based on the expertise of scientists and engineers in the active institutions of the Consortium.

A fifth thrust area, design and evaluation, was also formed in the initial years of the project. The membership of this thrust area included engineers from the U.S. Army Engineer District, Baltimore, and ERDC. Both of these institutions have extensive experience in economic evaluations and field implementation of the remediation technologies. As a result, the goal of this thrust area was to assist the researchers in other thrust areas in periodic evaluation of impact of their own research (and development) on the overall design and economics of their proposed technologies. In the later years, as the projects approached the field activity, this design and evaluation activity ceased to be a separate thrust area and became an integral part of activities of the remaining thrust areas.

The philosophy of process development in each thrust area involved a broad project mix during the flask phase of the project, followed by focus on selected projects deemed to be sufficiently advanced to reach a field implementation stage in the later years. Each of the initial projects was targeted to a specific technology. The Consortium Director regularly evaluated progress from the quarterly and annual projects reports and presentations at the annual project meetings. Yearly presentations to the SERDP SAB and to the SERDP staff also provided opportunities to get external feedback. As the projects progressed, a Technical Advisory Committee (TAC) was formed by ERDC and the Consortium Director to assist the Director in the selection and focusing of the project(s) during further development. The various members of the TAC during the course of the project are listed in Table 3. The TAC first met in May 1997 and from then on continued to meet twice a year (until December 2000) to evaluate the continuing projects and to provide their evaluations to the Consortium Director. TAC thus represented transformation of review process of the consortium projects from an informal to a formal and regular review.

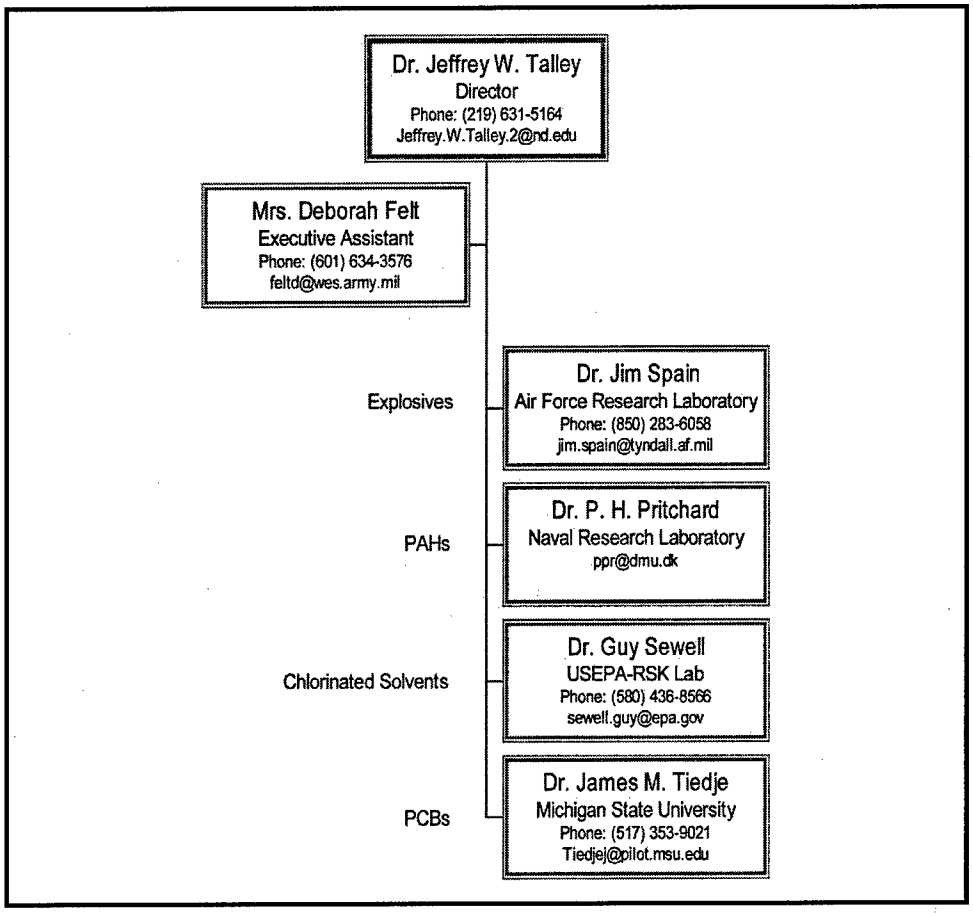


Figure 1. Consortium organization

The TACs provided a valuable input by evaluating the progress of research at different stages and influencing the Consortium resources to research and developments most likely to be field implemented. Since the advisory committee had a number of practicing engineers in its membership as well, the technologies being developed by the Consortium members were broadcast to the user community as well. A key advantage of the multiyear Consortium-type project was the high-quality technical review of progress by the different projects on a continuous basis and potential of sustained funding for outstanding projects.

A schematic of the biotreatment process development in the Consortium is presented in Figure 2. The technical approach within the Consortium was to develop the most promising biotreatment processes at the bench scale and then validate the technology at the pilot and field scale. Engineering groups worked closely with scientists in evaluating the potential of the resulting technologies and in the transfer of technologies from bench scale to field. The TAC periodically reviewed projects for technical merit. The TAC was most active from 1997 to 2000 in assisting the Consortium Director in focusing the Consortium resources on projects with the most potential for process engineering and pilot/field application. As a result, several laboratory projects were discontinued in spite of satisfactory progress, and resources were redirected to

Table 3
TAC Members of FIBRC

Name	Company	Years Served
Dr. Al Bourquin	Camp Dresser & McKee	1997 – 2000
Dr. Richard Brown	ERM, Inc.	1997 – 2000
Mr. Glenn Celerier	US Environmental Protection Agency	1997 – 2000
Dr. Frank Chappelle	US Geological Survey, Research and Development	2000 – 2000
Mr. Richard Conway	Union Carbide (retired)	1999 – 2001
Dr. Ron Crawford	University of Idaho	1998 – 2000
Dr. Craig Criddle	Stanford University	1998 – 2000
Dr. Robert Hinchee	Parsons Engineering Sciences	1997 – 2000
Mr. Ron Hoeppel	U.S. Navy/Naval Facilities Engineering Service Center	1997 – 1999
Dr. Joseph Hughes	Rice University	2000 – 2000
Dr. Gary Klecka	Dow Chemical Company	2000 – 2000
Dr. Donna Kuroda	U.S. Army Corps of Engineers	1997 – 2000
Dr. Perry McCarty	Stanford University	1997 – 1998
Dr. Anna Palmisano	U.S. Department of Energy	1997 – 2000
Dr. Roger Prince	Exxon	1998 – 2000
Ms. Cathy Vogel	U.S. Air Force	1997 – 1998
Dr. C. Herb Ward	Rice University	1997 – 2000
Dr. David White	University of Tennessee	1997 – 1999
Dr. Joseph Zelibor	National Research Council	1997 – 1998

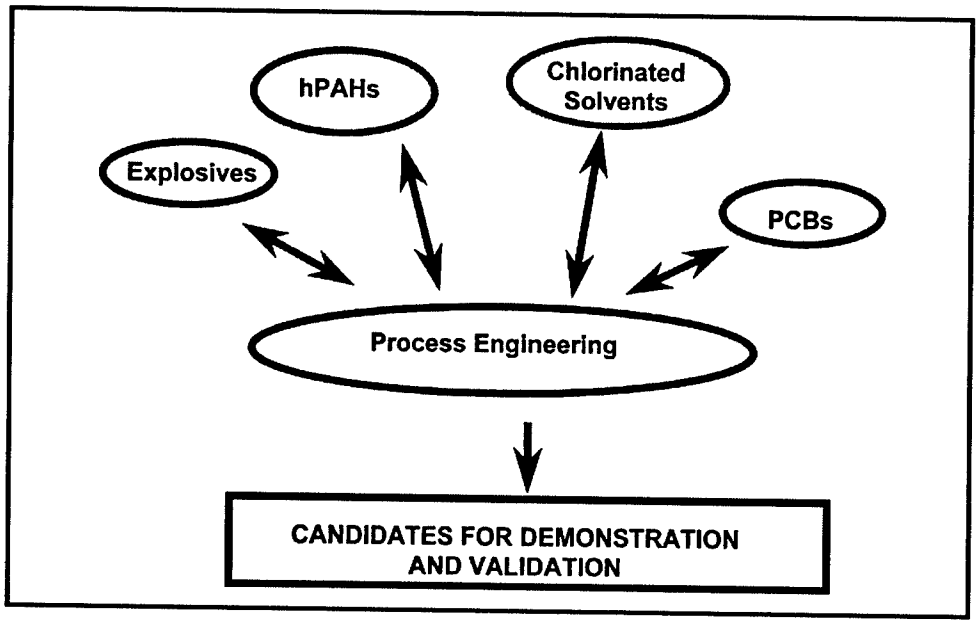


Figure 2. Biotreatment process development in FIBRC

projects in pilot and field stages of evaluation. This regular evaluation and redirection of resources remained a continued activity until December 2000, when the project moved on to the stage of final reporting.

This report focuses only on those projects that went to the pilot and/or field evaluation stage. Detailed reports on each of these projects are included in this final report as appendices. The following section on highlighted accomplishments summarizes the achievements in each contaminant class. The

discontinued projects were described in the annual reports to SERDP and resulted in several peer-review publications. A list of all the projects funded by the Consortium between FY 1994 and FY 2001 is presented in Table 4.

Table 4
List of Projects Funded by FIBRC

Thrust Area	Principal Investigator	Title
Explosives	Spain	Discovery of Novel Enzymatic Reactions and Determinations of Biodegradation Mechanisms
	Sleeper	Completion of FBR Pilot Demonstration for Biodegradation of DNTs in Groundwater at VAAP
	Mello	Enhanced TNT Biodegradation Through Genetic Modification
	McCutcheon	Phytoremediation of Munitions Contaminated Soils and Sediments
	Miller	Phytoremediation of Explosives Contaminated Groundwater using Wetland and Aquatic Plants
	Qasim	Enhancing TNT Biodegradability by Chemical Transformation of TNT
hPAH	Pritchard Jones-Meehan	Bioremediation of HMW PAHs: Effectiveness of Specialized Techniques in Bioaugmentation and Bioavailabity Enhancement
	Fredrickson	Bioremediation of HMW PAHs: Effects of Contaminant Mixtures on hPAH Biotreatability
	Weber	Intermittently Mixed Reactor Systems for Enhancement of Mass Transfer and Bioavailability in the *In-Situ* Treatment of DNAPL-Contaminated Soil and Sediments
Chlorinated Solvents	Sewell	Anaerobic Bioactive Wall for In-Situ Reductive Bioremediation of Chloroethenes using Induced Electrical Potential
	Sewell	Solvent Extraction Residual Biotreatment (SERB)
	Tiedje	Distinguishing the Microbial Communities Active in the Enhanced Anaerobic Treatment of Chlorinated Ethenes
	McCutcheon	Phytoremediation of Shallow Chlorinated Solvent Plumes: Engineered Tree Plantations and Constructed Wetlands
PCBs	Tiedje, Pennell, Fredrickson	Enhancing PCB Bioremediation
Design	Sleeper	Design and Cost Evaluation
Support	Talley, Bajpai	Project Management, General Support to PIs and TAC; Contingencies for Initiating Field Activities in hPAH or PCB Thrust Areas

5 Highlighted Accomplishments

Energetics and Explosives

This thrust area was led by Dr. Jim C. Spain of the Air Force Research Laboratory (AFRL), Tyndall Air Force Base, FL. A detailed summary of the accomplishments of the group under Project CU-720 funding is provided by the thrust area leader in Appendix A. Major contributors to the effort at the AFRL are identified in the thrust area leader's report. In addition, Baltimore District, Envirogen, Inc., Malcolm Pirnie, Inc., and Stone and Webster Environmental Technology and Services also contributed to pilot and field-scale investigations. The work conducted under the project resulted in 38 peer-reviewed publications, 6 technical reports, 3 conference/symposium proceedings papers, 44 published technical abstracts, and 8 published textbooks/chapters.

The following were key accomplishments in the explosives thrust area:

a. *Characterization of novel metabolites formed from TNT by a nitrobenzene-degrading strain of* Pseudomonas pseudoalcaligenes. Strain JS45, isolated for its ability to grow aerobically on nitrobenzene (NB), was found to transform TNT rapidly into previously unknown metabolites using a novel pathway. Nitrobenzene nitroreductase from JS45 partially reduces nitro groups to hydroxylamines but not to amines. The enzyme catalyzed the reduction of TNT to yield 2,4-dihydroxylamino-6-nitrotoluene (DHANT). In whole cells, a nonspecific nitroreductase catalyzed the conversion of DHANT to 2-hydroxylamino-4-amino-6-nitrotoluene (2HA4ANT). 2HA4ANT was further oxidized to unknown products that disappeared during subsequent incubation, and a major portion of the carbon was incorporated into the cells. Identification of the end products and evaluation of kinetics of the reaction(s) are continuing in Dr. Spain's laboratory.

b. *Pathways and genetic studies for microbial degradation of mono- and dinitrotoluenes*. Bacteria able to utilize 2-nitrotoluene (2-NT), 4-nitrotoluene (4-NT), 2,4-dinitrotoluene (2,4-DNT), and 2,6-dinitrotoluene (2,6-DNT) as sole carbon and nitrogen sources have been isolated and the degradative pathways have been identified both prior to and under the auspices of Flask to Field. Three different pathways for degradation of 4-NT have been identified in bacteria, but only a single 2-NT degradation pathway has been found. Several strains degrading the DNTs have been isolated. The DNT degradation pathways found in all

isolates to date start with an initial oxidative attack that results in the release of one nitro group from the DNT ring. The pathways for degradation of 2,4- and 2,6-DNT then diverge. 4-Methyl-5-nitrocatechol formed from 2,4-DNT undergoes a monooxygenese attack at the remaining nitro-substituted position to form 2-hydroxy-5-methyquinone with the release of the second nitro group. The quinone undergoes ring cleavage following conversion to 2,4,5-trihydroxytoluene by a quinone reductase. In contrast, 3-methyl-4-nitrocatechol formed from 2,6-DNT is attacked by an extradiol ring-cleavage-dioxygenase to yield 2-hydroxy-5-nitro-6-oxo-2,4-heptadienoic acid. The acid is then hydrolyzed to 2-hydroxy-5-nitro-2,4-pentadienoic acid and acetic acid. The second nitro group is released in a subsequent reaction. The genes involved in the pathways have been cloned and sequenced.

c. *Bench-scale groundwater studies for ex situ bioremediation of DNT mixtures.* A small fluidized-bed reactor (FBR) with sand as carrier for microorganisms was used to treat an artificial groundwater containing 2,4- and 2,6-DNT. Complete and simultaneous mineralization of mixtures of DNTs was demonstrated at various DNT loading rates. The hydraulic retention times and oxygen requirements to support degradation were established. During the process, a "dual degrader" capable of degrading both DNTs simultaneously was isolated. When the artificial groundwater was replaced with contaminated groundwater from Volunteer Army Ammunition Plant (VAAP), Chattanooga, TN, containing mixture of NTs and DNTs, >98 percent of all the nitrotoluenes were removed at a hydraulic retention time of 3 hours with the exception of 2,6-DNT, for which the removal efficiency was 62 percent. When sand was replaced with granular activated charcoal (GAC) as the cell-carrier, the mixed microbial community attached to GAC was capable of degrading all of the nitroaromatic compounds (NTs, DNTs, TNT), and the attached community could be recovered after storage. However, the GAC needed to be saturated with all the nitroaromatics before inoculation with degradative bacteria in order to have a successful start-up of the reactor.

d. *VAAP pilot study for treatment of groundwater contaminated with nitrotoluenes.* A pilot-scale field study based on the bench-scale studies was conducted for 5 months in spring-summer 1998 at VAAP. The objective was to demonstrate the feasibility of using an FBR to remediate nitrotoluene-contaminated groundwater and to establish the parameters for designing full-scale FBR systems for nitrotoluene remediation. The groundwater at the site contained a mixture of mono-, di-, and trinitro-toluenes. The groundwater required pH adjustment but no other amendments. Temperature control was also not necessary. The removal efficiencies for 2-NT, 3-NT, 4-NT, and 2,4-DNT were high throughout the test period. Removal efficiencies for TNT varied between 33 and 73 percent depending on the hydraulic retention time. Removal of 2,6-DNT was low during the initial phases of the study, but improved dramatically after 4 months of operation. The cause for initial inhibition of degradation of 2,6-DNT was attributed to some nonpolar organic component present in VAAP groundwater to which the system

acclimated after several months. Cost analysis, based on treatment rates limited by degradation rates of 2,6-DNT, showed that the aerobic FBR technology is more cost-effective than ultraviolet (UV)-ozone treatment or simply GAC treatment when the total nitrotoluene removal rate exceeds 120 lb/day (54 kg/day).

e. *Bioremediation for treatment of a 2,4-DNT contaminated industrial site.* Preliminary studies indicated that added DNT-degrading microorganisms rapidly mineralize DNT present in the seep water from the waste gypsum stack of a former phosphate fertilizer plant. Indigenous DNT degraders were isolated from the holding ponds on the site, and pH and nutrient conditions for DNT degradation in seep water were established in the laboratory. Artificial wetlands are being planned as the treatment alternative for DNT contamination at the site.

f. *Biodegradation of DNT in soil slurries.* Remediation of DNT in contaminated soil was demonstrated in bench-scale slurry reactors. Both 2,4- and 2,6-DNT were repeatedly removed from highly contaminated soil by inoculated DNT-degrading bacteria. Forty-three to sixty percent of radiolabeled DNT was recovered as $^{14}CO_2$. In studies with contaminated soil in pilot-scale airlift reactors, coarse sand particles needed to be removed in a preprocessing wash step. At low loading rates of soil (continuous feed), both the DNT isomers were degraded, but the operation was limited by the degradation rate of the more recalcitrant isomer. Sequential treatment of 2,4- and 2,6-DNT reduced the overall treatment time. Nitrite toxicity to 2,4-DNT degradation limited the soil loading to 40 percent. Cost analysis showed that sequential slurry treatment system is competitive with thermal treatment with estimated costs of $100-200 per cubic yard.

g. *In situ bioremediation of DNT in soil.* Feasibility of in situ bioremediation of DNT-contaminated soil was investigated with soil obtained from Badger Army Ammunition Plant (BAAP), Wisconsin. The soils contain indigenous populations of DNT-degrading bacteria that are adapted to degrade DNT at high soil pH. Addition of phosphate and moisture were sufficient to initiate DNT degradation in field-contaminated soils within 2 weeks at room temperature and within 2-3 months at normal soil temperature of 13 °C. No other macronutrient was required for complete degradation of DNT. Phosphate addition stimulated but was not necessary for DNT degradation. Low temperature, lack of moisture, and accumulation of nitrite are the main deterrents to rapid biodegradation of DNT in the BAAP soils. A demonstration of in situ degradation of DNT was performed by Stone and Webster Engineering for the U.S. Army Corps of Engineers and BAAP. Air was delivered by converted soil-vapor extraction vents, moisture and macronutrients were supplied by groundwater recharged through infiltration pipes, temperature was manipulated by heating the recharged groundwater, and nitrite was destroyed in an artificially created downgradient anaerobic zone. In a 5-month study that started in April 2000, the concentration of 2,4-DNT in recirculating groundwater dropped from 16,000 to 130 mg/L and the ratio of 2,4- to 2,6-DNT changed from 50:1 to 1:5. Cost savings for

cleanup of DNT-contaminated soil at BAAP using this technology has been estimated to be $60-65 million with a time saving of 10 years.

h. *Nitrobenzene (NB) degradation pathways.* An oxidative pathway for biodegradation of NB, a model nitroaromatic compound, was elucidated. Extensive work was done to purify the enzymes and identify the genes of both the oxidative pathway and the previously identified partially reductive pathway in order to better understand the mechanisms by which nitroaromatic degradation pathways evolve.

Polycyclic Aromatic Hydrocarbons

This thrust area was led by Dr. P. H. Pritchard of the Naval Research Laboratory, Washington, DC. A detailed summary of accomplishments by this group under the Project CU-720 funding is provided by the thrust area leader as Appendix B. Major contributors in this effort included Dr. Joanne Jones-Meehan and Dr. William Straube (Naval Research Laboratory), Mr. Lance Hansen (ERDC, Vicksburg, MS), Ms. Cathy Nestler (Applied Research Associates Inc., Southern Division, Vicksburg, MS), and Drs. William Jones and John Hind (Maryland Biotechnology Institute, Baltimore, MD). The work conducted under this project contributed to 3 peer-reviewed publications, 2 technical reports (an additional 2 reports under preparation), 4 conference/symposium proceedings papers, 11 published technical abstracts, 2 international presentations, and 1 patent application.

The following were key accomplishments in this thrust area:

a. *Isolation and characterization of bacteria capable of degrading high-molecular-weight PAHs.* Isolates capable of growing on PAHs were obtained from PAH-contaminated soil using enrichment culture techniques. These isolates did not grow on higher molecular weight PAHs (pyrene and above) but demonstrated an ability to degrade these compounds, suggesting that cometabolism was occurring. Most of the isolates were also able to degrade low-molecular-weight PAHs. Using a modified enrichment procedure and a plate screening technique, a variety of cultures capable of using fluoranthene or pyrene as growth substrates were isolated. Many of these isolates transformed other 4-, 5-, and 6-ring PAHs as well, and belonged to *Sphingomonas* and *Mycobacterium* spp. When the isolates were grown in the presence of supplemental carbon sources, the rates of degradation of PAHs were faster suggesting that the capability to degrade PAHs was not affected by alternative growth substrates. EPA 505 was highly effective in removing 4-, 5-, and 6-ring PAHs (67 percent removal compared to 28 percent removal by the next best strain, *Mycobacterium* strain PRY-1) and showed the highest rate of removal (91 percent) of the PAHs overall. Only two or three other isolates were able to grow on or cometabolize PAHs as well as the *Sphingomonas paucimobilis*, strain EPA 505.

b. *Pyrene degradation.* Pyrene, a nongrowth PAH for EPA 505, is cometabolically degraded by this strain, forming two different partial degradation products. Both of these compounds suggested opening of an

aromatic ring, and both indicated an inability to cleave carbon moieties to use for growth. The ability to transform a compound by two different actions was accredited to a very loose specificity of the dioxygenase enzymes responsible for metabolizing phenanthrene and fluoranthene.

c. *Phenanthrene.* Phenanthrene and pyrene are cometabolized by *S. paucimobilis* EPA 505, which prefers the former to the latter PAH. Phenanthrene inhibits pyrene metabolism, but does not suppress it completely. However, pyrene metabolism in cells pre-exposed to phenanthrene was faster than in the cells not pre-exposed to phenanthrene. This observation was recognized to have great significance for the PAH degrader if it needs to be added to the soils in order to enhance PAH biodegradation.

d. *Chemical surfactants.* The chemical surfactant Triton X-100 was found to be toxic to PAH degraders belonging to *Sphingomonas* spp. and *Mycobacterium* spp. The nonionic surfactant Tween 80, however, had a stimulatory effect on the mineralization of fluoranthene by both the microorganisms. When the degradation of a mixture of PAHs by *S. paucimobilis* EPA 505 was studied in presence of Tween 80, an increase in availability of all the PAHs to the microorganisms was observed. At the same time, an ordered sequential degradation of phenanthrene, fluoranthene, and pyrene was observed. The sequential degradation was probably due to specificity of the initial dioxygenase system in the cells to the three PAHs.

e. *Evaluation of biosurfactants for enhancing bioavailability of sorbed PAHs and isolation/characterization of bacteria producing the biosurfactants.* Using extraction experiments for phenanthrene and fluoranthene, it was established that rhamnolipid biosurfactant was much more effective at emulsifying PAHs from soil than was lichenysin biosurfactant. A rhamnolipid-producing strain, *Pseudomonas aeruginosa* strain 64, was isolated in ERDC using enrichment cultures from TNT-contaminated soil. The cell-free culture broth of *P. aeruginosa* strain 64, grown on glycerol, was found to cause a significant enhancement in solubilization of PAHs from contaminated soils (5-6 times more HMW PAHs in solution than in control). The enhancement in solubilization of phenanthrene, fluoranthene, and pyrene by rhamnolipid was also greater than that produced by chemical surfactant, Triton X-100, as illustrated in Figure 3 (Figure 9 in Appendix B).

f. *Development of carrier technology for bioaugmentation of contaminated soil.* Laboratory studies showed that vermiculite-carried *P. aeruginosa* strain P64 and *S. paucimobilis* strain EPA 505 cells remain viable for several months when stored on the biocarrier at room temperature. The ability of EPA 505 to mineralize fluoranthene in soil was not affected by immobilization of the cells on vermiculite. Dried blood fertilizer was found to be an effective source of degradable carbon and nitrogen for *P. aeruginosa* strain 64 and resulted in production of rhamnolipid biosurfactants in liquid cultures. For soil use, the loading rate was established at 2 percent vermiculite inoculated with *P. aeruginosa* (by

weight of the soil) and 2 percent dried blood fertilizer (by weight of the soil).

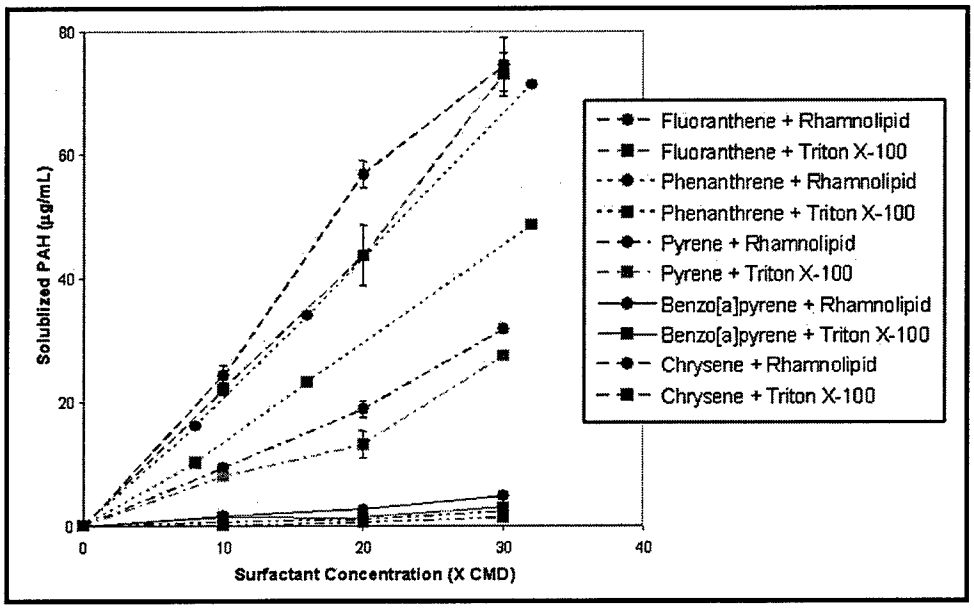


Figure 3. The solubilization of PAHs by *P. aeruginosa* strain 64 rhamnolipid and Triton X-100 (a synthetic surfactant)

g. *Implementation and testing of bioremediation of HMW PAHs in microcosms and pilot-scale field tests.* Microcosm studies were conducted with 100 g pentachlorophenol (PCP)- and PAH-contaminated soil from the Popile, Inc., site, a former wood treatment facility in El Dorado, AR (13,000 mg/kg PAHs and 1,500 mg/kg PCP). Both the biostimulation and the bioaugmentation soil samples were mixed with ground rice hulls as bulking agent, and dried blood fertilizer. Bioaugmentation was accomplished using *P. aeruginosa* strain 64 on vermiculite carrier. All samples were placed in sterile plastic cups with tight-fitting snap lids. Water content in the soil was maintained at 30-40 percent by weight. The cup contents were stirred thoroughly weekly to simulate tilling of field units. A dramatic decrease (87 percent in total PAHs and 67 percent in total Benzo(*a*)pyrene (B*a*P) equivalents) was observed in the *P. aeruginosa* bioaugmented microcosms compared with decreases of 34 percent in total PAH and 57 percent in total B*a*P equivalents in cups that were simply biostimulated (i.e., supplied only with bulking agent, dried blood fertilizer, and stirring) after 11 months of incubation. The corresponding decreases in control cups were 23 percent and 48 percent, respectively.

(1) Since *P. aeruginosa* strain 64 does not degrade phenanthrene or HMW PAHs, the enhanced degradation of PAHs in bioaugmented microcosms was attributed to stimulation of the autochthonous PAH-degrading communities already present in the soil, and to an enhanced bioavailability of the PAHs to these communities. After a

period of 3-4 weeks, the color of the bioaugmented PAH-contaminated soil was different from that of the biostimulated soil, presumably due to production of pyocyanin pigment by P64 cells. Formation of the pigment is coordinately regulated with production of rhamnolipid biosurfactant in *P. aeruginosa* strain 64 cells. The soil from such microcosms, when suspended in saline solution, showed fine particles that remained suspended in aqueous phase for hours to days longer compared to soil from microcosms that were not bioaugmented. Neither the biodegradation activity by the autochthonous communities nor the growth of *P. aeruginosa* cells was inhibited by the high concentration of PCP present in soil.

(2) Although strain EPA 505 was previously shown to degrade HMW PAHs in pure culture, its addition to soil microcosms did not cause any enhancement in PAH removal. The addition of EPA 505 to improve PAH degradation was problematic due to its sensitivity to soil conditions (i.e., toxic creosote components, competition with indigenous microbial communities, etc.). No EPA 505 cells were detected in Popile soil after a couple of days of incubation. Clearly more research is required to make it a useful and practical tool to increase cometabolic degradation of HMW PAHs.

h. *Pilot-scale field tests in land treatment units (LTU)*: These studies were conducted in two open trenches (20 by 4 by 1.5 ft (6 by 1.2 by 0.5 m)) containing contaminated soil to a depth of 1.0 ft (0.3 m). This two-phase study was designed to evaluate several cultivation and management strategies for landfarming of HMW PAHs. In both the LTUs, soil moisture was adjusted to 50-80 percent of the field moisture capacity. Leachate from the LTUs was collected on a regular basis. Soil was cultivated using a rear-tine rotary cultivator. LTU1 was cultivated initially and on day 71. LTU2 was cultivated on a bimonthly basis for the first 6 months, and then quarterly for an additional 24 months. Nitrogen was amended to the LTUs twice during the initial 6 months. Maintaining nitrogen content in the desired range was difficult as even slow-release nitrogen was easily washed out following each rain event. As a result, biostimulation was not achieved effectively. After 30 months of operations, the concentration of PAHs in both the units had decreased by 69 percent starting from an initial value of 13,000 mg/kg. Disappearance of PAHs in both the LTUs followed a first-order kinetic pattern, with half-life of PAHs of 14.7 months. Respiration analysis, coupled with reduction in heavy PAHs (4-, 5-, and 6-ring), demonstrated significant biological activity even at the unusually high contaminant concentrations involved.

i. *Pilot-scale field tests in troughs*: These studies were conducted in three galvanized steel troughs (10 by 3 by 2 ft (3 by 0.9 by 0.6 m)) with 12-in. (0.3-m) depth of contaminated soil or soil/amendment mix. The objective of this study was to confirm, at pilot scale and under landfarming conditions, the improved biodegradation of HMW PAHs using both biostimulation and bioaugmentation as observed at flask scale. In all the troughs, soil moisture was regularly checked and adjusted to 50-80 percent of field moisture capacity and tilling was done

using front-tine rotary cultivators. The troughs were in an environment where temperature was not controlled. Soil temperature in winter months (months 5-8 of the study) averaged 12 °C. Trough 1 (control) did not receive any amendments other than adjustment of moisture. Trough 2 (biostimulated) received powdered rice hulls (1:1 volume ratio with the soil) and nitrogen (dried blood at 2 percent weight per weight (w/w) of the soil). Trough 3 (bioaugmented) received vermiculite (2 percent w/w of the soil) inoculated with *P. aeruginosa* strain 64, in addition to rice hulls and dried blood. The study was conducted for 16 months. The control (Trough 1) showed only 12 percent reduction in total PAH concentration based on initial values, and no removal of HMW PAHs. The reduction of total PAH concentration in the biostimulated (Trough 2) and bioaugmented (Trough 3) troughs was 86 percent and 87 percent, respectively. The initial rate of biodegradation of PAHs was higher (50 percent reduction in the first 6 months) in the bioaugmented trough compared to that in the biostimulated trough (50 percent reduction in the first 8 months).

(1) The results of trough and LTU studies are presented in Figure 4 (Figure 37 in Appendix B). Both the biostimulated and bioaugmented troughs achieved significantly higher removal of PAHs than the LTUs. PAH degradation in both the pilot-scale projects occurred in patterns, with lower molecular weight PAHs degrading first, followed by consecutively larger molecules. Degradation of next higher ring PAHs did not require that the lower ring compounds be completely degraded first. The PAH-degrading bacteria did not appear to be sensitive to temperature changes as both the increases in biomass and respiration activity continued vigorously even during winter conditions.

(2) Together, the two pilot-scale projects allowed comparison of several field treatment variations using the same contaminated soil. Trough studies clearly indicated that both biostimulation and bioaugmentation are beneficial at pilot scale as well as in flasks. When properly conducted, these can result in significant reduction in the time required to achieve the desired reduction in PAHs.

This research has demonstrated the existence of indigenous microorganisms for degradation of HMW PAHs. As a result, biodegradation of PAHs from contaminated soils is dependent on the availability of these compounds to the microorganisms. This research has also demonstrated that biosurfactant producers can play an important role in bioremediation of PAH-contaminated soils. When managed properly, bioaugmentation and supplementation of the soil with nutrients would result in significant enhancements in the rates of biodegradation of the contaminants.

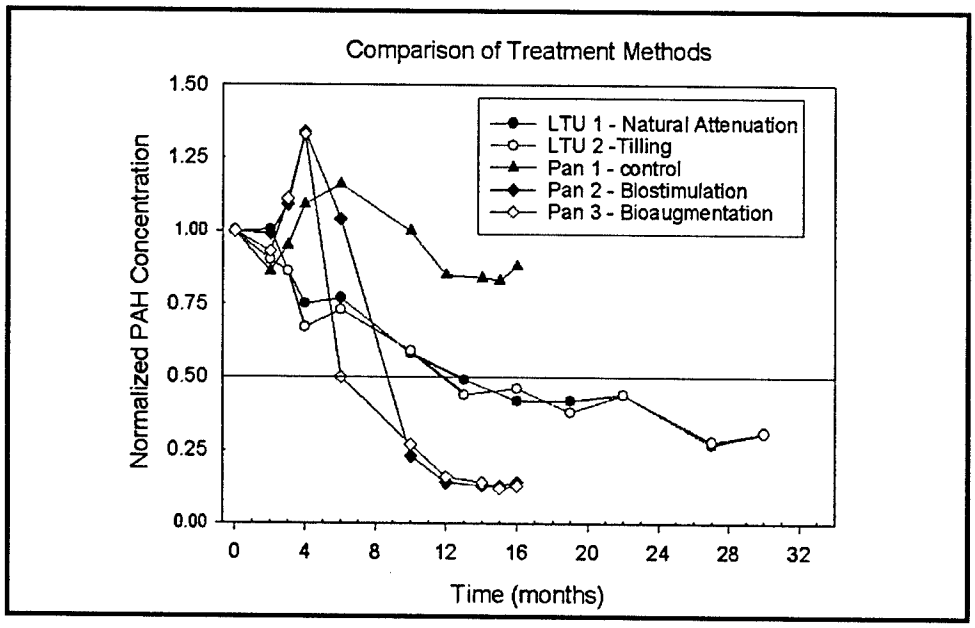


Figure 4. A comparison of PAH degradation between traditional land farming units (LTUs) and enhanced land farming troughs

It is traditionally believed that, for landfarming treatment, cultivation of the contaminated soil through intensive tilling is required for successful bioremediation. This study demonstrated that this might not always be the case. While tilling appears to enhance the biodegradation of PAHs initially, primarily by increasing the microbial biomass, overall, landfarming of PAHs does not appear to be driven by soil mixing. Soil moisture content also does not seem to be as important as previously thought. Therefore, preliminary, pilot-scale studies should be done in order to optimize conditions favorable to the full-scale effort. High contaminant concentration usually eliminates landfarming immediately as a remediation option. Again, this study demonstrated that this might not be necessary, especially if time is not a consideration. Even at the high PAH concentrations associated with the Popile soils, successful bioremediation was possible.

When setup costs and operation and maintenance (O&M) costs are calculated, overall remediation expenditures could be significantly reduced if a PAH landfarming site was treated quarterly instead of weekly. As an example, based on 1999 cost estimates for the northeastern United States, traditional landfarming of this soil would require a minimum $3.5 million for a single year of treatment. One alternative, 6 months of intensive treatment followed by 6 months of minimal treatment, would require approximately $1.6 million. A second alternative, 12 months of minimal treatment, would require less than $500,000. Therefore, choices in landfarming options could provide significant savings at sites where time is not a factor. As well as decreasing costs, limiting the intervention at a site decreases the potential for human exposure to the contaminant, the production of fugitive dust (air particulates), and the possibility of accidental groundwater contamination.

The significance of these positive results is further enhanced in light of the excessive PCP cocontamination, common to wood treatment facilities, but rarely studied in conjunction with PAH degradation in the laboratory. Biological degradation of seemingly high concentrations of PAHs has not been successfully demonstrated in previous efforts. As this study showed, microbiological analysis coupled with contaminant degradation analysis demonstrated biological degradation of the recalcitrant compounds. Biological degradation of PAHs with previously reported inhibitory PCP concentrations is possible, and appropriate for sites where time is available for long-term active bioremediation.

Chlorinated Solvents

This thrust area was led by Dr. Guy W. Sewell, Subsurface Protection and Remediation Division (SPRD) of the National Risk Management Research Laboratory (NRMRL), EPA, Ada, OK. A detailed summary of accomplishments by this group under the Project CU-720 funding is provided by the thrust area leader and as Appendix C. Major contributors in this effort included Ms. Susan Mravik and Dr. Lynn Wood of NRMRL, Dr. Jim Tiedje of Michigan State University, Dr. Michael Annable and associates from the University of Florida, Gainesville, and Mr. Kevin Warner of LFR Levine Fricke. The work conducted under this project contributed to 7 peer-reviewed publications, 6 technical reports, 2 conference/symposium proceedings papers, 13 published technical abstracts, 1 thesis, and 12 conference presentations.

A proposed pathway for biotransformation of chloroethenes is shown in Figure 5 (Figure 1 of Appendix C). This pathway suggests serial dechlorination of PCE to form end products of ethane and carbon dioxide.

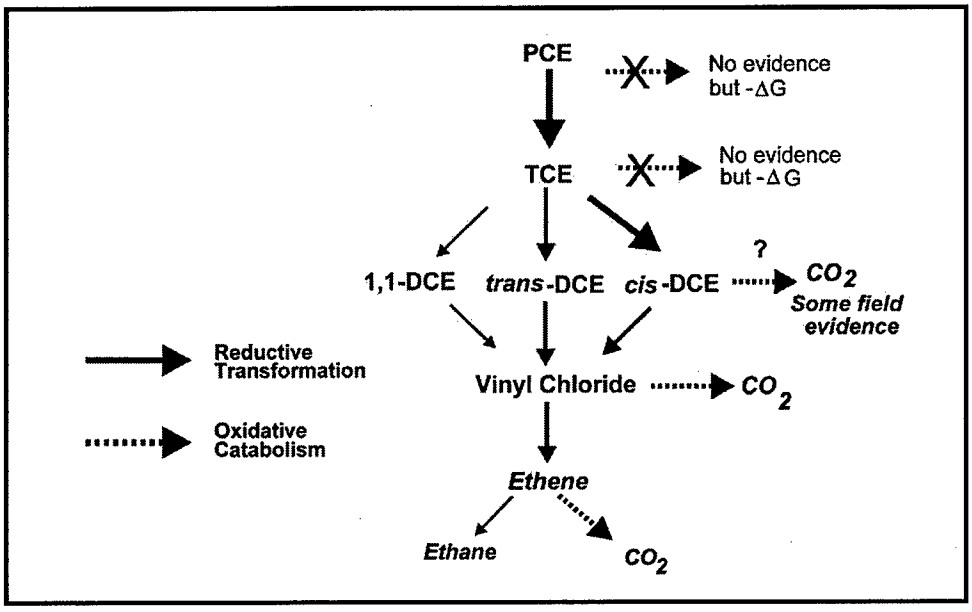


Figure 5. Biotransformation pathways for chloroethenes

The following were key accomplishments in this thrust area:

a. *Development of tools to monitor microbial potential of reductive dehalogenation of chlorinated solvents at contaminated sites.*

(1) Source material for evaluation of microbial ecology and molecular probes for microorganisms capable of reductive dechlorination of chloroethenes was obtained from a number of chloroethene-contaminated sites. The enrichments that completely dechlorinated PCE were further enriched on the lesser chlorinated ethenes, cis-DCE, and vinyl chlorides separately. The resulting subcultures completely dechlorinated their respective chlorinated ethene. The ability to biotransform these compounds was maintained by the subcultures for more than 10 transfers in basal salts mineral medium. Subcultures were subjected to an analysis of the 16S ribosomal ribonucleic acid (rRNA) genes by denaturating gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). The changes in community structure were evaluated by PCR (polymerase chain reaction) amplification of 16S rRNA genes from deoxyribonucleic acid (DNA) extracted from a microbial community in which one of the primers is labeled with a fluorescent molecule. After digesting the resulting fluorescently labeled PCR product by restriction enzyme, the fragments are resolved using an automated DNA sequencer. The resolved terminal fragments provide a fingerprint of the community, and an estimate of the number of ribotypes in a community. 16S ribosomal deoxyribonucleic acid (rDNA)-based PCR methods for detection of PCE-dechlorinating *Dehalococcoides* species and *Desulfuromonas* sp. Strain BB1 were developed and used to monitor their presence in environmental samples. The obligate hydrogenotrophic organism *Dehalococcoides ethenogenes* is the only known pure culture capable of complete reductive dechlorination of PCE to ethane using hydrogen as the ultimate electron donor. *Desulfuromonas* sp. Strain BB1 is a dechlorinator that utilizes acetate as an electron donor to support the reductive dechlorination of PCE and TCE.

(2) The pilot site material was subjected to direct and postamplification ribosomal gene probing with probes developed from known PCE-dechlorinating bacteria. A sample from one location at the pilot site resulted in visible amplification in the initial PCR with the universal primers. Nested PCR with the *Dehalococcoides*-targeted primers yielded a positive signal with the sample. Presence of the dechlorinators was confirmed using microcosm studies. Evaluation of the initial samples from the test site indicated "inadequate" to "limited" evidence for naturally occurring reductive dechlorination of chlorinated solvents based on the observation of aerobic conditions, lack of sulfate reduction or methane production, and low levels of dechlorination products, such as chloride, ethane, and ethene. Based on this assessment, the conclusion was that remediation by natural attenuation had limited potential as a significant component of the overall remedial strategy for effective

cleanup of the site. Molecular analysis and microcosm studies using material from the site indicated potential for complete reductive dechlorination of PCE to ethene. Additional samples collected prior to the cosolvent extraction test and 1 and 2 years following the test are being analyzed to determine the changes in microbial ecology that may have occurred. These samples are currently being analyzed and the data are not available at this time.

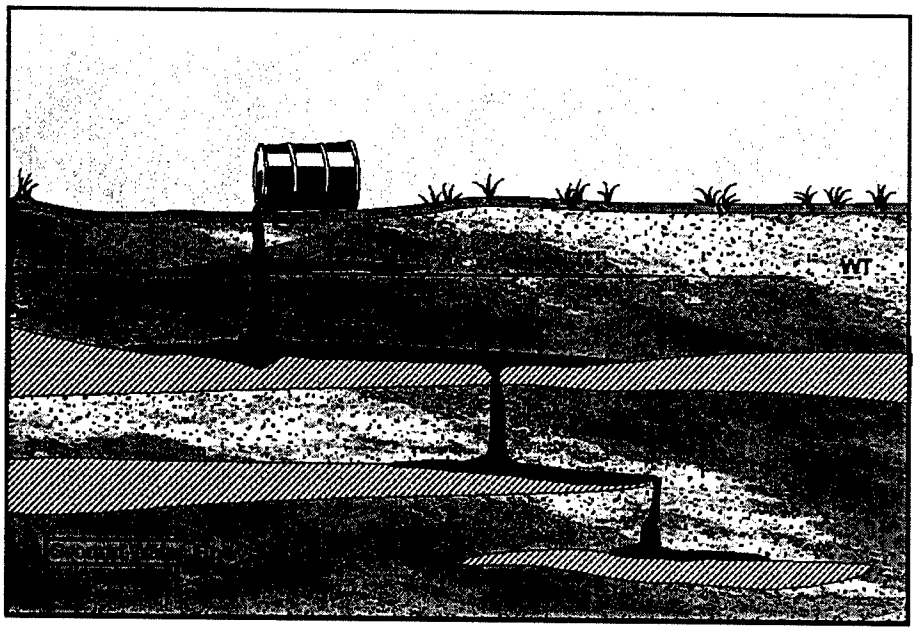
b. *Implementation and evaluation of postextraction reductive halogenation at a solvent extraction field site based on preestablished flask-level potential for ethanol-enhanced reductive dehalogenation at a given site.*

(1) The cosolvent flushing pilot test activities at a former dry cleaner site (Sage's) in Jacksonville, FL, were completed on September 10, 1998, with the collection of posttest groundwater samples. The site had extensive PCE contamination, and a subsection also suspected of having separate phase dense non-aqueous phase liquids (DNAPL) contamination was selected for pilot demonstration of Solvent Extraction Residual Biotreatment (SERB). Schematic drawings illustrating the application of SERB technology are shown in Figure 6. Comparison of core material collected prior to and following the cosolvent-flushing test indicated a PCE removal effectiveness of 65 percent. The hydraulic containment system was adequate to maintain capture within the testing zone. The cosolvent extraction pilot at the Sage's site was a successful demonstration of the removal of a significant mass of DNAPL contaminant from a selected zone.

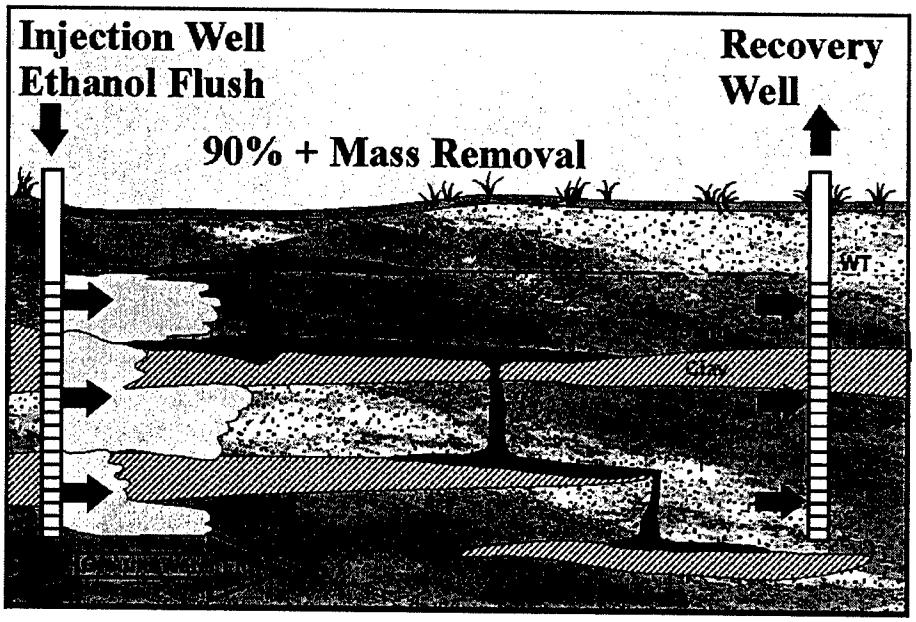
(2) The postflush assessment of the ethanol-enhanced in situ microbial degradation of the chlorinated solvents has been going on for 2-1/2 years. The contour plots of the electron donor, ethanol, show decreasing concentrations and downgradient movement of ethanol over time. The data also show that a significant amount of electron donor is still available after 2-1/2 years for subsurface microbial activity. Hydrogen, the ultimate electron donor, has been detected at the site. Similarly, acetic acid, an intermediate of the microbial degradation of ethanol, has also been detected. At some locations, acetic acid concentrations have peaked and are now decreasing, suggesting that the microbial population at these locations is shifting from those utilizing ethanol to those consuming acetic acid. The contour plots of acetic acid show that a significant amount of the ethanol is being completely degraded to carbon dioxide and hydrogen gas.

c. *Dissolution of DNAPL by cosolvent flushing technology.* Separate and mixed areas of DNAPL and cosolvent exist in the subsurface following cosolvent extraction.

d. *Development of bioactive zones conducive to the reductive dechlorination of DNAPL.* The formation of the daughter products of PCE at the site since solvent flushing suggests that in situ reductive dechlorination is

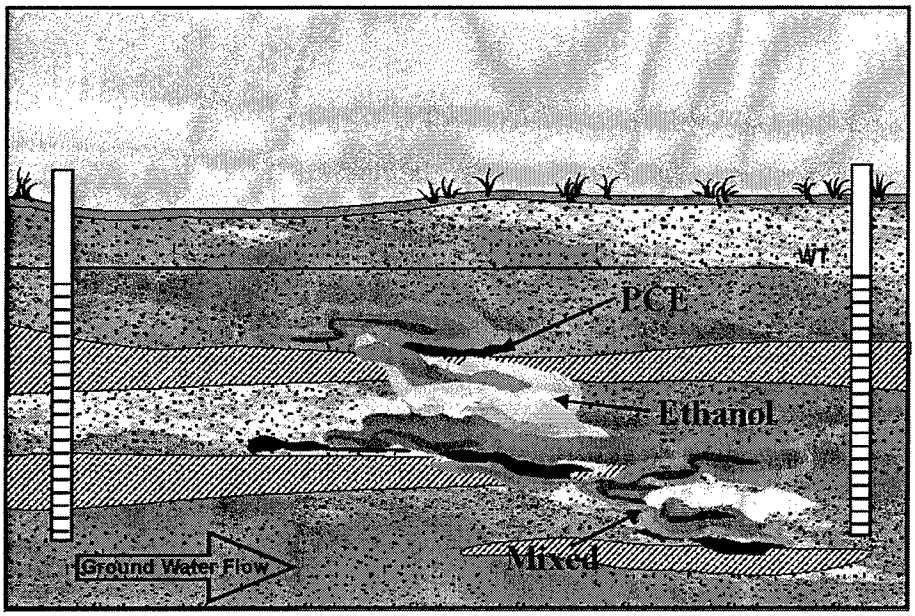


a. Complex distribution of DNAPL in subsurface

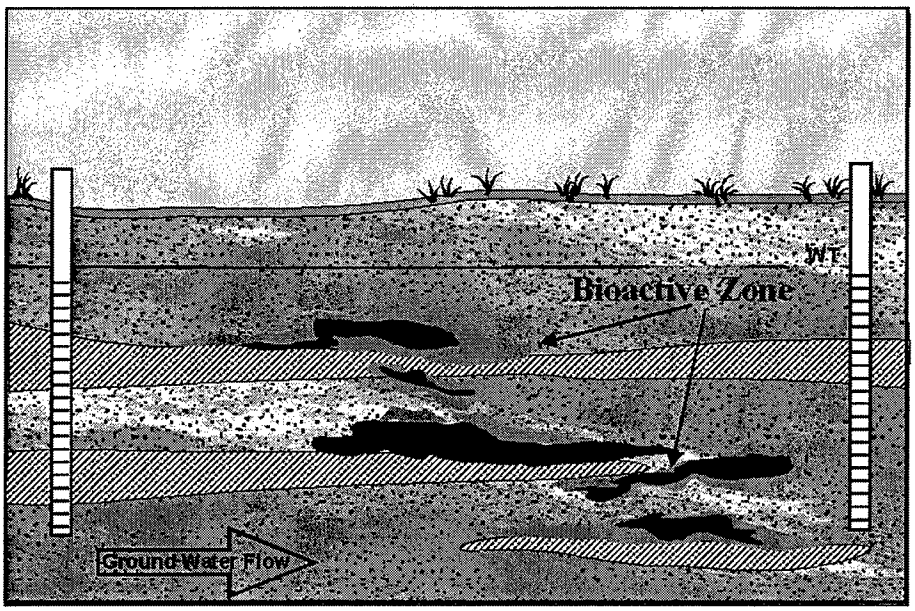


b. Dissolution of DNAPL by cosolvent flushing technology

Figure 6. Application of the SERB technology (Continued)



c. Separate and mixed areas of DNAPL and cosolvent exist in subsurface following cosolvent extraction



d. Development of bioactive zones conducive to the reductive dechlorination of DNAPL

Figure 6. (Concluded)

occurring. The process appears to have begun initially in the area where the cosolvent extraction was conducted and later at locations downgradient of the targeted source area.

(1) PCE concentrations began to decrease after approximately one year of monitoring in all the injection and return wells. In general, the downgradient monitoring wells showed an increase in PCE (from 10 to 60 mg/L) for the first 6 months following the cosolvent flush and then a decrease in concentrations to less than 5 mg/L after 2 years of monitoring. The concentrations of TCE and *cis*-DCE, the first and second daughter products of dechlorination of PCE, increased in the groundwater following the cosolvent flushing test. TCE concentrations increased in all wells during the first 19 months of monitoring, up to as high as 15 mg/L, and then decreased to relatively low concentrations (<4 mg/L). After 6 months, significant *cis*-DCE (up to 4 mg/L) had formed in the area of the cosolvent extraction and immediately downgradient. After 19 months significant *cis*-DCE formation (>10 mg/L) was detected in the wells further downgradient. Groundwater samples, analyzed for the isomers of *cis*-DCE, *trans*-1,2-DCE and 1,1-DCE, showed that these compounds made up less than 10 percent of the DCE analyzed. This ratio is indicative of biological transformations, which seem to favor cis-DCE as the predominant product under most conditions. Vinyl chloride, the next dechlorination product after DCE and a known carcinogen and regulated contaminant, was not detected in any groundwater samples collected from the site during the monitoring period. Ethylene, which is the final dechlorination product in the biotransformation of PCE, was detected at noticeable levels within 6 months following the cosolvent flushing test. This confirms the activity of the obligately hydrogenotrophic organism *D. ethenogenes,* which is the only known strain capable of complete dechlorination of chloroethenes to ethylene.

(2) All locations showed a decrease in sulfate concentrations over the monitoring period. The contour plots of sulfate concentrations give a strong indication that sulfate-reducing bacteria are becoming active and creating conditions conducive to the reductive dechlorination process. Along with the removal of sulfate there was a production of methane, which indicates that methanogenesis has been enhanced. Methane production began approximately 6 months after flushing was initiated and was measured in all wells. As with the sulfate removal, methane production began in the area near the cosolvent flushing test and concentrations downgradient increased after 14 to 19 months.

e. *A design guidance document for implementation and monitoring of Solvent Extraction Residual Bioremediation (SERB).* This design guidance document is the first draft based on the experiences with the technology of solvent extraction residual bioremediation at Sage's site in Florida. It discusses the regulatory aspects of SERB, its cost considerations, and implementation. Methods for evaluating the potential of natural attenuation of contamination at the site and the

suitability of subsurface ecology for sustained biotreatment have been detailed. Cosolvent selection has been discussed from the viewpoint of physical/chemical as well as biological considerations. The document describes the injection/extraction network design, operation, and options for solvent recovery. Parameters for monitoring and evaluation of performance of SERB have been detailed in order to assist potential users of the technology. These include mass removal of the DNAPL during solvent extraction, estimation of residual DNAPL and cosolvent in the subsurface at the start of bioremediation, determination of fate of cosolvent, and the rate and extent of biodechlorination. Methods of prediction of site closure including calculations of cosolvent electron donor half-life, dechlorination rates, and time to achieve the maximum contamination level (MCL) and acceptable contamination level (ACL) values have been described. Options for performance augmentation have also been discussed. It is anticipated that this document will be utilized by vendors of the technology during their design and monitoring of future SERB processes and their experiences will be incorporated in subsequent revisions of the document.

The goal of the chlorinated solvents thrust area was to develop and demonstrate biological remediation technologies for chlorinated solvents. This project focused on one such technological approach for source area treatment, a subarea for which current technologies are limited, but which must be addressed if viable, cost-effective approaches are to be implemented at solvent-contaminated sites. The results showed that solvent extraction removed a significant amount of dense-phase nonaqueous phase contaminant. The electron donor left in the contaminated soil matrix after the solvent extraction stimulated the microbial reductive dechlorination processes where none were apparent before the solvent extraction. The amount of electron donor left behind by the solvent extraction was still quite sufficient to continue providing the ultimate electron donor, hydrogen, for the reductive dechlorination process even in a situation where competing sinks of electron donor are present. These field pilot-scale results are very encouraging for cleaning up of DNAPL contaminations. The molecular probes developed in this work for identification of microorganisms capable of carrying out reductive dechlorination can be used at other locations where chloroethene contaminations occur. The design-implementation guidance manual prepared for the project should serve as a basis for similar activities at other locations; and with appropriate mechanism for incorporating the experiences at other sites into the manual, it should continue to improve as the technology transfer occurs.

The SERB technology has several advantages for remediation of free-phase DNAPL in the subsurface:

- Combination of an active in situ process, solvent extraction, with an essentially passive in situ process, residual biotreatment.
- Rapid removal of large masses of DNAPL in a very short time.
- Cosolvent injection/extraction overcoming transport and mixing limitations of standard bioremediation infiltration and injection techniques.
- DNAPL mass removal reducing toxicity to microbes for bioremediation.

- Continued removal of dissolved contaminants following cessation of pumping.

The ability to remove large masses of contaminant in a relatively short time is attractive to both regulators and stakeholders, who might be reluctant to support an extremely long natural attenuation option for the duration of natural DNAPL dissolution (assuming that natural attenuation processes were functioning in a sufficiently protective manner). It is also attractive to responsible or potentially responsible parties who might otherwise have no options other than expensive long-term and energy-intensive pumping and treating operations for containment. The in situ treatment train approach improves on either natural attenuation or enhanced bioremediation in the DNAPL scenario by both removing large masses of DNAPL and reducing toxicity for long-term biological processes to operate on the dissolved contaminants.

There are also challenges to the SERB technology, as there are for any remediation scheme:

- The technique is innovative and remains to be proven at sites having a variety of characteristics.
- Both cosolvent and surfactant in situ floods have the potential and are implemented to mobilize and/or dissolve the contaminants. If hydrodynamic control is inadequate, the potential exists for contaminating larger volumes of the aquifer and the groundwater.
- The cosolvent flood itself will probably not reduce contaminant concentrations to the needed regulatory requirements. Hence, a successful residual biotreatment phase is very important.
- The choice of cosolvent is critical for both the solvent extraction and residual biotreatment phases of the technique, and success or failure can depend upon this choice. Laboratory studies must be considered before going to field scale.
- There is considerable regulatory resistance to the injection of foreign materials (e.g., cosolvents) into the groundwater, which may require a significant effort, large amounts of documentation, and persuasion to overcome.
- The residual biotreatment phase of SERB might still take a very long time to come to completion following the cosolvent flooding phase, depending upon the conditions created and the mass of contaminant remaining at site.

The findings from this study provide ideas for addressing these challenges.

Polychlorinated Biphenyls

This thrust area was led by Professor James M. Tiedje of Michigan State University, East Lansing, MI. A detailed summary of accomplishments by this group under the Project CU-720 funding is provided by the thrust area leader as Appendix D. The work was performed jointly by Michigan State University, Georgia Institute of Technology, and ERDC, and the major contributors in this

effort are identified in the thrust area leader's report. The work conducted under this project contributed to 18 peer-reviewed publications (6 submitted or under preparation), 3 technical reports, 34 presentations at national and international conferences/symposia, one doctoral dissertation, 1 masters thesis, and 1 patent request.

The following were overall key accomplishments in this thrust area:

a. *Characterization of aerobic PCB metabolism by biphenyl degrading organisms*. Eight *o*- and *o* + *p*-chlorinated PCB congeners account for up to 80 mole% of the total Aroclor 1242 products in anaerobically dechlorinated sediments and are primary targets for the aerobic phase of the PCB bioremediation scheme. PCB metabolism by biphenyl-degrading Gram-positive *Rhodococcus erythreus* NY05 and *Rhodococcus* sp. RHA1 and Gram-negative *Comamonas testosteroni* VP44 and *Burkholderia cepacia* LB400 were studied for the range of substrates, rates of PCB oxidation, intermediates, dead-end products, and specificity of biphenyl ring oxidation. Two principal and complementary modes of PCB metabolism (preferential oxygenation of *o*- or *p*-chlorinated ring of biphenyl moiety) were demonstrated by *Rhodococcus* strains NY05 and RHA1. The consumption rates and products of metabolism of the eight PCB congeners by strains NY05, VP44, and LB400 were determined. Cometabolism of the targeted congeners was studied by using defined mixtures simulating most extensive (pattern C) and average (pattern M) anaerobic dechlorination products in PCB-contaminated soils and sediments. Ortho-directed strains were equally efficient in depletion of PCBs not only from the pattern C, but also from the pattern M product.

b. *Construction of recombinant variants metabolizing PCBs*. The *fcb*-operon for hydrolytic dechlorination of *p*-chlorobenzoate (isolated from *Arthrobacter globiformis* strain KZT1) and the *ohb*-operon for oxygenolytic dechlorination of *o*-halobenzoate (isolated from *P. aeruginosa* strain 142) were successfully expressed in host *C. testosteroni* VP44. Recombinant strains VP44(pE43) containing the *ohb* DNA fragment on plasmid pE43 and VP44(pPC3) containing the *fcb* DNA fragment on plasmid pPC3 were obtained. Both could grow on chlorobenzoates (CBA) and chlorobiphenyls (CB) as sole carbon source at concentrations as high as 10 mM. These strains grew on monochlorinated biphenyls with short doubling times (in hours) and were able to grow on congeners with both rings substituted as well with substantial dechlorination depending on the substrate. In contrast, the parent strain, VP44, grew on low concentrations of monochlorobyphenyls with accumulation of stoichiometric amounts of chlorobenzoate and no dechlorination. The parent strain did not grow on chlorobiphenyls containing halogen atoms in both the rings.

A broad host range vector pRT1 (with *Escherichia coli lac*-promotor) was developed for transferring dehalogenase gene cassettes into Gram-positive *Rhodococcus* as well as Gram-negative strains. A plasmid pRHD34 with the gene cassette carrying *fcbABC*-operon capable of degradation of 4-chlorobenzoate

(4-CBA) was constructed. Recombinant strain *Rhodococcus* RHA1(pHRD34) was able to grow on 4-CBA as sole carbon source and resulted in complete consumption of 4-CBA from the medium. The parent *Rhodococcus* RHA1 did not grow on 4-CBA. The *fcb*-operon appeared to be stable in the *Rhodococcus* strain. The 4-CBA grown cells of the recombinant strain grew well on 4-chlorobiphenyl (4-CB) as the only carbon source also, releasing stoichiometric quantities of chloride in solution and without accumulation of 4-CBA. When exposed to mixture M congeners, the recombinant strain accumulated no 4-CBA in broth. When introduced to soil systems, the *fcb*-operon was found to be stable in both sterile as well as nonsterile soil.

For degradation of the most environmentally important *o*-chlorinated PCB congeners, *ohb*-operon from *P. aeruginosa* strain 142 was used and two plasmids (pRO41 and pOCC1) were constructed starting from plasmid pRT1. Plasmid pRO41 contained *ohb*ABCR genes coding for ISP_{OHB}, potential ABC transporter and putative transcriptional regulator. Plasmid pOCC1 contained additionally chlorocatechol pathway clc genes from pAC27 under control of *lac*-promotor. These plasmids were introduced in strains VP44, LB400, NY05, and RHA1. LB400(pRO41) was the best growing genetically engineered microorganism (GEM) on 2-CBA up to the concentration of 3 mM and stoichiometric release of chloride ions. Parent strain LB400 did not grow on 2-CBA. When precultured on biphenyl (Bph), LB400(pRO41) caused complete mineralization of 2-CB. History of preculturing determined the degradation of complex congeners. While the degradation of a simple congener such as 2-CB was not affected by whether the inoculum was grown on Bph or 2-CBA, it made a significant impact on degradation of the higher chlorinated 2,2'-CB. Complete mineralization was achieved only when the inoculum was grown on 2-CBA.

While strain LB400 showed no growth on any artificial or naturally occurring PCB dechlorination products, Bph-grown LB400(pRT1) cells grew very well on 1 mM mixture C and resulted in formation of mainly Cl-, 2-CBA, 4-CBA, 2,4-dCBA. On the other hand, 2,5-dCBA grown cells of GEM LB400(pRO41) not only grew very well on mixture C, it resulted in nearly total mineralization of the PCB congeners. GEM LB400(pRO41) cells grew well on heavier mixture M as well, with higher biomass production and chloride release and greatly diminished accumulation of CBAs and potentially toxic intermediates such as Cl-HOPDA, compared with parent strain LB400. The GEM also sustained the high rates of PCB consumption characteristic of the host LB400. The following are milestones for GEMs:

a. *Development and testing of tools for tracking of GEMS in situ.* Real-time PCR (RTm-PCR) techniques were used to follow population dynamics of genetically engineered *Rhodococcus* strain. Both the 16S rRNA gene and the engineered 4-CBA (*fcb*) degradation operon were quantified with primers designed using Primer Express Software. When the probe specific for *fcb*B gene was used against total DNA from the same closely related species, quantitative amplification of the sequence was possible only for the strain RHA1(*fcb*), indicating high specificity of the technique. When the recombinant strain RHA1(*fcb*) was tested in the background of soil community DNA, the native *Rhodococcus* soil provided a response below the detection limit. A linear relation was also observed between the probe response and the isolated colony-forming

units (CFUs) per gram of soil, albeit with a reduced sensitivity compared with the pure culture standard curve. The sensitivity decrease was 1.1 orders of magnitude for *fcb*-probe and 1.3 orders of magnitude for 16S rRNA probe. Both the probes performed equally well when the number density of RHA1(*fcb*) cells per gram soil was measured against CFUs of the rifampicin-resistant cells incubated in soil for 30 days.

b. *Validation of PCB remediation strategy in soil (flask and microcosm studies).* Laboratory experiments in flasks and microcosms were conducted to validate the two-phase anaerobic-aerobic remediation and to determine the most effective GEMs. Using defined PCB mixtures M and C, Aroclor toxicity, and survival of GEMs in soil microcosms, a combination of two GEMs, a longer surviving Gram-positive *Rhodococcus* RHA1(*fcb*) and the more active PCB-tolerant Gram-negative *Burkholderia* LB400(*ohb*), was established to be the most effective for achieving maximum PCB degradation. An Aroclor 1242 contaminated sediment was inoculated with microorganisms eluted from River Raisin sediment and subjected to anaerobic conditions for one year before use in the aerobic phase. Recombinant cells, RHA1(*fcb*) and LB400(*ohb*), were grown on Bph and 2,5-dCBA for inoculation of sediment containing 50 percent solids. The inoculated sediment was continuously shaken for 30 days at 30 °C at 150 rpm. PCB removal was 57 percent and 54 percent in flasks inoculated with high and low inoculum densities, respectively. The corresponding degradation in uninoculated flasks was 4 percent. In sediments containing 20 percent solids, inoculation with the GEMs resulted in 73 percent PCB degradation in 15 days.

In another experiment, enhanced anaerobic treatment using Hudson River inoculum resulted in a shift in the congener profile from highly to lower chlorinated congeners. When the anaerobic treatment was followed by aerobic treatment with the aerobic GEMs, 78 percent PCB was eliminated from the system (Figure 7; Figure 26 of Appendix D). Inoculation with GEMs was essential for efficient PCB removal. The following are milestones for the Hudson River Project:

a. *Development of protocol for delivery of inoculum.* Laboratory tests showed that the survival of *Rhodococcus* sp. Strain RHA1 *fcb* and *Burkholderia cepacia* strain LB400 *ohb* cells in soil was greatly improved when vermiculite was used as the inoculum carrier. Growth of cells took place in the presence of CB in soil with and without vermiculite. However, the growth was an order of magnitude greater when vermiculite was used as a carrier. A protocol for delivery of cells to the soil was developed.

b. *Compatibility of anaerobic and aerobic phases during remediation process.* Effect of $FeSO_4$, used to enhance anaerobic reductive dechlorination, on the two GEMs was studied. It was established that neither of the organisms was adversely affected by FeS or $FeSO_4$.

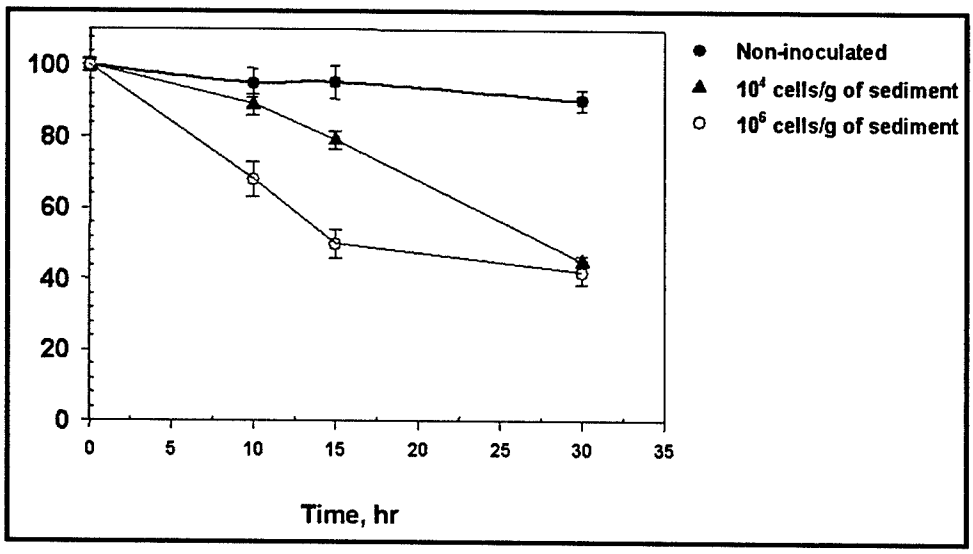


Figure 7. Bench-level anaerobic-aerobic treatment of PCB-contaminated soil used for scale-up to pilot-level demonstration

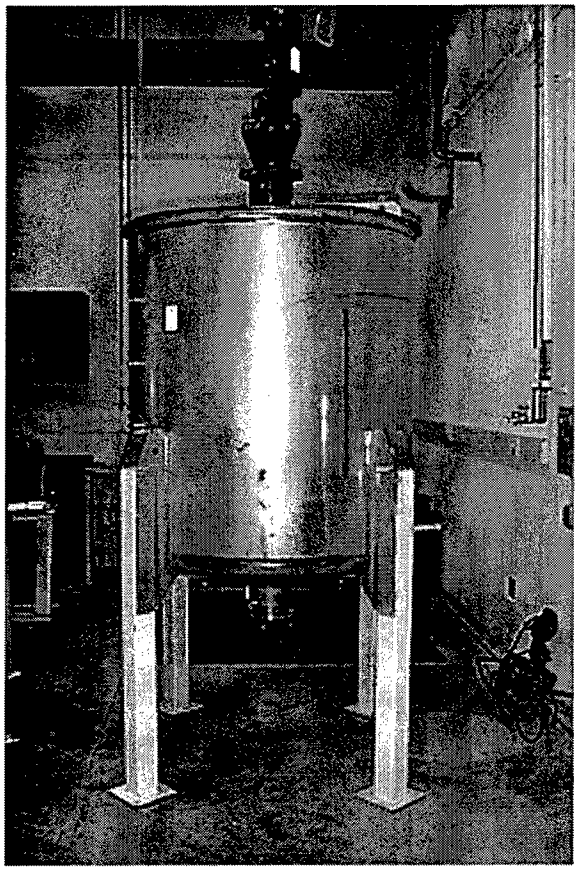
c. *Evaluation of surfactants for use in PCB bioremediation.* Solubilization capacities of two surfactants, Tween 80 and Tergitol NP-15, with respect to 4-CB, were determined at 22 °C and at 30 °C. The capacities were expressed as molar solubilization ratio (MSR, moles of PCB per mole of surfactant). MSR values were 0.33 (22 °C) and 0.38 (30 °C) for Tween 80 and 0.14 (22 °C) and 0.22 (30 °C) for Tergitol. A model was developed for predicting the effect of surfactant additions on the distribution of PCB congeners in a solid-liquid system. Growth experiments for strains NY05 and VP44 on the two surfactants showed that neither surfactant was inhibitory to cell growth on Bph or on 4-CB. The cells were able to use Tween 80 for cell growth, but not Tergitol. The presence of surfactants in solution did not contribute to any loss of plasmids in the GEMs, nor did they adversely influence the growth of cells or the disappearance of PCBs.

d. *Pilot-scale evaluation of bioremediation for PCB-contaminated soil.*

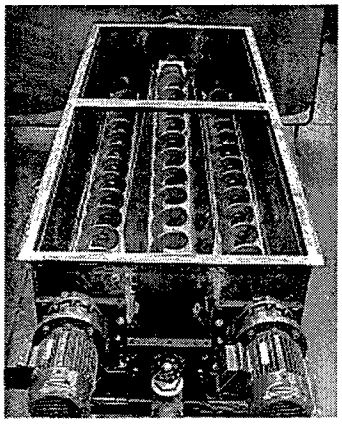
(1) Pilot-scale activities were planned to demonstrate the flask-level success of the PCB degraders in reducing PCB concentrations in contaminated soils under controlled conditions. For this purpose, a two-phase anaerobic/aerobic treatment strategy was designed. This activity is currently ongoing due to the long treatment periods required for the anaerobic phase of the process.

(2) Since the planned treatment involves use of genetically engineered microorganisms, permission was obtained from the appropriate regulatory agencies. A document, prepared for submission to the regulatory agencies seeking permission for use of GEMs in ex situ pilot-scale reactors for treatment of PCB-contaminated soils/sediments, is included in Appendix D as attachment D-3.

(3) Shaftless screw reactors used for low, medium, and high solids mixing evaluated in this study are illustrated in Figure 8 (Figures 35, 36, and 37 in Appendix D). They were tested to evaluate their ability to move the contaminated soil within the reactor, to study the effect of water on conveyor performance, and to quantify mixing of solution and solids within the matrix. It was found that these reactors provide efficient movement and mixing of thick soil slurry at a reasonable power input of 2-4 kW/ m^3 contaminated soil. Continued research to evaluate the frequency and duration of agitation at pilot scale is required.



a. Low solids reactor



b. Medium solids reactor

c. High solids reactor

Figure 8. Reactors used in PCB pilot demonstration and evaluation

These accomplishments show that several naturally occurring microorganisms aerobically metabolize the products of anaerobic dehalogenation of PCBs, but produce dead-end products. These dead-end products are toxic to the microbes

as well. As a result, complete microbial degradation of PCBs does not occur in nature. The researchers have, however, created aerobic GEMs that, by utilizing a slightly different pathway, transform the products of anaerobic dehalogenation of PCBs to compounds that serve as growth and energy sources for the cells. Thus, not only are the PCBs degraded to innocuous compounds, the need for a supply of an additional carbon and energy source is alleviated easing the problems of management of biotreatment strategy. The researchers have also shown that use of carriers for delivery of microorganisms enhances the survival of the GEMs in unsterile soils.

Another difficulty in developing a biotreatment technology stems from the low solubility of PCBs in aqueous media in which the microorganisms thrive. It has been demonstrated here that properly chosen chemical surfactants can be used to enhance the solubility of PCBs in aqueous media without adversely affecting the activity of the PCB degraders.

Since the genetically engineered organisms utilize only the lower chlorinated PCBs formed in the process of reductive dehalogenation of highly chlorinated PCBs, the treatment technology has to be a sequential two-step process. Further, since the process involves GEMs, it would be necessary to conduct the biotreatment under controlled conditions. Bioreactors for mixing high-solids slurry have been developed and tested. These should allow onsite ex situ biotreatment of PCB-contaminated soils.

6 Conclusions

Twelve research and application groups (including five from various universities) participated in the effort that resulted in the development of new biotreatment technologies for several recalcitrant compounds found at military sites. In the area of explosives and energetics, this has contributed to awareness that microbial ability to metabolize mono- and dinitrotoluenes is widespread at the contaminated sites. These organisms grow on the nitrotoluenes under aerobic conditions and release nitrites in the environment as a result of the degradation process. While the cells do not grow on trinitrotoluene (TNT), TNT is cometabolically degraded in presence of other nitrotoluenes. Hence, the biological treatment of contaminated soils and groundwater can be easily conducted provided delivery of electron acceptor (oxygen) and macronutrients can be ensured and the released nitrites can be managed. The process has been demonstrated for treatment of contaminated groundwater in a pilot plant at a field site and is being used to treat contaminated soil at several other locations. At one industrial location, the research has resulted in considerations of natural attenuation to handle the liquid waste stream containing nitrotoluenes. At one of the sites alone, the use of bioremediation technology for treatment of contaminated soil has been projected to yield savings of $60-65 million and reduction of 10 years in treatment time compared with other technologies under consideration.

Microorganisms capable of transforming 4-, 5-, and 6-ring polycyclic aromatic hydrocarbons (PAHs) were also shown to be naturally present at the contaminated sites. Biodegradation of PAHs by indigenous microorganisms is a slow process. *Sphingomonas paucimobilis* strain EPA 505 was found to be most effective in biodegradation of PAHs in pure cultures, and it metabolized high-molecular-weight (HMW) PAHs in a cometabolic fashion. Prior exposure of EPA 505 to low-molecular-weight PAHs increased the rates of degradation of HMW PAHs, but the simultaneous presence of low- and high-molecular-weight PAHs resulted in a reduction in HMW PAH degradation rates. In addition, when introduced into contaminated site soil, EPA 505 failed to survive. However, the biosurfactant-producing microorganism *Pseudomonas aeruginosa* strain 64 grew well in the site soil. It was shown to enhance the solubilization of PAHs in both aqueous medium and soil and thus increase the rate of biodegradation of PAH by the indigenous microbial communities. Techniques to deliver the microorganisms on carriers were developed in order to increase survivability of the added bacteria, improve ease of handling, and decrease remediation costs. The use of the vermiculite carrier also resulted in considerable increase in the shelf life of the carrier-based microorganisms.

Studies in pilot-size land treatment units demonstrated that the rate of biodegradation is influenced neither by the intensity of soil mixing nor by the moisture content of the soil. As a result, a passive or low-intensity landfarming operation is advisable for treatment of HMW PAH contaminated soils if time of treatment is not a consideration. Since the frequency of cultivation is a major cost factor in operation of the landfarming sites, reduction of intensity of cultivation from a normal of twice monthly year-round to normal operation for half a year followed by passive operation for the rest of the year would reduce the cost of operation to half. Further reduction of intensity to minimal after initial mixing would reduce the operating costs to 17 percent of those under normal operation. In order to enhance the rates of biodegradation by the soil microbial community, biostimulation by delivery of nutrients and carrier-based bioaugmentation by biosurfactant-producing microorganisms could be recommended based on flask as well as pilot studies.

Chlorinated solvent research focused on problems caused by the dense nonaqueous phase liquids (DNAPL) pollution sources that are difficult to treat by conventional in situ biotreatment techniques. Since anaerobic microbial degradation of chloroethanes has been demonstrated in the presence of electron donors, residual bioremediation after solvent extraction of DNAPLs was followed at a field treatment site. Probes to monitor the microbial communities capable of reductive dehalogenation were developed in the laboratory using samples from the solvent (ethanol) extraction site containing carbon tetrachloride contamination. It was demonstrated that reductive dehalogenation activity was quickly restored at the site after solvent extraction and significant microbial degradation of carbon tetrachloride continued over the period of operation. The results suggested that microbial degradation of chloroethanes by reductive dehalogenation is viable at the sites of solvent extraction and thus can supplement the solvent extraction process. A draft design guidance manual for the operation/monitoring of solvent extraction residual bioremediation (SERB) was prepared for assisting those interested in this technology. Further refinements in the technology by continued monitoring and feedback of bioremediation from the solvent extraction sites are recommended. This combination of technologies is anticipated to raise the expected end points in the solvent extraction process, a region of operation marked by decreasing efficiencies of extraction process. The combined process has potential for reducing the costs of treatment of DNAPL-containing sites.

Polychlorinated biphenyls (PCB) research focused on aerobic biodegradation of PCB fractions commonly found at old sites where reductive dehalogenation activity is observed. It was found that the aerobic microbial activity is commonly present but results in toxic dead-end products. Formation of the dead-end products was eliminated by metabolic engineering of microbial degradation pathways in the aerobic PCB degraders. The genetically engineered microorganisms (GEMs) thus developed showed an ability to grow on dichloro-PCBs and completely metabolize them. These GEMs were shown to be capable of surviving in contaminated soil systems when delivered in vermiculite carrier particles. Biomarkers for the aerobic PCB degraders were developed, and their effectiveness in tracking the GEMs in nonsterile contaminated soils was demonstrated. Chemical surfactants were screened to enhance solubilization of PCBs in aqueous phase, and effects of these surfactants on growth of microorganisms and PCB degradation were quantified. When the GEMs were

used to inoculate an aerated contaminated sample subjected previously to reductive dehalogenation, 78 percent reduction in PCB concentration was demonstrated. The control (uninoculated) aerated contaminated samples showed no reduction, demonstrating the necessity of using GEMs. The anaerobic-aerobic treatment using GEMs is currently ongoing in controlled pilot-scale bioreactors specially developed for handling high-solids slurries.

A number of these results were possible because of the sustained funding of research and collaborations of different scientists, engineers, practitioners, regulators, and site managers associated with Project CU-720. The Consortium provided the basic scientists with access to field samples for research and to the practical aspects of developing new technology. The practicing engineers and site management personnel had an opportunity to work with the scientists developing the technology, and the interactions resulted in bidirectional flow of information in several projects. The technical advisory committees provided a valuable input in terms of evaluating the progress of research at different stages and influencing the direction of the Consortium resources to research and developments most likely to be field implemented. Since the advisory committee had a number of practicing engineers as well in its membership, the technologies being developed by the Consortium members were broadcast to the user community as well. A key advantage of the multiyear Consortium-type project was the high-quality technical review of progress by the different projects on a continuous basis and potential of sustained funding for outstanding projects.

7 Recommendations for Future Transitional Research

During the course of Project CU-720, researchers and engineers in FIBRC conducted basic research as well as developed treatment processes, many of which were scaled up to the pilot- and/or field-scale implementation. In the course of this work, a number of research and developmental issues were identified as worthy of following up, but were not pursued due to a desire to move the technology with the most potential to pilot and/or field scale. The following issues were identified in each of the thrust areas as frontiers of science and technology in bioremediation.

Explosives and Energetics

The work conducted so far has resulted in characterization of pathways for the bacterial degradation of 2,4-DNT, 2,6-DNT, and NB. The enzymes involved in the pathways have been purified and characterized, and several of the genes involved in the pathways have been identified and cloned. Preliminary field tests for the application of bacterial degradation of DNT at contaminated sites were conducted, but some difficulties with scale-up tests occurred due to a lack of fundamental understanding of the induction of the pathways involved. Induction and control mechanisms of the involved pathways need to be elucidated in order to ensure reliable scale-up. Nonetheless, full-scale application of in situ bioremediation of DNT has been approved for BAAP following a successful pilot demonstration. Industrial producers of DNT have adopted bioremediation as clean-up alternatives for contaminated sites based on discoveries made so far.

Nothing is known about the regulation of the enzymes that remove the nitro groups from nitroaromatic compounds. Insight about the enzymes involved in the initial oxidative attack on nitroaromatic compounds and their regulation should lead to improved strategies for the degradation of mixtures of nitrotoluenes and an improved basis for predicting natural attenuation of DNT. At many sites, soil and groundwater are contaminated by mixtures of DNT and TNT. Laboratory and field tests conducted to date with such mixtures of contaminants have not indicated that TNT is removed effectively during the aerobic degradation of DNT. It may be possible to develop systems that partially

reduce TNT prior to oxidative attack. Understanding the regulation and biochemistry of the initial oxidative reactions of the DNT-degrading bacteria can provide the basis for metabolic engineering to develop a new biochemical strategy for the initial attack on TNT. Research with the nitramine explosive tetryl has demonstrated that nitroreductase enzymes can remove the nitramine nitro group. Whether that may prove to be a universal mechanism for degradation of nitramine explosives remains to be answered.

Little work has been published concerning the biodegradation mechanisms of other energetic compounds such as hexahydro-trinitro-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), CL-20, and perchlorate. Initiatives need to be taken to identify microorganisms capable of degrading these compounds and understand the metabolic pathways for degradation and the regulatory mechanisms for the enzymes and genes involved in these pathways. Another critical research need is in the area of biomarkers for identification/ quantification of indigenous microbial capability to degrade energetic compounds.

Polycyclic Aromatic Hydrocarbons

In theory, soils contaminated with PAHs may be treated utilizing various clean-up strategies. However, many proposed strategies have significant economic and feasibility problems. What is needed is an effective technology that supports the economics of disposal, eliminates adverse contaminant impacts, and supports the reuse of treated contaminated soils. Regardless of whether the biotreatment system is passive (augmented natural attenuation) or engineered (in-place treatment), a pragmatic solution is to focus active biotreatment on the available contaminant fraction. Research is needed to identify the factors affecting the bioavailability of PAHs on soil and how this affects treatment rates and acceptable toxicological end points. Research is then needed to use the bioavailability information to develop a technical base for enhancing natural recovery processes involved in in situ biotreatment of PAH-contaminated soils. Such research could result in guidelines for the assessment and prediction of the bioavailability of PAHs for in situ biotreatment.

Beyond availability, the issue of residual contamination is still unresolved in the case of biological treatment of PAHs where an understanding of the complex interactions between hydrophobic organic contaminants and soil is key to establishing realistic risk assessment criteria. Cleanup criteria based only on the chemical properties of the contaminant lead to an overprediction of the risks associated with the contamination. However, leaving an immobile, bound residue in the soil after cleanup needs to be justified scientifically. On the other hand, if residuals are released, the rate should be sufficiently slow to allow consumption by the microbial community. Thus, an alternative environmental end point (clean-up level) may be appropriate, rather than basing decisions solely on total concentration of contaminant in the soil or sediment. Thermal programmed desorption (TPD) is one new technology available to study the first of these issues, the mechanism of PAH binding to the soil, the process that affects their bioavailability in the environment.

Phytoremediation (defined as the use of green plants to remove, contain, or decrease toxicity) is a cost-effective technology with many advantages over

highly engineered solutions, including public acceptance. It is only in the last decade that research has been conducted on phytoremediation of PAHs. Recent studies have shown that plants are capable of removing not just the smaller molecular weight PAHs, such as anthracene, but also HMW compounds, such as chrysene and benzo(g,h,i)pyrene. In contrast to phytoremediation of metals, which is an extraction process, large organic compounds like the PAHs are degraded in the rhizosphere (root zone) of the plant. The rhizosphere comprises the top 3-6 ft (0.9-1.8 m) of soil. Various elements of the rhizosphere appear to play a role in the degradation. First, the soil around the roots is very different in chemistry and physical structure from the bulk soil. The root system of the plant is a moist, aerobic environment, which promotes microbial activity. Secondly, an exudate is released from the roots into the surrounding soil. The exudate is composed of simple sugars, amino acids, enzymes, aliphatic and aromatic compounds, and vitamins, and has been shown to increase the dissipation of soil-bound PAHs when applied to soil as a single treatment. Third, active microbial and fungal communities are associated with the root system. These are enhanced by the root exudate in ways that are not completely understood. Growth, with the development of biomass, is certainly one response, but in some plant species the exudate appears to favor selection of microorganisms that degrade the contaminant.

Plants can be incorporated into current treatment strategies in several ways. The most recent development is an alternative agricultural technique, plant-based biotechnology. The goal of this "molecular farming" is to produce large quantities of root exudate to be used as a soil amendment. As these exudates vary between species and soil conditions, a systematic study needs to be undertaken before this technology is feasible.

The most promising method of incorporating phytoremediation into soil PAH remediation is to develop a new paradigm of treatment that utilizes rhizospheric degradation as part of a treatment train. Once a contaminated soil has been treated by landfarming, for example, a plant cover would be established. This method has the following advantages:

- Plant cover reduces both wind and water erosion from the treated soil.
- The improved soil structure allows continued bioremediation.
- Naturally occurring nitrogen-fixing microorganisms reduce the need for chemical fertilizers, reducing costs.
- The roots establish an optimal environment and furnish energy for growth of microorganisms.
- The exudate contains oxidative enzymes that contribute to PAH degradation.

Current research indicates that the establishment of plants on contaminated sites may be an economic, effective, low-maintenance approach to complete soil PAH remediation.

Chlorinated Solvents

The SERB technology developed under this Consortium has been specifically targeted for contamination at sites containing separate-phase DNAPLs. Molecular probes for two microorganisms capable of reductive dechlorination of chloroethenes have been developed. The experience over the last two decades with recalcitrant compounds suggests that there are perhaps many more microorganisms in nature capable of reductive dechlorination that have so far not been discovered. The techniques developed and described herein can be used to continue to search for such potential microorganisms and for their isolation, cultivation, and monitoring.

Since chloroethenes are still commonly used, the potential for fresh spills and environmental contaminations exists. These being mostly man-made chemicals, the fresh sites are not likely to possess extensive dechlorination capability. In such cases, it may be desirable to deliver known dechlorinators in the environment. In order to ensure successful introduction of the microbes in the surface and confidently utilize the SERB technology, it is necessary to develop a better understanding of methods of delivery of the microorganism in the desired locations. Understanding of the movement and survival of augmented microorganisms in the subsurface needs improvement as well.

Pilot-scale and full-scale demonstrations at different sites are needed to refine the techniques developed and presented in the design guidance manual. The design guidance document that describes the SERB technology should be promoted among technology vendors and the user community. It is suggested that this be done using the Air Force model for the promotion and improvement of in situ bioventing technology. The following additional considerations need attention:

- Further characterization of shifts in microbial ecology in response to SERB.
- Evaluation of mixtures of optimal cosolvent/electron donor solutions.
- Modeling of impact of source removal on long-term economics for site remediation costs.

Polychlorinated Biphenyls

Metabolic engineering of pathways involved in aerobic biodegradation of mono- and dichlorinated PCBs has shown that genetically stable GEMs capable of growing on PCBs can be generated. Such organisms have been found to be able to survive in contaminated soils for a considerable time, especially when delivered in a suitable carrier. Biomarkers for these GEMs have also been developed for tracking them in engineered systems. The metabolic engineering approach that produces microbial strains that thrive on complex recalcitrant contaminants, rather than strains that transform contaminants using cometabolism, is very interesting. The ability of GEMs to extract energy from recalcitrant compounds simplifies management of treatment strategies for such contamination. This approach should be pursued for other more complex PCBs.

Anaerobic dechlorination of highly chlorinated PCBs is a very slow process, requiring several years; and it is a major bottleneck in any demonstration project. The microbial strains capable of doing anaerobic dechlorination have not been isolated and characterized. Their isolation from the contaminated sites and maintenance in laboratories/culture collections are highly desirable in order to promote this technology. Similarly, the GEMs generated in the present work should also be registered with suitable culture collections in order to make them more widely available to potential users. Due to the slow nature of the degradation process, pilot demonstration of the technology has been lagging. Validation of this technology should be more extensively demonstrated.

8 Recommendations to Facilitate Technology Transfer

The major contribution of the work described in this report is the discovery that many recalcitrant compounds including certain nitroaromatics and explosives are biodegradable. The understanding of the mechanisms, strategies, and requirements for biodegradation provides the basis for a variety of field applications. Several bench- and field-scale demonstrations have shown that the principles apply in field-scale applications as well. The major payoff of the work will come in the future when bioremediation based on the principles described here will be used routinely for cleanup of sites contaminated with explosives, PCBs, PAHs, and chlorinated solvents. Further technology efforts will facilitate this broader use.

An effective example of technology transfer is the organization in September 1999 by AFRL of an International Symposium on the Biodegradation of Nitroaromatic Compounds and Explosives. The symposium was funded by the Air Force Office of Scientific Research and the Defense Threat Reduction Agency. The work funded under the Flask to Field program was showcased at the symposium. In a related effort, the work on biodegradation of explosives for the past 5 years was critically reviewed in Spain, Hughes, and Knackmuss.[1] The book comprises peer-reviewed chapters by a group of international experts who not only analyzed ongoing work in their areas of expertise, but also identified areas where important questions remain. A summary of the consensus on areas where additional research is needed was provided to SERDP and other funding agencies. Similar activities in other thrust areas are highly recommended.

An important way to transfer technology is to develop and publish testing protocols. Site-specific laboratory testing is essential prior to selection and design of a bioremediation system. Key issues for laboratory tests include (a) appropriate chemical and physical conditions; (b) verification of the presence of bacteria capable of mineralizing the major contaminants; (c) presence of indigenous bacteria capable of removing or converting excess inorganic nitrogen compounds to environmentally acceptable forms; and (d) acceptability of achievable end points in terms of toxicity and risk. Laboratory tests may also be needed to estimate degradation rates, to establish maximum contaminant

[1] J. C. Spain, J. B. Hughes, and H.-J. Knackmuss, ed. (2000). *Biodegradation of nitroaromatic compounds and explosives*. Lewis Publishers, Boca Raton, FL.

concentrations that can be biodegraded, and to design strategies to biodegrade mixtures of compounds to optimize the efficiency of the remediation system. Examples of protocols developed are given in each thrust area discussed.

Another technology transfer thrust involves devices. For example, a device to monitor microbial respiration during bioremediation and natural attenuation was designed, tested, and submitted for patent during this study. This device is a low-cost, low-technology means to decrease the costs associated with the operation and maintenance of landfarming facilities without sacrificing human health and safety. More devices should be identified from this work, refined, and made available.

Technologies for bioremediation and natural attenuation of organic compounds have developed dramatically over the past 15 years due to extensive programs carried out in the United States and in Europe. The possible breadth of application of bioremediation continues to be inhibited by significant data gaps in the ability to apply the principles of biotechnology to chemical contamination. A considerable amount of field experience is available and protocols have been developed for the bioremediation of easily degradable compounds such as petroleum hydrocarbons and chlorinated aliphatic solvents. However, until now, the development of similar state-of-the-art protocols for bioremediation of the more recalcitrant PAHs (particularly at high concentrations) has continued to lag. One such document has been developed for SERB technology. It is desirable to develop and promote similar documents dealing with other contaminant classes as well among vendor and user communities.

Demonstrating improved bioremediation of these more toxic, HMW PAHs through bioaugmentation and biostimulation has enhanced the potential for more frequent and successful use of biotreatment as a viable option for PAH cleanup. The combination of the use of known surfactant-producing bacteria in conjunction with vermiculite-carried bioaugmentation, enhanced nutrient delivery, and appropriate bulking agent application provides the basis for a variety of field applications on higher concentrations of toxic PAHs not previously thought possible. The advances described herein allow the vast body of knowledge and experience in bioremediation and natural attenuation to be extended to hydrophobic organic compounds previously thought recalcitrant, such as the PCBs and pesticides. An application manual, such as that developed for the SERB process, would enhance the usage of these findings.

This research has provided a succinct progression of bench- and pilot-scale demonstrations that applied sound scientific principles and assessed practical engineering issues in the development of enhanced parameters for contaminant remediation. The major payoff of the work will come in the future when successful bioremediation based on the success described herein is used routinely to enhance cleanup of sites contaminated with PAHs, PCBs, nitroaoromatics, chlorinated ethenes, and other recalcitrant compounds. A book is being prepared to tie all of this together for wider distribution.

The environmental challenge posed by separate phase chlorinated solvents in the subsurface is a problem found at a large number of both military and industrial sites. The application of the SERB technology could have a significant effect on cleanup efforts for both DoD and private industry. Subsurface restoration practitioners should be able to make direct use of the results of the

laboratory and field investigation. The results are also directly applicable to the needs of the regulatory community and should promote the acceptance of innovative technologies for subsurface remediation. The SERB project was focused on a controlled field test, which demonstrated the capabilities of a treatment train technology that incorporated both active and passive remediation. The reports and presentations from this work will provide technical guidance as to the feasibility for implementation at full scale. The field demonstration was conducted in conjunction with an ongoing collaborative field research project, which involved the State of Florida Department of Environmental Protection, University of Florida, LFR Levine Fricke (site operations contractor), EPA Technical Innovation Office, and the EPA NRMRL. The involvement of this diverse group of collaborators should facilitate technology transfer efforts.

The SPRD of the NRMRL, Ada, OK, houses the Superfund Technology Support Center (TSC), which provides a mechanism for technical assistance and technology transfer to move research results to the private sector. The TSC has provided technical assistance on over 300 Superfund sites since 1987 and has conducted numerous technology transfer seminars for EPA regional personnel, state personnel, and private contractors who are responsible for subsurface remediation at hazardous waste sites. The TSC provides a very effective means for transferring the results of this research to the user community.

A very effective approach for technology transfer is the "tool-kit" approach the Air Force used to promote and improve in situ bioventing technology and natural attenuation. Protocols and case histories were developed and widely publicized. For new test sites, feedback of results was solicited. All were assembled in a tool kit that was available upon request.

Another approach is to form groups of persons working in a given technology who meet regularly under the sponsorship of a governmental agency. Work groups can be established from researchers, consultants, users, and providers (vendors).

The work funded under SERDP described in this report already has been showcased in many venues, including national and international conferences focusing on recalcitrant compounds, bioremediation, and microbiology, and in peer-review journals and technical reports. These are listed in Table 5.

Table 5
Technology Transfer Products Resulting from SERDP Project CU-720

Explosives
Peer-Reviewed Journals
An, D., D. T. Gibson, and J. C. Spain. 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. *J. Bacteriol.* 176:7462-7467.
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He, Z., and J. C. Spain. 2000. One-step production of picolinic acids from 2-aminophenols catalyzed by 2-aminophenol 1,6-dioxygenase. *J. Ind. Microbiol. Biotechnol.* 25:25-28.
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Appendix A
Explosives Biodegradation: Novel Enzymatic Reactions, Mechanisms, and Pathways for Nitroaromatic Compounds

Strategic Environmental Research and Development Program

Explosives Biodegradation: Novel Enzymatic Reactions, Mechanisms and Pathways for Nitroaromatic Compounds

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Air Force Research Laboratory
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Draft report

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Preface

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1 Introduction

Contamination by explosives is an enormous problem at ammunition production and handling facilities in the U.S. and worldwide. The estimated cost for cleanup of defense sites in the U.S. alone is $2.66 billion. Explosives contamination at active installations is estimated to include 750,000 cubic yards of soil and 530 billion gallons of groundwater (Annual Report to Congress for Fiscal Year 1995). Because much explosives contamination occurred during the manufacturing process, cleanup of contaminated sites frequently also deals with starting materials, solvents, production intermediates, and byproducts.

We have recently reviewed the basic research and practical applications of aerobic bacterial degradation of nitroaromatic compounds (Nishino et al., 2000b) in the context of an overview of biodegradation of nitroaromatic compounds and explosives (Spain et al., 2000) and will therefore largely confine this report to investigations that have been pursued under the Flask to Field initiative funded by the Strategic Environmental Research and Development Program (SERDP).

The most common explosive used by the U.S. military has been 2,4,6-trinitrotoluene (TNT). The production of TNT involves the nitration of toluene to form mononitrotoluenes and dinitrotoluenes (DNT) in turn prior to the final nitration step. As a result, 35 active and 23 formerly used TNT manufacturing facilities are extensively contaminated with mono-, di-, and trinitrotoluenes. To date, there is inadequate evidence to evaluate TNT for its carcinogenicity in either humans or animals (International Agency for Research on Cancer, 1997a), but a considerable variety of acute and chronic toxic effects to humans and animals have been reported (Hazardous Substances Data Bank, 1985). As a consequence, TNT is the most widespread explosive contaminant of interest to DoD. A considerable amount of work in a number of laboratories worldwide has led to the development of a wide range of strategies for the cometabolism of TNT (Ahmad and Hughes, 2000; Bruns-Nagel et al., 2000; Burken et al., 2000; Fritsche et al., 2000; Lenke et al., 2000). DNT is a second major environmental contaminant that resulted from the production of TNT, but many of the cometabolic strategies developed for the bioremediation of TNT have proved to be much less effective for DNT.

Although TNT has only limited solubility in water, dissolved TNT is transformed under aerobic, anaerobic or alkaline conditions, by biological and abiological processes, to complex mixtures of TNT and reduced products (Honeycutt et al., 1996; Lenke et al., 2000). TNT has not been produced in the U.S. since the end of the Vietnam war, but DNT isomers are also major production intermediates for the manufacture of the toluenediamines that are precursors to toluene diisocyanate. Small amounts of toluene diisocyanate are used in the manufacture of adhesives, sealants and coatings, but the majority is used in the production of the ubiquitous polyurethane foams that go into furniture, car seats, carpeting underlays, insulating and packaging materials. Currently, the annual worldwide DNT production is 2.7 billion pounds. Exposure to DNT results in acute and long term toxicity and possible low level carcinogenicity (International Agency for Research on Cancer, 1997b). Both 2,4-DNT and 2,6-DNT are listed as U.S. EPA priority pollutants in the Clean Water Act, the Safe Drinking Water Act, the RCRA, and the CERCLA (Environmental Statutes).

Environmental contamination at TNT manufacturing sites is very common. During TNT production 2,4- and 2,6-DNT are produced in a ratio of 4:1. TNT manufacturing plants are often contaminated with di- and trinitrotoluenes and the original 4:1 ratio of 2,4- and 2,6-DNT persists at contaminated sites long after the manufacture of DNT or TNT has ceased. This suggests that DNT is not being effectively degraded at such sites. Volunteer Army Ammunition Plant (VAAP) is one of numerous sites in the US and Europe that are extensively contaminated with DNT. There is also extensive DNT contamination at the BLM Sparks site in Nevada, at Badger Army Ammunition Plant (BAAP), at West Virginia Ordnance Works, at Weldon Spring Ordnance Works in Missouri, and at a number of firing ranges and EOD sites where DNT was used as a propellant in howitzer ammunition. Several sites in Germany including Werke Tanne have large areas of DNT contamination. Radford Army Ammunition Plant currently handles large quantities of DNT and the waste treatment system suffers periodic problems with effluent treatment.

Two of the nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are frequently found as co-contaminants with TNT, either as a result of the manufacture of both explosives at the same production plant, or because they were used as ingredients in mixtures of explosives known as compositions. Various mixtures of TNT and RDX were used in cyclotols and composition B type explosives, while mixtures of TNT and HMX made up the octols. The result is that cleanup and remediation schemes for explosives contaminated sites often have to deal with the two common nitramine explosives as well as with TNT.

Contaminated material at former explosives manufacturing sites is currently treated either by incineration or by composting. In some cases treatment has not begun and contaminated soils have been warehoused pending installation of treatment systems. Costs for incineration are estimated at \$400-600/yd^3 of soil (Griest et al., 1998), and for composting only slightly less at \$404-467/yd^3 of soil (Kruchoski, 1997), although economies of scale may reduce those costs considerably (Jerger and Woodhull, 2000). Costs for cometabolic bioslurry treatment of TNT contaminated soil are estimated at \$290-350/yd^3 (Manning et al., 1996). All currently accepted biological treatment strategies for explosives remediation rely on cometabolic processes which require the addition of primary growth substrates and complex control strategies (Jerger and Woodhull, 2000). Such systems include various composting schemes (Bruns-Nagel et al., 2000; Jerger and Woodhull, 2000), anaerobic reactors (Lenke et al., 2000), phytoremediation (Burken et al., 2000), and fungal treatments (Fritsche et al., 2000).

All the cometabolic systems also generate large amounts of product that must eventually be landfilled. In the cases of composting and phytoremediation, the volume of product to be landfilled is increased substantially. In composting, no more than 10 to 30% of the compost pile can be made up of contaminated soil (Bruns-Nagel et al., 2000; Sims, 1990), with the bulk made up of primary carbon and nitrogen sources, and materials to promote aeration. In contrast to cometabolic strategies, strategies that rely on mineralization of contaminant compounds are self-sustaining and can lead to significant cost savings. Over the last 20 years it has become apparent that nitroaromatic compounds in the environment create intense selection pressure that has led to the evolution of microorganisms able to degrade a growing number of nitroaromatic compounds. Our initial effort under the Flask to Field initiative focused on the examination of bacteria able to grow on simple nitroaromatic compounds. The novel enzymes discovered in the biodegradative

pathways were screened for the ability to transform TNT. Later efforts focused on the discovery of bacteria that mineralize DNT, the catabolic pathways involved, and the exploitation of the pathways for bioremediation and waste minimization. Major effort was directed towards development of pilot and field scale systems for degradation of mixtures of nitrotoluenes. Another critical component of the research has also been to elucidate the bottlenecks that inhibit efficient DNT degradation in above ground reactor systems as well as *in situ* field applications.

2 Objectives

2.1 Potential for biological treatment of explosives and nitroaromatic compounds

The overall objective of our research has been to discover and understand biological systems able to destroy nitroaromatic contaminants with the ultimate goal of adapting such systems for remediation of environmental contamination, minimization of wastes in conventional manufacturing processes, and for the development of novel biocatalysts that are both more cost effective and have fewer deleterious environmental consequences than traditional chemical processes.

Research into biodegradation of explosives dates back to the 1970s (Bringmann and Kühn, 1971; Carpenter et al., 1978; Isbister et al., 1984; Kaplan and Kaplan, 1982; McCormick et al., 1978; McCormick et al., 1981; McCormick et al., 1976; Naumova et al., 1984; Naumova et al., 1982; Nay et al., 1974; Parrish, 1977; Won et al., 1974) (Bringmann and Kühn, 1971; Carpenter et al., 1978; Isbister et al., 1984; Kaplan and Kaplan, 1982; McCormick et al., 1978; McCormick et al., 1981; McCormick et al., 1976; Naumova et al., 1984; Naumova et al., 1982; Nay et al., 1974; Parrish, 1977; Won et al., 1974) when a number of workers identified reduced transformation products of explosives that appeared to be as toxic as the parent compounds, and resistant to further biological breakdown. The early results discouraged the hope that biodegradation would be a useful tool to remediate explosives contamination, but breakthroughs in research on the degradation of other toxic aromatic compounds spurred new research efforts with nitroaromatic compounds and explosives. New strategies including anaerobic reduction, composting, sequential anaerobic-aerobic treatment, phytoremediation, and fungal-based treatment have all gained acceptance (Spain et al., 2000). During the last decade microorganisms that can use a variety of nitroaromatic compounds (Lenke et al., 2000; Nishino et al., 2000b; Russ et al., 2000), nitrate ester explosives (Williams and Bruce, 2000), and nitramine explosives (Hawari, 2000) as carbon or nitrogen sources or both have been identified.

2.2 2,4,6-Trinitrotoluene

Because TNT was the most widely produced and used military explosive in an era when environmental regulations did not exist, environmental contamination from the manufacture and subsequent processing of TNT has caused extensive and worldwide contamination. TNT is the primary explosive contaminant of concern to DoD, therefore the early efforts under the Flask to Field initiative involved screening of bacterial isolates able to degrade simpler nitroaromatic compounds for their activity against TNT. Bacterial strains that produced novel metabolites from TNT were identified (Fiorella and Spain, 1997), however; no isolate that was identified was able to use TNT as a primary growth substrate.

2.3 Mono- and Dinitrotoluenes

Direct selection for aerobic TNT-degrading bacteria was unsuccessful, therefore a strategy was adopted to search for organisms that could degrade the mono- and dinitrotoluene precursors to TNT in order to exploit the possibility of expanding the catabolic potential of such bacteria to include TNT. Equally important was the fact that in many areas of TNT manufacturing plants, mono- and dinitrotoluenes are the predominant contaminants because they are more soluble in water than TNT. Contaminated material at former TNT manufacturing sites is not, but could be segregated according to which nitrotoluenes are the major contaminants. The basis for the work was the idea that a cost effective treatment strategy for destruction of mono- and dinitrotoluenes in appropriate areas could greatly reduce the total cost of cleanup. The major advance was the discovery of bacteria able to use mono- and dinitrotoluenes as growth substrates. We elucidated the degradation mechanisms and demonstrated the effectiveness of the aerobic mineralization strategy. Subsequent work led to pilot and field scale studies that demonstrated the utility of the strategy for both *ex situ* and *in situ* applications.

The work has ultimately led to the full scale application of *in situ* bioremediation of DNT in waste pits at BAAP, and to feasibility studies for the use of natural attenuation of DNT at BAAP as well as at industrial DNT manufacturing sites in Texas and Florida. A wetlands system for biodegradation of DNT has been designed for a phosphate fertilizer manufacturing site in Canada based upon the finding of DNT degrading bacteria in the DNT contaminated holding ponds on the site.

2.4 Nitrobenzene

Nitrobenzene was adopted as a model compound because it is the simplest of the nitroaromatic compounds, industrially important, and a hazardous substance in its own right. Work has focused on the evolution and genetic organization of the nitrobenzene degradation pathways as well as for that of 2,4-DNT. The objective was to understand how bacteria able to degrade nitroaromatic compounds have evolved so that we might predict the occurrence and distribution of such bacteria. Further work on the purification and characterization of the enzymes involved in the catabolic pathways has led to studies involving application of the novel enzymes to the inexpensive production of monomers and end-capping materials for composite materials useful to DoD. A final objective has been to determine possible reactions that may be recruited to engineer a pathway for mineralization of TNT.

3 Technical Approach

3.1 Discovery of novel organisms

The basis for much of the work done under Flask to Field was the discovery of bacteria able to use nitroaromatic compounds as growth substrates under aerobic conditions. Standard enrichment and selection techniques were used for isolation of bacteria able to degrade nitroaromatic compounds (Nishino and Spain, 1996), but the successful inocula predominantly came from contaminated explosives manufacturing sites or waste treatment plants operated specifically for nitroaromatic compounds rather than more general municipal or industrial waste treatment plants (Nishino et al., 2000a; Nishino and Spain, 1995). Various enrichment cultures were provided nitroaromatic compounds as sole carbon sources, sole carbon and nitrogen sources, or as sole nitrogen sources. For nitrobenzene, mononitrotoluenes, and dinitrotoluenes, only their use as sole carbon and nitrogen sources yielded isolates able to grow on nitroaromatic compounds.

Isolates were obtained from enrichment cultures by plating onto both selective and non-selective media, then testing individual colonies for the ability to grow on the desired nitroaromatic compound. The release of either nitrite or ammonia from cultures provided with the nitroaromatic compound as the sole carbon and nitrogen source along with the disappearance of the nitroaromatic compound from culture fluid was taken as proof of mineralization. For selected isolates, the accumulation of $^{14}CO_2$ from cultures provided with ^{14}C-U-ring labeled nitroaromatic growth substrates confirmed mineralization (Nishino and Spain, 1995; Nishino et al., 1999).

3.2 Characterization of pathways, enzymes, and reactions

Degradative pathways were worked out using standard techniques including oxygen uptake assays in resting cells, assays with crude cell extracts, and purification and identification of pathway intermediates (Haigler et al., 1994a; Haigler et al., 1994b; He and Spain, 1997; He and Spain, 2000b; Jain et al., 1994; Nadeau and Spain, 1995; Nishino et al., 2000a; Nishino and Spain, 1995; Spiess et al., 1998; Vorbeck et al., 1998). Individual enzymes were purified and characterized with regard to size, sequence, structure, substrate range, inhibitors, cofactors, and possible reaction mechanisms (An et al., 1994; Haigler et al., 1999; Haigler et al., 1996; He et al., 1998; He et al., 1999; He et al., 2000; He and Spain, 1998; Kadiyala and Spain, 1998; Lendenmann and Spain, 1996; Somerville et al., 1995). Clones carrying structural pathway genes and specific probes for nitroaromatic dioxygenases furthered our understanding of the evolution and control of nitroaromatic degradation pathways and enabled detailed study of specific steps and reaction mechanisms of each pathway (Davis et al., 1999; Davis et al., 2000; Haigler et al., 1999; He et al., 2000; Johnson et al., 2000; Suen and Spain, 1993).

3.3 Applications in bioreactors and scale-up

Specific strains able to degrade nitroaromatic compounds were tested in bioreactors (chemostats, airlift reactors, fixed bed, and fluidized bed reactors) for the ability to continuously degrade artificial and authentic waste streams (Goodall et al., 1998; Lendenmann et al., 1998; Reardon and Spain, 1993; Smets et al., 1999). Slurry reactor systems were also tested for the degradation of nitroaromatic compounds in soils (Nishino et al., 1999). Pilot and field scale tests for the degradation of mixtures of mono- and dinitrotoluenes were conducted in the laboratory and field to develop the engineering parameters and the cost analyses necessary to develop full scale remediation systems for ground water (Spain et al., 1999) and soil (Zhang et al., 2000).

Bench scale treatability studies (Nishino and Spain, 2001b) have also resulted in the development of first a pilot field system (Cuffin et al., 2001a) and now a full scale system for *in situ* biodegradation of DNT at BAAP.

4 Project Accomplishments

4.1 2,4,6-Trinitrotoluene

4.1.1 Overview

TNT was manufactured by sequential nitrations of toluene. The first nitration produced almost equal amounts of 2-nitrotoluene (2-NT) and 4-nitrotoluene (4-NT) due to the *ortho*- and *para*-directing methyl group. The second nitration, also *ortho*- and *para*-directed, primarily resulted in 2,4- and 2,6-DNT depending upon which carbon was still available for nitration. The third nitration filled the remaining *ortho* or *para* ring position. For reasons of safety, the three separate nitration steps were normally carried out in different reactors housed in separate buildings, and the buildings were often built in low lying areas between hills so that the hills between the reactors could direct accidental explosions upwards and away from other buildings (Fig. 1). The result is that soil contamination at explosives manufacturing sites is patchy and heterogeneous. In many areas, the large amounts of nitric and sulfuric acid used in the TNT manufacturing process, have lowered the pH of the soil and helped to preserve the nitrotoluene contaminants from biological activity.

Known biological reactions with TNT (Fig. 2) include reduction of one or more nitro groups under both aerobic and anaerobic conditions (Rieger and Knackmuss, 1995), oxidation of the methyl group (Bruns-Nagel et al., 1999), and reduction of the aromatic ring to form a Meisenheimer complex with the subsequent spontaneous release of nitrite (Vorbeck et al., 1994; Vorbeck et al., 1998). Under anaerobic conditions, TNT can be used as a sole nitrogen source (Boopathy and Kulpa, 1992; Esteve-Núñez and Ramos, 1998) and as a terminal electron acceptor (Esteve-Nuñez et al., 2000). Nonetheless, mineralization has not been reported as the major result of such transformations, and the aromatic ring generally remains untouched.

The commonly identified transformation products (Kaplan, 1990) include: 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 4-amino-2,6-dinitrotoluene (4ADNT), 2-amino-4,6-dinitrotoluene (2ADNT), 2,4-diamino-6-nitrotoluene (24DANT), and under strict anaerobic conditions 2,4,6-triaminotoluene (Hwang et al., 2000; Preuss et al., 1993). The azoxy compounds, 2,2',6,6'-tetranitro-2,2'azoxytoluene and 4,4',6,6'-tetranitro-2,2'azoxytoluene are believed to result from nonenzymatic reactions of the hydroxylaminodinitrotoluenes (Haïdour and Ramos, 1996; McCormick et al., 1976), but recent evidence suggests that the tetraamino analogs are produced from triaminotoluene by biological activity (Hawari et al., 1998). All the above compounds are considered dead end products of TNT transformation.

4.1.2 Survey of nitroaromatic-degrading strains/enzyme activities

Bacteria able to attack the aromatic ring of TNT and add hydroxyl groups preparative to ring cleavage have been elusive, but during the past decade a number of strains able to completely degrade aromatic compounds with only one or two nitro groups have been discovered. Bacterial isolates that degrade nitrobenzene (NB) (Nishino and Spain, 1993; Nishino and Spain, 1995),

4-nitrophenol (Spain and Gibson, 1991), 2-NT (Haigler et al., 1994b), 4-NT (Haigler and Spain, 1993), 2,4-DNT (Spanggord et al., 1991) and 2,6-DNT (Nishino et al., 2000a) through oxidative pathways were tested for their ability to degrade or transform TNT after growth with and without the nitroaromatic substrate that they had been selected to degrade. The strategy was to identify any activity that led to other than reduced products. With a single exception, rigorous mass balances indicated that all of the cultures stoichiometrically transformed TNT to the common reduced products. The studies also demonstrated that the unique nitroarene oxygenases possessed by the strains were not involved in TNT transformation. Rather the metabolites identified were the products of nonspecific nitroreductase activity. As a result we discontinued screening for oxygenase enzymes that catalyze novel transformations of TNT.

4.1.3 Reduction of TNT by JS45 and production of novel metabolites

Pseudomonas pseudoalcaligenes strain JS45 grown on NB catalyzed the rapid transformation of TNT to previously unknown metabolites (Fiorella and Spain, 1997). Strain JS45, isolated for its ability to grow aerobically on NB, uses a novel pathway that begins with a partial reduction of the nitro group to the hydroxylamine level (Nishino and Spain, 1993; Somerville et al., 1995). The hydroxylaminobenzene is subsequently converted to 2-aminophenol by a novel mutase (Davis et al., 2000; Davis et al., 1997; He et al., 1999; He et al., 2000) and ring fission is catalyzed by an unusual dioxygenase (Davis et al., 1999; Lendenmann and Spain, 1996).

Rather than the sequential reductions of TNT to amino derivatives seen in other systems, strain JS45 reduced two nitro groups sequentially to the hydroxylamine level (Fig. 3). 4HADNT, the first product to appear, is converted directly to 4ADNT in a number of bacterial, plant and fungal systems (Hawari et al., 1999; Khan et al., 1997; Michels and Gottschalk, 1994; Pavlostathis and Jackson, 1999; Van Aken et al., 1997), but in the JS45 system a second partial reduction yielded 2,4-dihydroxylamino-6-nitrotoluene (DHANT). Experiments with purified nitrobenzene nitroreductase made it clear that the nitrobenzene nitroreductase catalyzed the reactions and was unable to reduce the hydroxylamino groups to amines. In whole cell experiments, a nonspecific nitroreductase catalyzed conversion of 4HADNT directly to 4ADNT, and DHANT to 2-hydroxylamino-4-amino-6-nitrotoluene (2HA4ANT). 2HA4ANT was oxidized to unidentified products that disappeared during subsequent incubation and a major portion of the carbon became associated with the cells. The unusual transformation pathway could provide the basis for a TNT treatment process and further research has begun to identify the end products of the reaction and evaluate the kinetics with the overall goal of developing strategies that will lead to cleavage of the aromatic TNT ring (Hughes, 2001).

4.2 Mono- and dinitrotoluenes

4.2.1 Pathways and genetic studies

The major contaminants found at many TNT manufacturing sites include the mono- and dinitrotoluene intermediates that lead to TNT, largely 2-NT, 4-NT, 2,4-DNT and 2,6-DNT. Only traces of 3-nitrotoluene, 2,3-dinitrotoluene and 3,4-dinitrotoluene were generated during the manufacture of TNT, and such compounds are rare at contaminated sites. Bacteria able to utilize 2-NT (An et al., 1994; Haigler et al., 1994b), 4-NT (Haigler and Spain, 1993; He and Spain, 2000b; Spiess et al., 1998), 2,4-DNT (Haigler et al., 1999; Haigler et al., 1994a; Spanggord et al.,

1991), and 2,6-DNT (Nishino et al., 2000a) as sole carbon and nitrogen sources have been isolated and the degradative pathways determined. Multiple pathways for 4-NT degradation, all involving an initial reduction, have been identified (Haigler and Spain, 1993; Rhys-Williams et al., 1993; Spiess et al., 1998) in three different bacteria (Fig. 4). To date only a single 2-NT degrading strain has been reported (Haigler et al., 1994b). It attacks 2-NT via an initial oxidation at the 2-nitro position (Fig. 5). A number of strains able to degrade 2,4-DNT or 2,6-DNT have been isolated (Nishino et al., 2000a; Spanggord et al., 1991). The degradative pathways (Fig. 6) used by all the isolates have been the same, although the genes and gene organization may vary considerably among the strains (Johnson et al., 2000). Degradation of 2,4-DNT and 2,6-DNT begins via an initial oxidative attack on the aromatic ring that results in the formation of a methylnitrocatechol and the release of one nitro group. The two pathways diverge at that point (Nishino et al., 2000a). The 4-methyl-5-nitrocatechol formed from 2,4-DNT undergoes a monooxygenase attack at the remaining nitro-substituted position to form 2-hydroxy-5-methylquinone with the release of the second nitro group. A quinone reductase converts the hydroxymethylquinone to 2,4,5-trihydroxytoluene which then undergoes ring cleavage. In contrast, the 3-methyl-4-nitrocatechol formed from 2,6-DNT is attacked by an extradiol ring cleavage dioxygenase to yield 2-hydroxy-5-nitro-6-oxo-2,4-heptadienoic acid which is then hydrolyzed to 2-hydroxy-5-nitro-2,4-pentadienoic acid plus acetic acid. The second nitro group is released as nitrite in a subsequent reaction.

A number of the genes involved in the pathways have been cloned and sequenced (An et al., 1994; Haigler et al., 1999; Haigler et al., 1997; Johnson et al., 2000; Johnson and Spain, 1997; Suen and Spain, 1993). Genetic analysis has shown that the initial dioxygenase that attacks 2,4-DNT at the 4-position is closely related to naphthalene dioxygenase (Table 1) (Suen et al., 1996), but an expanded survey of the small ISP subunits from a number of nitroarene dioxygenases and naphthalene dioxygenases showed that the nitroarene dioxygenases and naphthalene dioxygenases cluster separately (Johnson and Spain, 1997). The closely related nitroarene dioxygenase ISP subunits are distributed throughout both the gram negative and gram positive bacteria suggesting that the initial dioxygenase evolved once and has spread horizontally through bacterial communities at contaminate sites (Nishino et al., 2000b).

4.2.2 *Ex situ* bioremediation of DNT mixtures

The isolation of specific mono- and dinitrotoluene degrading bacteria allowed the development of strategies for *ex situ* remediation of mixtures of mononitrotoluenes and DNT. Early research conducted before the start of Flask to Field, focused on treating 2,4-DNT with pure cultures in bioreactors. The studies showed that complete and rapid removal of 2,4-DNT depended on retention of the slow-growing 2,4-DNT-degrading biomass within the bioreactor (Heinze et al., 1995; Reardon, 1992). After the start of Flask to Field, our program concentrated on developing strategies for treating ground water and soil contaminated with mixtures of nitrotoluenes. Much of the work described in the following sections has been incorporated into U.S. Patent 6,248,580 (Spain et al., 2001) describing bioremediation systems for DNT. A technology status review for bioremediation of DNT is available on the ESTCP web site (Nishino and Spain, 2001c).

Bench scale ground water studies. An artificial ground water containing 2,4- and 2,6-DNT was used to feed a mixed culture of 2,4- and 2,6-DNT degrading bacteria in a bench scale fluidized bed reactor (FBR, Fig. 7) throughout a series of experiments spanning a 6 month period

(Lendenmann et al., 1998; Smets et al., 1999). Sand was used as the biomass carrier so that rigorous mass balances could be determined. The bench scale tests demonstrated the complete and simultaneous mineralization of mixtures of DNT at various DNT loading rates and hydraulic retention times (Fig 8). Minimum oxygen requirements to support complete DNT degradation were determined. A nitrite oxidizing population that converted most of the excess nitrite to nitrate became established during the first month of the reactor operation. Federal regulatory standards for nitrate in drinking water are 10 mg/L vs. 1 mg/L for nitrite, making nitrate the more desirable form for discharge.

During the course of the bioreactor experiments, it became possible to isolate bacteria able to degrade both isomers of DNT. No such strains were found in initial samples, less than 1% of isolates obtained after 2 months of operation could degrade both DNT isomers, and 98% of the isolates obtained after 4 months of operation could degrade both 2,4- and 2,6-DNT. How such "dual degraders" arose and the specific pathways by which they degrade the two DNT isomers are currently under investigation.

The artificial ground water was replaced by contaminated ground water from VAAP containing 2-NT and 4-NT in addition to the DNT. A 7-day acclimation period was required before 2,4-DNT removal rates returned to >98% at a 6 hr hydraulic retention time. The hydraulic retention time was reduced to 3 hr, and after an additional 12 days, the removal of rates of the nitrotoluenes were > 98% with the exception of 2,6-DNT for which the removal rate was 62% (Lendenmann et al., 1998).

Field study at VAAP. The bench scale tests established the parameters necessary to design a pilot scale system for tests in the field at VAAP. The field demonstration was a combined effort of AFRL, Envirogen, Inc., Malcolm Pirnie, Inc., and the USACE Baltimore District (Envirogen Inc., 1999; Spain et al., 1999). A FBR was operated with ground water at VAAP for 5 months during 1998 to determine the feasibility of using a FBR inoculated with specific nitrotoluene-degrading bacteria to remediate ground water contaminated with mixtures of mono-, di- and trinitrotoluene. Additional objectives were to characterize removal efficiencies and rates, and to establish operating costs and the necessary parameters for designing full scale FBR systems for nitrotoluene degradation.

Because the FBR at VAAP was to use granular activated carbon (GAC) rather than sand as the biomass carrier, experiments were conducted in the laboratory to determine how the strong sorption of nitroaromatic compounds by GAC affects the bioavailability of nitroaromatic compounds. It was not known whether bacteria remained active and viable in the absence of their substrates. We designed experiments to evaluate the effect of various strategies for saturation of the GAC and inoculation. Three assays were developed to distinguish biodegradation from sorption. The first compared contaminant concentrations in the FBR influent and effluent. The second strategy measured oxygen depletion, and the third measured nitrotoluene degradation in batch experiments. The results demonstrated that a mixed microbial community attached to GAC is capable of degrading all of the nitroaromatic compounds and that the community can be recovered from storage. However, it is essential that the GAC be saturated prior to inoculation with degradative bacteria. The most sensitive populations were the 2,6-DNT degraders which may be lost or inactivated if unsaturated carbon is used. Saturation of the GAC also occurred at different times for each compound in the mixture of chemicals. That is, saturation of GAC with

2,4-DNT did not also mean that the GAC was saturated for 2,6-DNT. An early misstep during the field study was inoculation with the degradative bacteria before the GAC was saturated by all the nitrotoluenes. As a consequence, the bacteria starved and the FBR had to be reinoculated with active cultures before biodegradation began.

After reinoculation, removal efficiencies for 2-NT, 3-NT, 4-NT, and 2,4-DNT were high throughout the test period (Table 2). Removal efficiency for TNT varied between 33 and 73% depending upon the hydraulic retention time. 2,6-DNT removal was low during the initial phases of the study, but improved dramatically after 4 months of operation. Because the slow degradation of 2,6-DNT limited the overall efficiency of the system, a simple tank for extended aeration of FBR effluent was tested in the laboratory. There was no pH or temperature control, and no agitation other than that supplied by an air stone. Effluent was added to the tank in 2.5 to 3 gallon batches and replaced with fresh effluent when 2,6-DNT was degraded (Fig. 9). The time required for the degradation of 2,6-DNT in successive batches of effluent became shorter, due in part to the lower concentration of 2,6-DNT in later batches of effluent. The lower concentration was due to the improvement in 2,6-DNT degradation in the FBR. The results demonstrate clearly that 2,6-DNT can be degraded rapidly during extended aeration.

The specific causes of the initial inhibition of 2,6-DNT degradation are unknown. A number of tests with artificial ground water containing the nitrotoluenes in the concentrations found in the VAAP ground water failed to show any effect of the presence of any combination of 2-NT, 4-NT, 3-NT, 2,4-DNT, or TNT on 2,6-DNT degradation. Addition of lead, copper, and zinc, the metals found in the highest concentrations in ground water from VAAP had no effect, nor did high concentrations of nitrate, also present in the ground water at VAAP. Onlt the total removal of organic compounds from VAAP ground water with a C_{18} solid phase extraction column, and addition of the extracted organic compounds back to the eluate inhibited 2,6-DNT degradation. An artificial mixture of nitrotoluenes added to the eluate at the concentrations originally found in the ground water failed to inhibit 2,6-DNT degradation. The result suggests that some non-polar organic component of the VAAP ground water was responsible for the observed inhibition of 2,6-DNT degradation. Despite the initial inhibition, the acclimation of the system at the termination of the field study and the lack of any undesired metabolic or transformation products in the reactor effluent demonstrated the feasibility of the technology for mixed nitrotoluene remediation. A steady state for 2,6-DNT degradation was not achieved during the final days of operation of the FBR. The concentration of 2,6-DNT in the effluent was still trending downwards when the FBR was shut down. With continued operation, it is likely that high 2,6-DNT removal rates could have been achieved at shorter hydraulic retention times. It is clear that the FBR could have been optimized for 2,4-DNT degradation if a second reactor in series had been operated for 2,6-DNT degradation. The extended aeration experiment demonstrated that the second reactor could have been as simple as the addition of aeration to the effluent holding tank.

Cost analysis showed that the aerobic fluidized bed reactor technology is more cost effective than UV/Ozone treatment or granular activated carbon treatment when the total nitrotoluene removal rate exceeds 120 lb/day (Envirogen Inc., 1999; Spain et al., 1999). Because the cost analysis was based on treatment rates that were limited by the degradation rate for 2,6-DNT, it is expected that costs would be lower in a treatment system that has adapted to rapidly degrade both isomers of DNT. The U.S. Army has estimated the cost for remediation of contaminated ground water at five

sites (Volunteer AAP, West Virginia Ordnance Works, Cornhusker AAP, Iowa AAP, Longhorn AAP) to be one quarter of the total cleanup cost at the sites (Annual Report to Congress for Fiscal Year 1995).

Bioremediation as a clean up alternative for an industrial site. The gypsum waste stack of a former phosphate fertilizer plant owned by Imperial Chemical Industries, Canada, is the source of about 1 M gallons of DNT-contaminated water. Seep water is extremely acidic as it exits the gypsum stack. The seep water is treated with lime to raise the pH to 11 in order to precipitate fluoride and drive off ammonia. Following lime treatment, the water is sent to a series of holding ponds for several years. The current remediation permit will expire in a year and a permanent solution is required by the Canadian regulatory agency. Preliminary studies indicated that added DNT-degrading strains will mineralize the DNT in the water once the pH is adjusted to neutral (Fig. 10.) (Nishino and Spain, 2000a). Based on the results of the preliminary studies, ICI Canada made the decision to use bioremediation for cleanup of the site. A CRADA (USAF, 2000) was negotiated between AFRL/MLQL and ICI Canada to support the bioremediation project.

Studies under the CRADA found that DNT-degrading bacteria have developed in some of the holding ponds on the ICI site (Nishino and Spain, 2001a), and further tests determined the pH and nutrient requirements for the DNT-degrading enrichments. The initial objective of the studies was to elucidate the necessary physical conditions to support DNT degradation in seep water from the gypsum stack in a field scale FBR and a permit was obtained from the Canadian authorities to operate such a reactor. The discovery, however, of rapid DNT degradation in the holding ponds has convinced ICI to create artificial wetlands for DNT degradation. The design for the artificial wetlands is currently being finalized, but it is expected to consist of a liming cell followed by a series of settling ponds and possible limestone filters prior to the actual deep water marsh and shallow water marsh treatment cells (Conestoga-Rovers & Associates, 2000). The wetland treatment cells will be planted with emergent plants to remove residual phosphate and ammonia, and to provide attachment sites for the DNT-degrading microbial biomass.

Biodegradation of DNT in soil slurries. We extended our research on mineralization of DNT to soil slurries and soil to address the problem of the large amounts of contaminated soil at U.S. defense sites. Remediation of DNT in soil slurries was demonstrated in bench scale studies of soils contaminated over 50 years ago with 9.6 g-DNT/kg-soil (Nishino et al., 1999). Draw and fill slurry reactors inoculated with DNT-degrading bacteria repeatedly removed both 2,4-DNT and 2,6-DNT from the field contaminated soil. Mineralization studies showed that from 43 to almost 60% of radiolabeled DNT was recovered as $^{14}CO_2$ (Fig. 11).

The success of the bench scale study led to supplemental SERDP funding to conduct scale up studies of DNT mineralization in soil slurries (Zhang et al., 2001; Zhang et al., 2000). DNT contaminated soils were obtained from VAAP and BAAP. The soils were dried, powdered, and passed through a 20 mesh sieve, and mixed. The composite soils were extracted with acetonitrile and analyzed for 2,4-DNT and 2,6-DNT (Table 3). Weighed amounts of soil were treated in a hot water wash prior to use in the 75-L Eimco reactors. The airlift design of the reactors prevented direct use of sandy soil in the reactors. The hot water wash removed better than 99% of the DNT from the heavier sand. The DNT-contaminated wash water and the light clay components were treated in the Eimco reactors. The reactors were inoculated with a mixed culture of 2,4-DNT and 2,6-DNT degrading bacteria that had been grown on a mixture of 2,4- and 2,6-DNT. The reactors

were run in both continuous feed and in batch modes in a series of experiments designed to determine the most effective operating conditions for DNT degradation.

2,4-DNT was degraded immediately and rapidly in both soils at concentrations up to 11 mM, but 2,6-DNT degradation in the presence of high concentrations of 2,4-DNT was inhibited. Continuous feeding of low volumes of soil wash was used to achieve degradation of both DNT isomers, but forced the reactors to be operated at a rate geared to degradation of the minor contaminant. Protocols were developed for sequential treatment of 2,4- and 2,6-DNT, thereby reducing the overall treatment time (Fig. 12). Nitrite release was stoichiometric with 2,4-DNT degradation. At high soil loading rates, accumulated nitrite both lowered the pH of the slurry and eventually inhibited 2,4-DNT degradation. The pH was adjusted with concentrated NaOH, but nitrite toxicity to 2,4-DNT degradation limited the soil loading rate to 40%. Nitrite did not inhibit 2,6-DNT degradation, but the high background of nitrite in the second reactor prevented the use of nitrite as a simple monitor for 2,6-DNT degradation. The DNT degradation rates achieved in the sequential batch reactors increased with higher DNT concentrations (Table 4).

The study demonstrated that highly contaminated soils can be treated without extensive dilution. That in turn allows for smaller reactors and lower volumes of residual materials for treatment and disposal. Although the airlift reactors were quite effective when the heavy sand was removed from the treatment system, the soil wash step could be eliminated through the use of other reactor designs. Cost analysis showed that the sequential slurry treatment system is competitive with thermal treatment with estimated costs of $100 to $200 per cubic yard (Zhang et al., 2000).

4.2.3 *In situ* degradation of DNT

In situ remediation has several potential advantages over traditional *ex situ* technologies all related to the fact that the contaminated material, whether soil or water, does not have to be removed from the site for treatment and disposal. That offers potentially huge cost savings for handling, transport, and disposal, particularly when large volumes of materials are involved. Minimal handling and transport also reduces the potential for accidental contamination of additional equipment and sites exposed to the contaminated materials. In spite of these advantages, few *in situ* technologies have been developed for non-volatile, non-dissolved contaminants. That is largely due to the difficulties in: 1) establishing precisely where and how much contaminated material must be cleaned up, 2) determining the presence and activity of indigenous degradative microbial populations, 3) developing effective delivery systems for amendments and nutrients, 4) predicting and controlling undesired side effects of the remediation technology.

Engineered approach for in situ *bioremediation at BAAP.* It is clear that bacteria able to degrade DNT are present at many DNT-contaminated sites. The finding raises questions about the reasons for persistence of DNT at such sites, and what can be done to encourage *in situ* degradation of contaminants by the native bacteria. We conducted preliminary microcosm experiments to determine the feasibility of *in situ* DNT degradation at BAAP which is located near Baraboo, WI. BAAP has two sites that were formerly used to dispose of wastes from the processing of single-base gun propellants and deterrents which consist of various mixtures of nitrocellulose, 2,4-DNT, dibutylphthalate, and diphenylamine (Table 5). The propellant residues were dissolved in organic solvents like benzene then disposed of in large in-ground waste pits. In some areas of the waste

pits, the soil contains up to 28% 2,4-DNT by weight (Stone & Webster Environmental Technology & Services, 1998).

Treatability studies. Soil from waste pits at two BAAP sites, the Propellant Burning Ground (PBG) and the Deterrent Burning Ground (DBG), were found to contain indigenous populations of DNT-degrading bacteria (Fig 13). At room temperature, the addition of phosphate and moisture was sufficient to initiate DNT-degradation in field contaminated soils within 2 weeks (Fig. 14). At normal soil temperatures, a year-round 13°C in the subsurface, degradation began in 2 months (Nishino and Spain, 1999)(Fig 15). The bacteria are uniquely adapted to degrade DNT at the high soil pH (8.5-9.3) at BAAP (Fig 16). The information was used as the basis for design of pilot scale *in situ* treatment systems for DNT-contaminated waste pits at the PBG.

Further bench scale treatability studies were conducted during the start up and operation of the pilot field study in order to define the critical parameters that affect *in situ* DNT degradation (Nishino and Spain, 2001b). Lack of DNT degradation can be attributed to a number of factors. We demonstrated the presence of indigenous bacteria with ability to grow on DNT in the preliminary studies and thus focused on physical conditions and potential inhibitors. Physical conditions can include such factors as soil pH, temperature, moisture, oxygen, and inorganic nutrients. Potential inhibitors are the presence of nitrite, or other compounds such as competing carbon sources that can cause catabolite repression.

Soil pH was eliminated as a limiting factor when it was demonstrated that the indigenous bacteria were most active at the soil pH. Starvation for oxygen at BAAP could not be addressed in the laboratory because only direct measurement of soil oxygen levels in the field can establish whether sufficient oxygen is present for DNT degradation. We had, however, previously established the minimum oxygen requirements for DNT degradation in bioreactor studies (Lendenmann et al., 1998) and provided that standard to Stone & Webster for monitoring purposes at the pilot study site. In addition to oxygen, bacteria require nitrogen and phosphorus as macronutrients. Tests in shake flasks with uncontaminated BAAP soil and ground water indicated that no macronutrient other than phosphorous had an effect on DNT degradation. Soil columns amended with ortho phosphate showed more rapid DNT degradation than columns without the amendment, but phosphate addition was not required for complete degradation of DNT (Fig. 17). Temperature effects were studied in shake flasks and columns. Initiation of DNT degradation was delayed in cultures incubated at the BAAP soil temperature of 13°C (Fig. 18), requiring up to 3 months before degradation began.

Previously, we found inhibition of 2,4-DNT degradation when nitrite concentrations exceed 20 mM (Nishino et al., 2000b). We constructed microcosms of PBG soil that was moistened with deionized water or phosphate buffer (10, 100, and 1000 mM) to varying percentages of field capacity (25, 50, and 100%). The headspace was approximately 4 times the volume of soil. The microcosms were inoculated with DNT-degrading bacteria and the microcosms were incubated at 30°C. Sets of microcosms were extracted and analyzed periodically. DNT degradation stopped in all microcosms at nitrite concentrations between 20 and 40 mM (Fig. 19) or the equivalent of 3 – 6 % of the DNT in the soil.

Small columns were constructed with PBG soil, and flushed twice weekly with one column volume of 20 mM phosphate buffer. Half the columns were inoculated with DNT degrading

bacteria. The columns were incubated at room temperature. The eluate was collected and analyzed. Nitrite was released immediately from the inoculated columns and after 2 weeks in the uninoculated columns. DNT was washed out of all the columns, but degraded in each eluate that contained nitrite. After 250 days, DNT was no longer detected in the eluate. Twice during the experiment, one of the twice weekly flushes was omitted and the columns dried out. In each instance there was a lag period before DNT degradation resumed.

The results of the treatability studies suggest that in the BAAP soils low soil temperatures, lack of moisture and accumulation of nitrite have the greatest impacts on DNT degradation. Moisture is not only necessary for bacterial activity, but also serves as a mechanism for removal of excess nitrite.

In situ treatment system. Stone & Webster Engineering designed, built and operated an *in situ* treatment system (Cuffin et al., 2001b)(Fig. 20) in one of three 30 ft diameter x 100 ft deep waste pits at the PBG under a cleanup initiative funded by the US Army Corps of Engineers, Omaha District. Ground water was pumped from the bottom of the waste pit into a holding tank on the surface. Water from the holding tank was returned to the waste pit through infiltration piping laid at the top of the waste pit. DNT-degrading bacteria were added to ground water during the early stages of the pilot test. Provision was made to heat the ground water in the holding tank and to add nutrients if necessary to stimulate DNT degradation. Existing soil vapor extraction vents were converted to deliver air to 5 levels within the waste pit. An anaerobic zone designed to destroy the nitrite released by DNT degradation was created down gradient from the waste pit by pumping an easily degradable carbon source, initially propylene glycol and subsequently ethanol, into the vadose zone. Initial tests of the treatment system began in early April, 2000 and ran through August, 2000. Several parameters indirectly indicate the success of the system. The concentration of 2,4-DNT in the recirculating ground water dropped from 16,000 to 130 µg/L between April 5 and July 17 and nitrate accumulation accompanied start up of the system. Oxygen became depleted in the ground water exiting the bottom of the waste pit. Finally, the initial ratio of 2,4-DNT to 2,6-DNT changed from 50:1 in the untreated soil to 1:5 in the treated water over the 4 month trial (Fig 21). The extreme shift in the ratios reflects the rapid removal of the 2,4-DNT .

Approval has been given to extend the treatment to the other two waste pits at the site. Costs for cleanup of DNT contaminated soil at BAAP using excavation to 110 ft below the surface and incineration were estimated to be $75 million per waste pit. Cost savings for successful *in situ* bioremediation is expected to be $60-65 million with a time savings of 10 years. If the technology is successful at Badger it can be expected to be applied at other sites in DoD and in civilian applications.

Validation studies. While results of the pilot-scale work were promising, the data gathered did not provide the information required to insure the successful use of the full scale systems and did not provide the information needed for site-specific analysis of *in situ* DNT bioremediation at other contaminated sites. A rigorous evaluation of the contribution of biodegradation to the overall decline in DNT contamination at BAAP could not be performed with the data collected during the pilot study because of the inability to conduct a mass balance on the DNT in that system. Questions that remain from the work at BAAP include: 1) the rates of DNT degradation, and how degradation may influence the longevity of the DNT source, 2) the level of 2,4-DNT degradation

that must be achieved before 2,6-DNT degradation begins, 3) the need/benefit of bioaugmentation – or other strategies to minimize DNT washout before rapid degradation begins, 4) the limits of treatment and whether they will meet remedial objectives. In order to resolve these questions, and determine the potential for widespread applications, highly controlled studies are needed in vadose-zone simulation systems where rigorous material balances can be performed.

In on-going experiments at Rice University the overall process implemented at BAAP is simulated in a scale where mass balance of DNT isomers is achievable and optimization of operational parameters can be tested. The soils used were obtained previously from contaminated areas at BAAP. The soil has been thoroughly characterized and packed in columns (4 in. I.D. x 6 in. length). Water (55.6 ml) and air (560 ml) are provided daily through metering pumps at volumes scaled from the BAAP process, and effluents analyzed for DNT isomers, nitrite, nitrate, and pH. Currently, four columns are in operation. Two receive sodium azide as an inhibitor of microbial activity. Effluent is not recycled in current studies.

Results to date have shown high levels of DNT washout with no statistical difference between systems that do and do not receive azide. The average DNT concentrations in the column eluates range between 844 and 903 μM for 2,4-DNT and between 371 and 396 μM for 2,6-DNT. Initial samples from systems without azide showed elevated nitrite levels (~100μM) that dropped to near detection limits after approximately 10 days. No significant nitrite production has been observed in the azide-treated controls. The dominant form of nitrogen leaving the active systems is nitrate at levels similar to that initially observed as nitrite. The columns are currently being run at room temperature, but additional columns will be run at the BAAP soil temperature.

Natural Attenuation. The occurrence of indigenous DNT-degrading bacteria is being used in support of monitored natural attenuation of a DNT-contaminated ground water plume also located at BAAP as well as at two commercial manufacturing sites for DNT. Air Products Inc., the world's largest commercial producer of DNT has two sites that are contaminated with several DNT plumes. Air Products requested that MLQL conduct the preliminary studies to identify the presence of indigenous DNT-degrading populations at the industrial sites in order to evaluate the contribution of natural attenuation to the cleanup strategy for the sites. Natural attenuation refers to the destruction, *in situ*, of contaminants by physical, chemical or biological means without human intervention. It has become clear that biological processes play the predominant role in natural attenuation (Williams et al., 1997), and the presence of active DNT-degrading bacteria at contaminated sites suggests that natural attenuation may play a role in the clean up of DNT contaminated sites. The U.S. Environmental Protection Agency recommendations define biological degradation processes directly demonstrated in contaminated site material to be sufficient evidence to propose monitored natural attenuation as a remediation alternative (US Environmental Protection Agency, 1999). When our studies made it clear that there are active DNT-degrading bacterial populations at both of the contaminated industrial plants (Nishino and Spain, 2000b) Air Products decided to have a remediation proposal designed to incorporate natural attenuation for DNT.

4.3 Nitrobenzene

NB has long served as a model compound, being the simplest of nitroaromatic compounds. It is also a widely used industrial chemical that serves as the starting material for a number of important industrial processes. We isolated NB degrading strains from a number of contaminated industrial sites and treatment plants (Table 6) (Nishino and Spain, 1995) and described two different NB degradation pathways (Fig. 22). The most widespread pathway, is the partial reductive pathway first described in *Pseudomonas pseudoalcaligenes* JS45 (He and Spain, 1997; Nishino and Spain, 1993), the organism described above with the ability to transform TNT to novel metabolites. A second oxidative pathway was found in *Comamonas* sp. JS765 which uses nitrobenzene dioxygenase to convert NB to catechol with the loss of nitrite. Catechol is then degraded via a *meta*-cleavage pathway (He and Spain, 1999a; Nishino and Spain, 1995).

When the partial reductive pathway in JS45 was described, only 4-nitrobenzoate (Groenewegen et al., 1992; Groenewegen and de Bont, 1992) and 4-NT (Haigler and Spain, 1993; Rhys-Williams et al., 1993) were known to be degraded via partially reductive pathways, but whereas those pathways involved eventual conversion to a catechol/protocatechuate, the NB pathway utilized a novel mutase to convert hydroxylaminobenzene to *ortho*-aminophenol. Since then partial reduction has been found in pathways for several other nitroaromatic compounds (Nishino et al., 2000b).

Extensive work has been done to purify the enzymes and identify the genes of both pathways, particularly for the partial reductive pathway (Davis et al., 1999; Davis et al., 2000; He et al., 1998; He et al., 1999; He et al., 2000; He and Spain, 1998; Lendenmann and Spain, 1996; Somerville et al., 1995). The genetic studies and comparisons of the two pathways have been done in order to better understand the mechanisms by which nitroaromatic degradation pathways might have evolved. Understanding such processes can help us to understand where and when we might expect such pathways to evolve in nature.

The novel reactions catalyzed by the enzymes of the partial reductive pathway have been exploited to produce novel *ortho*-aminophenols (Nadeau et al., 2000), picolinic acids (He and Spain, 2000a; Lendenmann and Spain, 1996), and other specialty chemicals (He and Spain, 1999b) (Fig. 23).

4.4 Nitramine Explosives

Nitramine explosives are compounds which have a nitro group attached to a nitrogen atom. Although RDX and HMX are the two most widely distributed nitramine explosives we began our work on tetryl (2,4,6-trinitrophenylmethylnitramine) a nitramine that also incorporates a nitrosubstituted aromatic ring, and thus has structural similarities to TNT (Fig 24). Enrichment cultures inoculated with soil from tetryl contaminated sites yielded some bacteria which could slowly grow on tetryl as a sole carbon and nitrogen source; however, the cultures could not be made to grow on tetryl concentrations greater than 25 μM and the experiments were discontinued. A metabolite that accumulated in tetryl fed cultures was identified as N-methylpicramide and had been previously described as a tetryl metabolite found in soil (Fellows et al., 1992). The commercially available ferredoxin NADP oxidoreductase from spinach was found to catalyze the same reaction at the nitramine nitro group to also produce N-methylpicramide (Shah and Spain,

1996). A mechanism was proposed for the nitroreductase mediated reaction with the suggestion that similar mechanisms should be investigated to determine whether they might lead to the elimination of nitrite from other nitramine compounds (Fig. 25).

5 Summary

The Explosives thrust of the Flask to Field initiative focused on the examination of bacteria able to grow on nitroaromatic compounds. We discovered bacteria that are able to mineralize 2,4-DNT, 2,6-DNT and nitrobenzene. The catabolic pathways used by the bacteria for growth on the above nitroaromatic compounds involve many novel reactions. The enzymes found in the pathways have been purified and characterized, and many of the genes involved in the pathways have been identified and cloned. The novel enzymes discovered in the biodegradative pathways were screened for the ability to transform TNT. Previously unidentified metabolites of TNT resulted from transformation by purified enzymes and whole cells of the nitrobenzene-degrading strain JS45.

Major effort was directed towards development of pilot and field scale systems for degradation of mixtures of nitrotoluenes. Bench scale studies established the parameters necessary to design a fluidized bed reactor for a five-month field test of ground water remediation at Volunteer Army Ammunition Plant. 2,4-DNT, 2-NT and 4-NT were degraded efficiently at all residence times. Removal efficiency for TNT varied between 33 and 73% depending on hydraulic retention time. 2,6-DNT removal was low initially but improved after four months of operation. Cost analysis showed that this technology was more cost effective than UV/ozone or granular activated carbon treatment when the total nitrotoluene removal rate exceeded 120 lb/day.

Other bench scale studies established the effectiveness of biodegradation of mixtures of DNT in soil slurry systems. Pilot scale studies demonstrated that 2,4-DNT degraded immediately and rapidly in up to 40% soil slurries where the soil contained 12 g/kg DNT, but nitrite toxicity is limiting to 2,4-DNT degradation. At high 2,4-DNT concentrations, 2,6-DNT degradation must be separated from 2,4-DNT degradation to optimize the overall process. Cost analysis showed that sequential soil slurry bioremediation systems are competitive with thermal treatment.

Another emphasis of the explosives research was on development of *in situ* pilot and field scale systems for degradation of mixtures of DNT in soil. A critical component of the research has been to elucidate the bottlenecks that inhibit efficient DNT degradation in above ground reactor systems as well as *in situ* field applications. Bench scale treatability studies using soil from Badger Army Ammunition Plant (BAAP) indicated that soil contains indigenous populations of DNT-degrading bacteria. Based on that finding, a pilot scale treatment system designed to stimulate the degradative activity of the indigenous bacteria was implemented at BAAP. The 2,4-DNT concentrations dropped from 16,000 to 130 µg/L during the first three months of the field test. Full scale application of *in situ* bioremediation of DNT has been approved for BAAP following the successful pilot demonstration. Cost savings per waste pit at the site is expected to be $60-65 million with time savings of 10 years. Industrial producers of DNT have also adopted bioremediation as clean up alternatives for contaminated sites based on the discoveries pursued under Flask to Field.

The Explosives thrust research has resulted in 38 peer-reviewed publications, 44 technical abstracts, and 8 book chapters. A technology status review of bioremediation of DNT has been

published on the ESTCP website at http://www.estcp.org/documents/techdocs/DNT_Report.pdf. United States patent 6,248,580 "Process for the biodegradation of dinitrotoluene" is based on the research pursued under Flask to Field.

6 Recommendations for Transitional Research

Nothing is known about the regulation of the enzymes that remove the nitro groups from nitroaromatic compounds. Insight about the enzymes involved in the initial oxidative attack on nitroaromatic compounds and their regulation should lead to improved strategies for the degradation of mixtures of nitrotoluenes and an improved basis for predicting natural attenuation of DNT. At many sites, soil and groundwater are contaminated by mixtures of DNT and TNT. Laboratory and field tests conducted to date with such mixtures of contaminants have not indicated that TNT is removed effectively during the aerobic degradation of DNT. It may be possible to develop systems that partially reduce TNT prior to oxidative attack. Understanding the regulation and biochemistry of the initial oxidative reactions of the DNT-degrading bacteria can provide the basis for metabolic engineering to develop a new biochemical strategy for the initial attack on TNT. Research with the nitramine explosive tetryl has demonstrated that nitroreductase enzymes can remove the nitramine nitro group. Whether that may prove to be a universal mechanism for degradation of nitramine explosives remains to be answered.

Other needed research that was identified at a recent symposium on nitroaromatic compounds and explosives is presented in Attachment A.

7 Conclusions Regarding Utility in Remediation

We have demonstrated that nitroaromatic compounds are readily degraded by specific bacteria that are widespread at contaminated sites. The bacteria, when supplied with appropriate nutrients and environmental conditions are capable of completely destroying the nitroaromatic contaminants more rapidly and cost effectively than traditional technologies. Because aerobic degradation strategies rely on bacteria that use nitroaromatic substrates as primary growth substrates, selection is direct and the degradation process is self-sustaining. Mineralization of the nitroaromatic compounds completely destroys the compound leaving only inorganic nitrogen, CO_2, and bacterial biomass. No additional growth substrates are necessary to support cometabolic processes, and undesired metabolites do not accumulate. We have also shown that when appropriate bacteria are not present at a contaminated site, bioaugmentation of soil or ground water with specific degradative bacteria is an effective treatment.

2,4-DNT is more easily degraded than 2,6-DNT, and sequential treatment systems may be needed to treat soil or water containing both isomers. TNT is far less biodegradable than these DNT isomers, and the presence of TNT may make bioremediation more difficult or expensive.

In situ bioremediation is possible at sites where: 1) aerobic conditions are present or can be engineered; 2) appropriate organisms are present or can be introduced effectively; 3) the potential for nitrite or nitrate accumulation can be managed. Ex situ bioremediation is more expensive but may be needed at sites that do not meet the above criteria. Fluidized bed reactors have been demonstrated for treatment of DNT in water, and soil slurry reactors are effective for contaminated soils.

8 Technology Transfer

The technology for bioremediation and natural attenuation of organic compounds has developed dramatically over the past 15 years due to extensive programs carried out in the US and in Europe. There is a considerable amount of field experience and protocols are available for the bioremediation of petroleum hydrocarbons and chlorinated aliphatic solvents. The technology for remediation of such compounds can, therefore, be considered mature with the possible exception of strategies for treatment of dense non aqueous phase liquids in source zones.

In contrast, there has been little work on bioremediation or natural attenuation of other synthetic organic compounds of commercial or military importance. The major contribution of the work described in this report is the discovery that many nitroaromatic compounds and explosives are biodegradable. The understanding of the mechanisms, strategies, and requirements for biodegradation provides the basis for a variety of field applications. The discoveries described here allow the vast body of knowledge and experience in bioremediation and natural attenuation to be extended to include nitroaromatic compounds. We have provided several bench- and field-scale demonstrations that the principles apply in practical applications. The major payoff of the work will come in the future when bioremediation based on the principles described here is used routinely for cleanup of sites contaminated with nitroaromatic compounds. Technology transfer activities during the conduct of the research are described in Attachment B.

In September 1999 AFRL organized an International Symposium on the Biodegradation of Nitroaromatic Compounds and Explosives. The symposium was funded by the Air Force Office of Scientific Research and the Defense Threat Reduction Agency. The work funded under the Flask to Field program was showcased at the symposium. In a related effort, the work on biodegradation of explosives for the past 5 years was critically reviewed in a book that was published in June, 2000 (Spain et al., 2000). The book comprises peer reviewed chapters by a group of international experts who not only analyzed ongoing work in their areas of expertise, but also identified areas where important questions remain. A summary of the consensus on areas where additional research is needed was provided to SERDP and other funding agencies (Appendix B).

Transition of biodegradation for clean up of DNT has begun. An *in situ* system for remediation of DNT is under construction at BAAP. ICI Canada has adopted biodegradation as the clean up alternative in a passive wetland treatment system for DNT-contaminated seep water at a former industrial site. Air Products is exploring natural attenuation as the clean up alternative for two DNT-contaminated industrial sites. First Chemical Corporation has recently made inquiries as to the feasibility of using natural attenuation for remediation of nitrobenzene at an industrial site.

Significant effort must be spent on the engineering design to ensure that treatment will be successful and cost-effective, particularly when mixtures of nitroaromatic compounds are present, the normal case at DNT contaminated sites. Site-specific laboratory testing is essential prior to selection and design of a bioremediation system. Key issues for laboratory tests include: 1) appropriate chemical and physical conditions; 2) verification of the presence of bacteria capable

of mineralizing the major contaminants; 3) presence of indigenous bacteria capable of removing or converting excess inorganic nitrogen compounds to environmentally acceptable forms; and 4) whether achievable endpoints are acceptable in terms of toxicity and risk. Laboratory tests may also be needed to estimate degradation rates, to establish maximum contaminant concentrations that can be biodegraded, and to design strategies to biodegrade mixtures of nitroaromatic compounds to optimize the efficiency of the remediation system.

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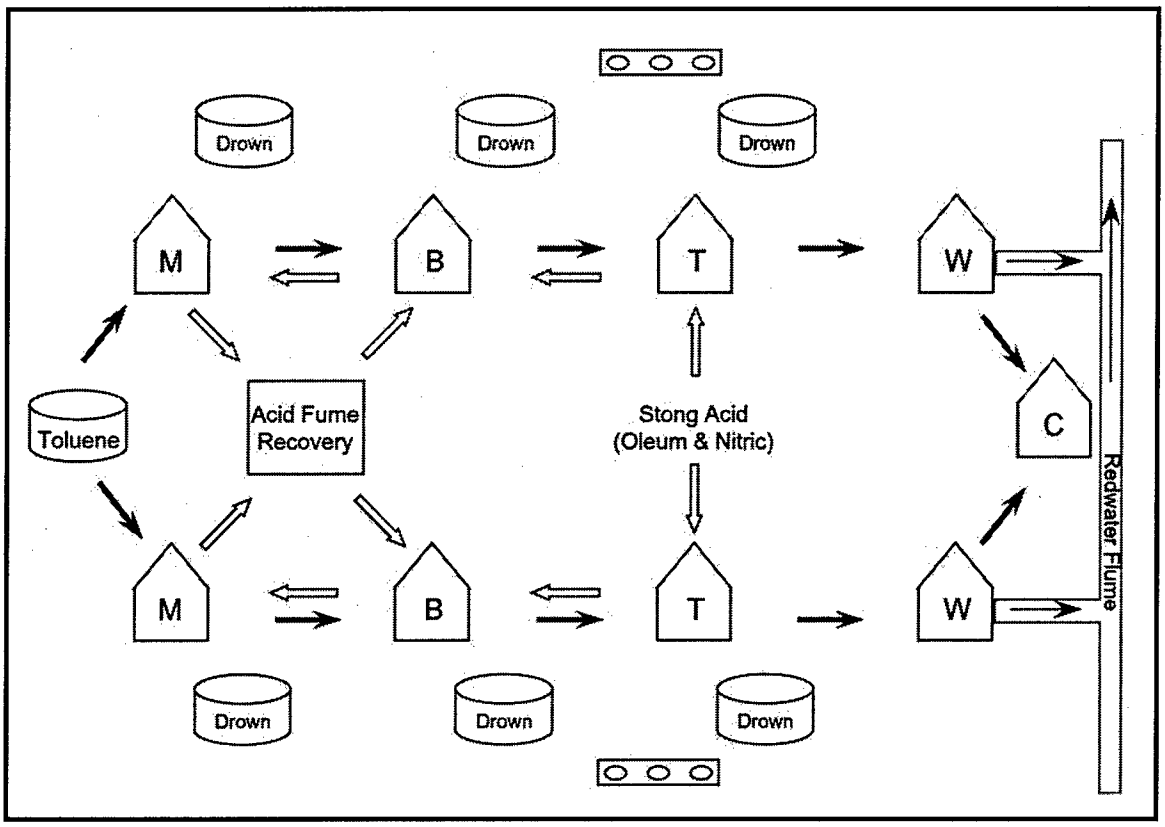

Figure 1. Typical TNT production line at VAAP

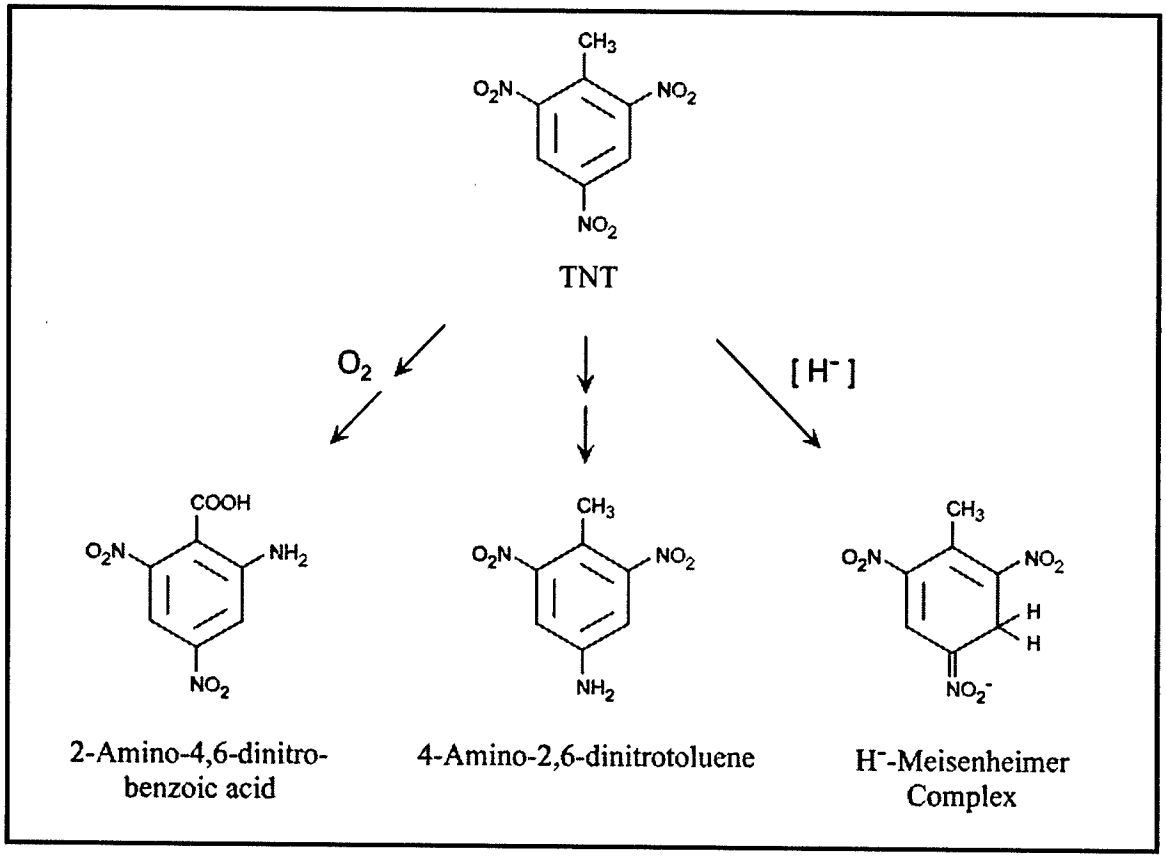

Figure 2. Biological reactions with TNT include A) reduction, B) oxidation of the methyl group, and C) formation of a Meisenheimer complex

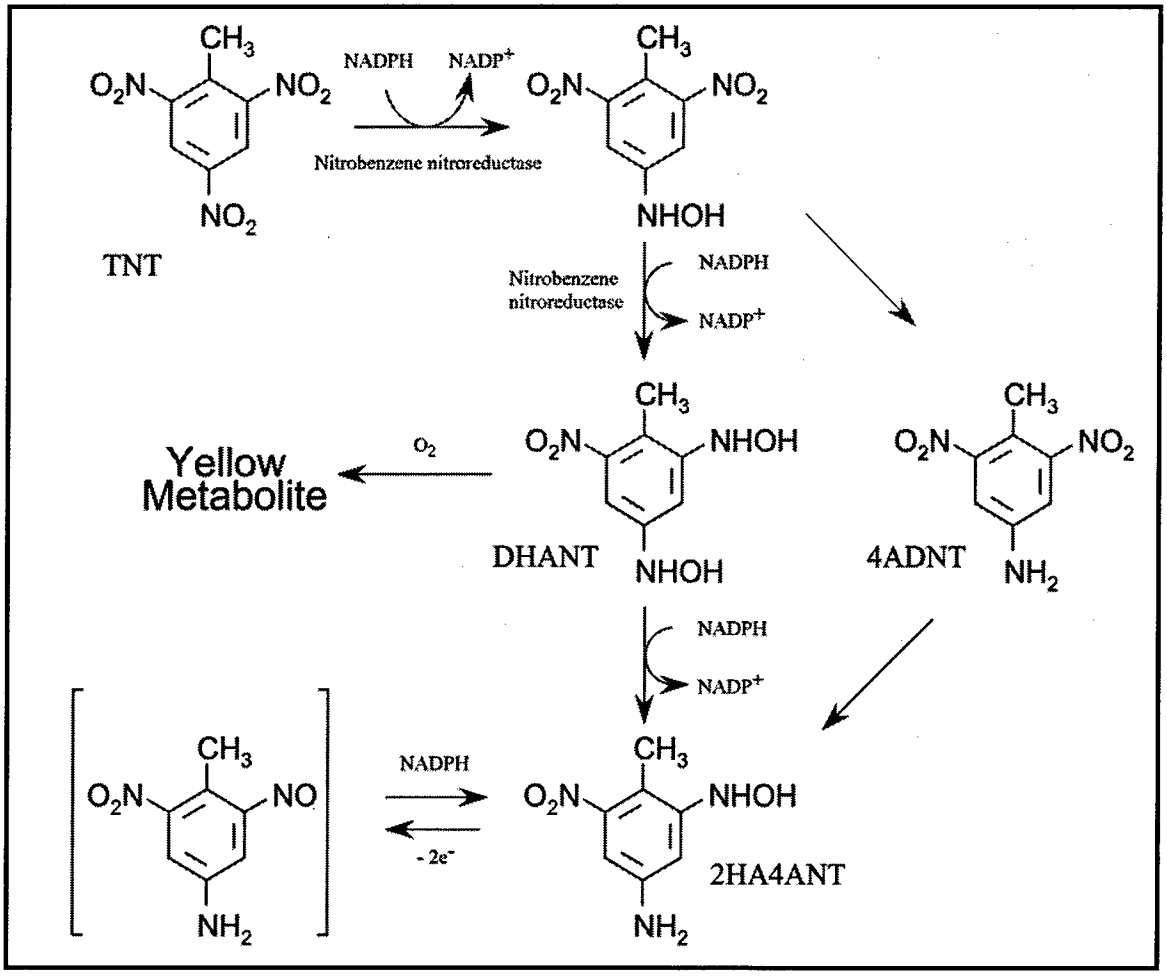

Figure 3. *Pseudomonas pseudoalcaligenes* JS45 attacks TNT to produce 2,4-dihydroxylamino-6-nitrotoluene

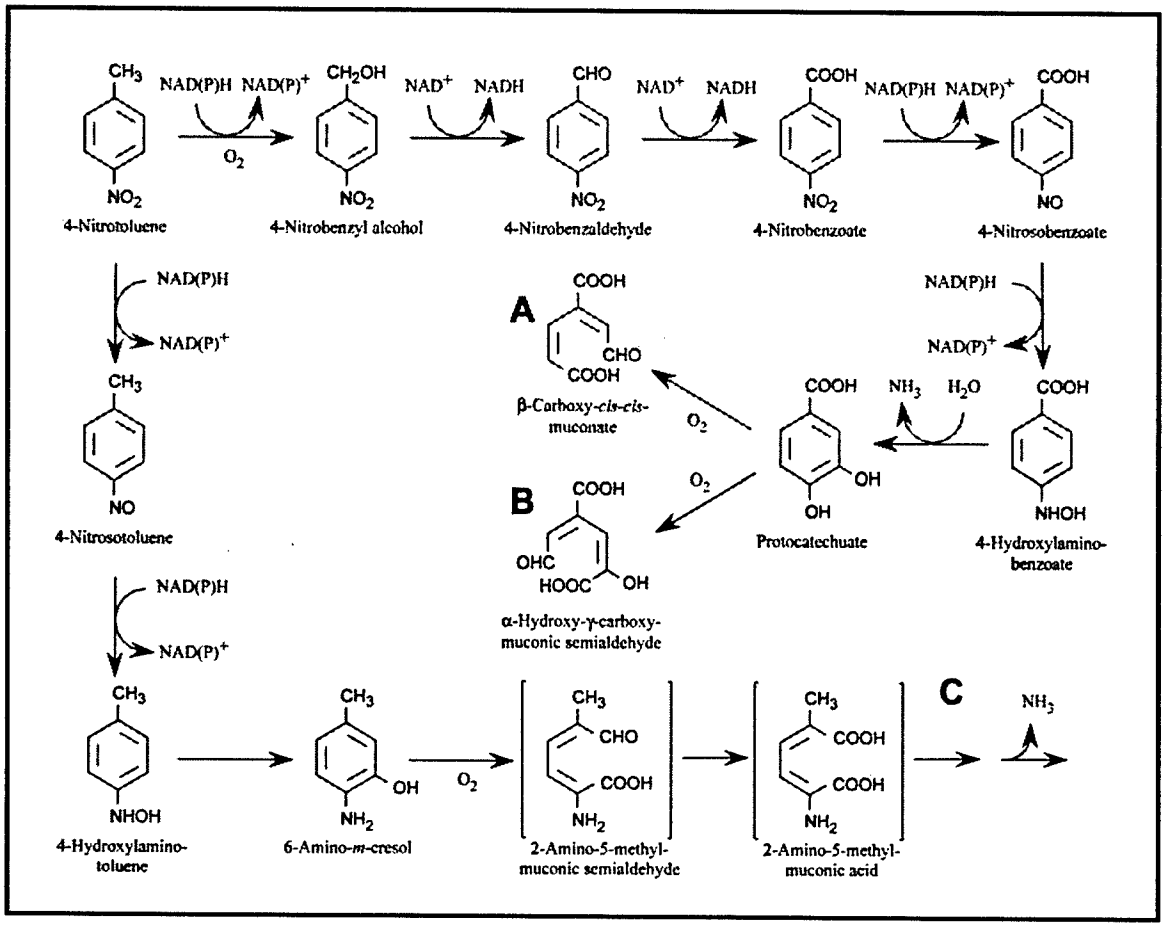

Figure 4. Pathways for biodegradation of 4-NT

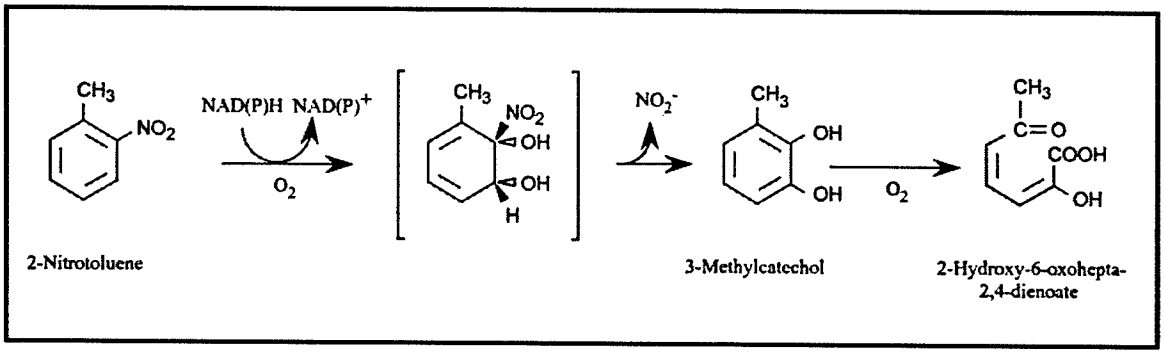

Figure 5. Pathway for biodegradation of 2-NT by *Pseudomonas* sp. JS42 (Haigler et al. 1994b)

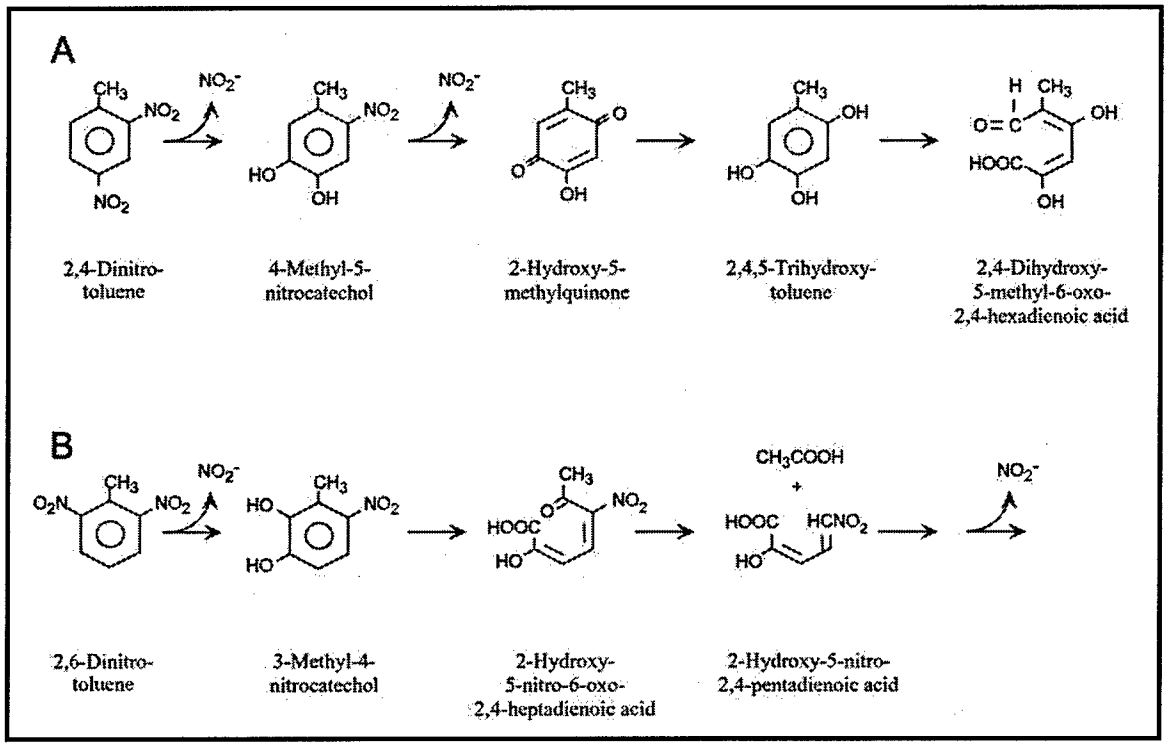

Figure 6. Pathways for biodegradation of (A) 2,4-DNT (Spanggord et al., 1991), and (B) 2,6-DNT (Nishino et al., 2000a)

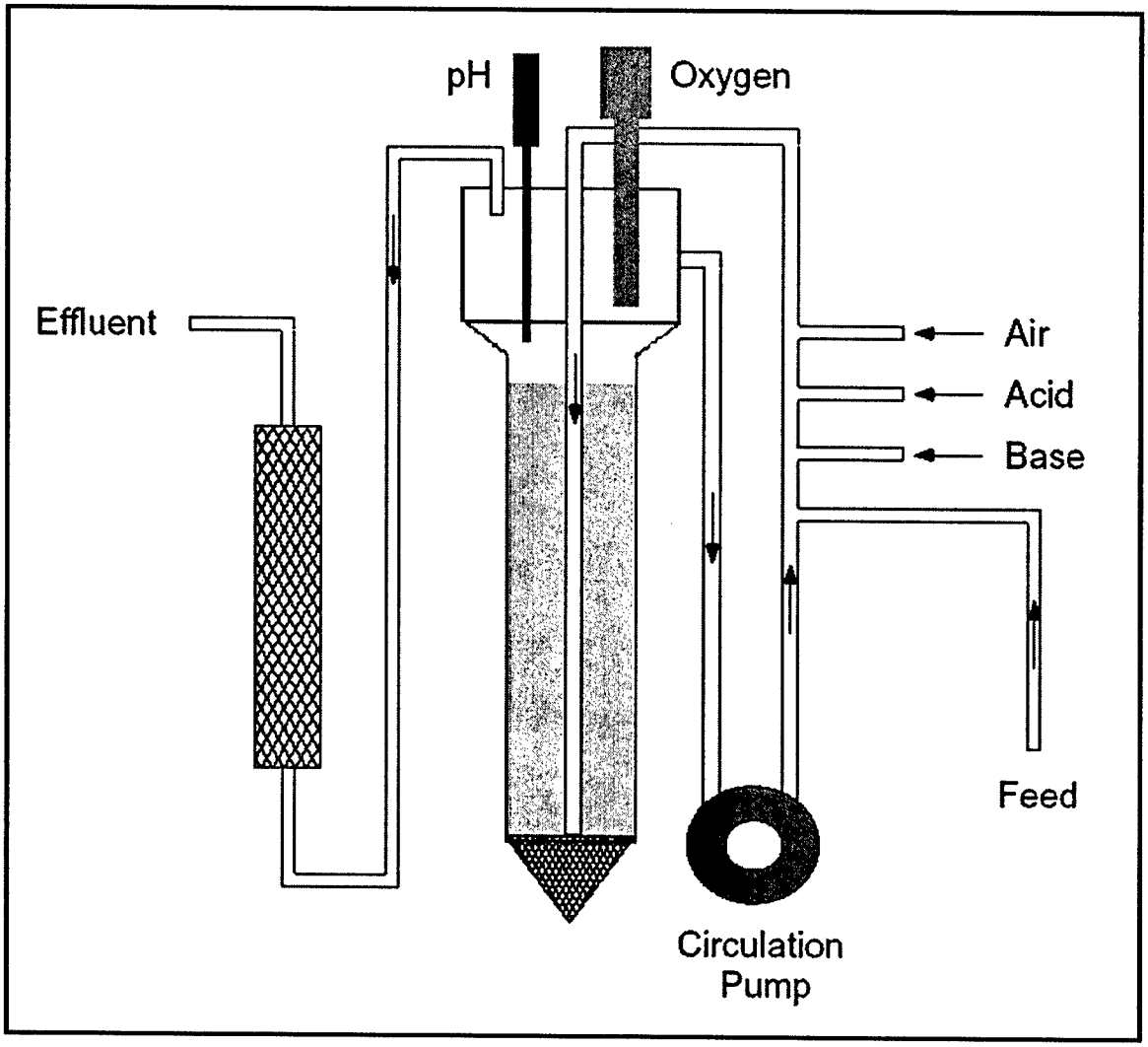

Figure 7. Schematic of bench scale FBR. Feed, air, acid and base are controlled independently of the recirculation rate

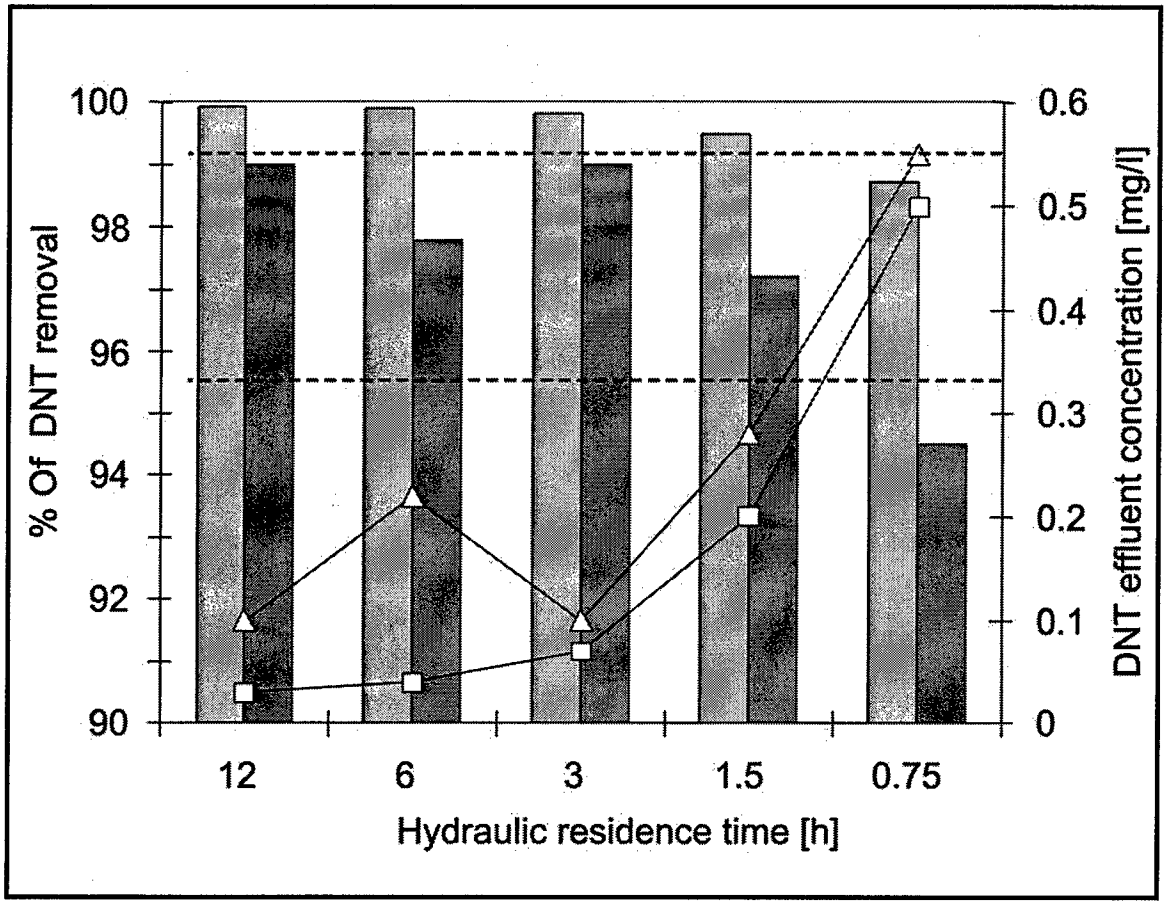

Figure 8. Steady state removal of DNT by the bench scale FBR

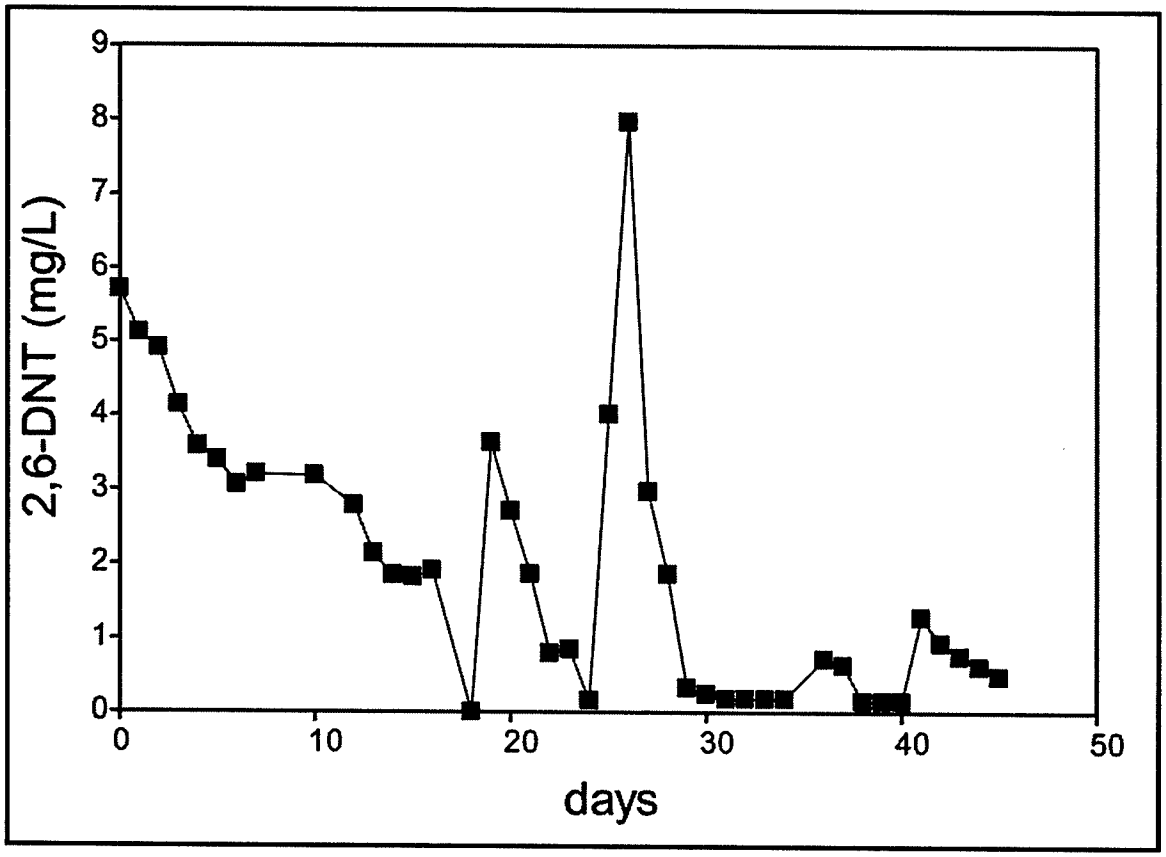

Figure 9. Extended aeration of the effluent from the pilot-scale FBR completed degradation of 2,6-DNT

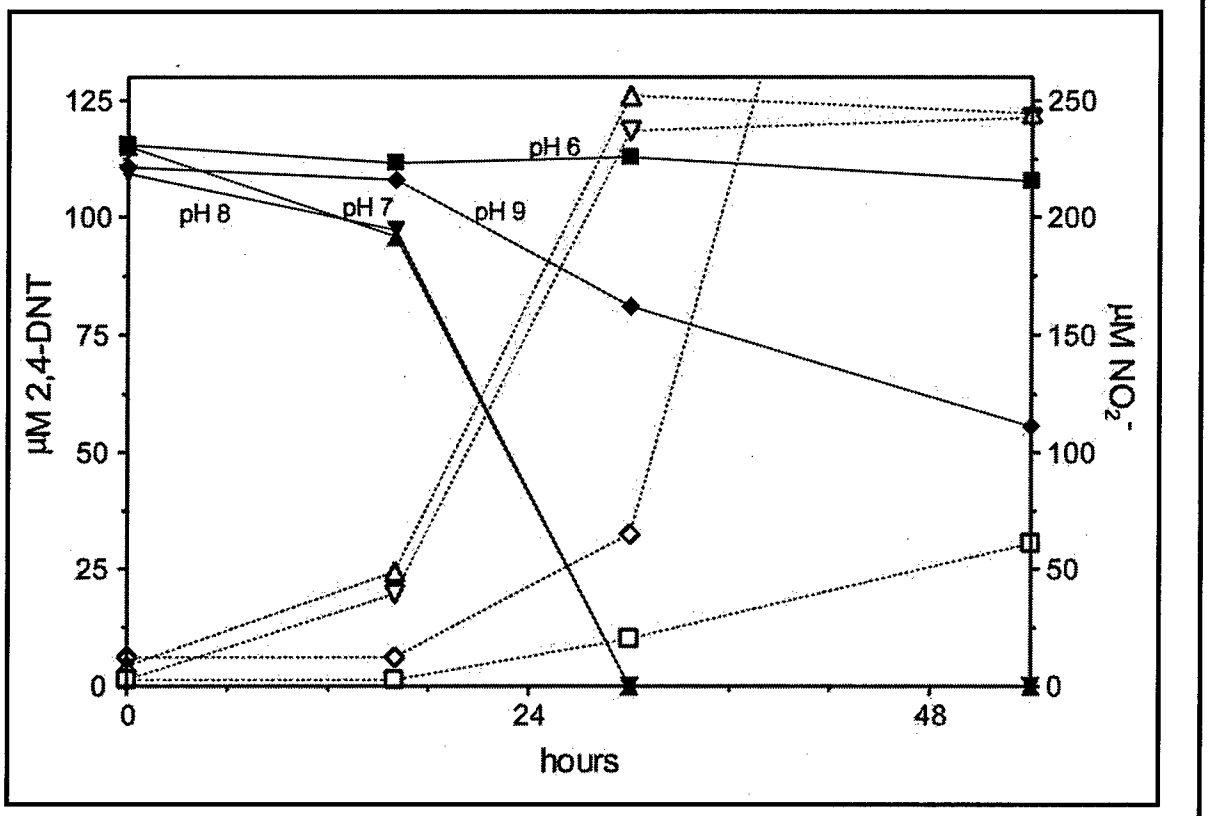

Figure 10. 2,4-DNT degradation in pH adjusted seep water from an industrial site

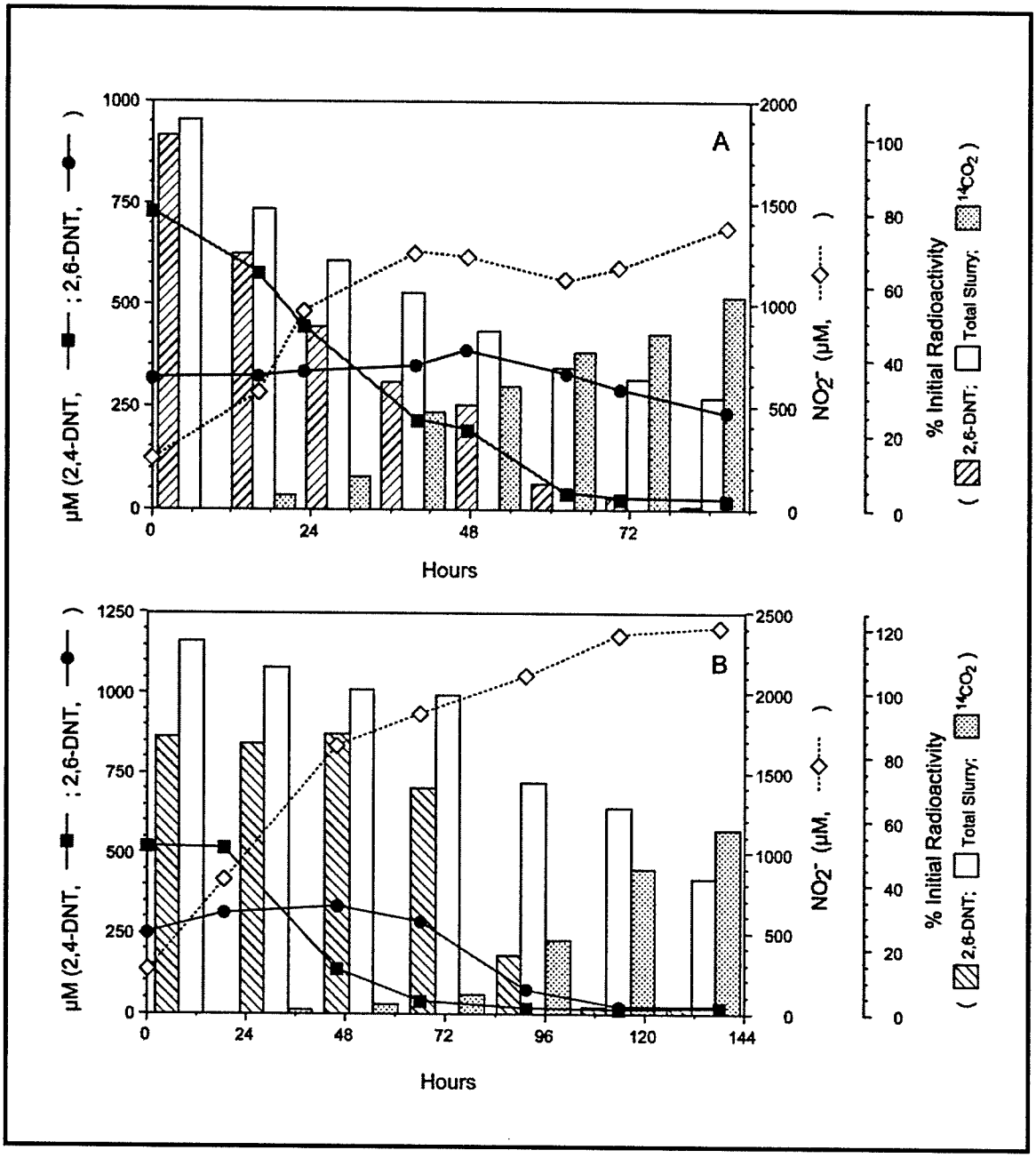

Figure 11. Mineralization of DNT in field contaminated soils (Nishino et al. 1999)

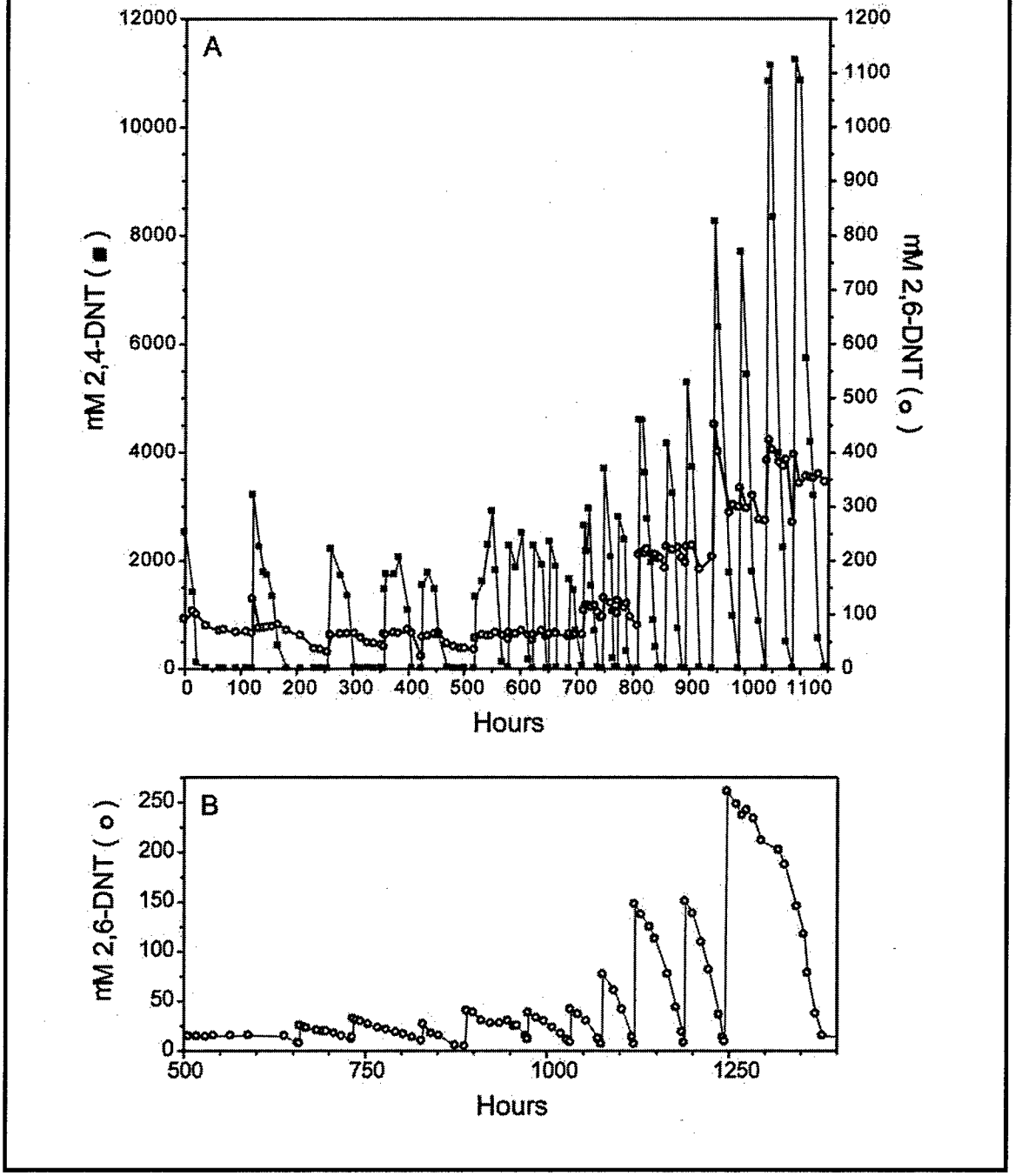

Figure 12. Biodegradation of DNT in soil slurries in pilot scale air lift reactors (Nishino et al. 2000b)

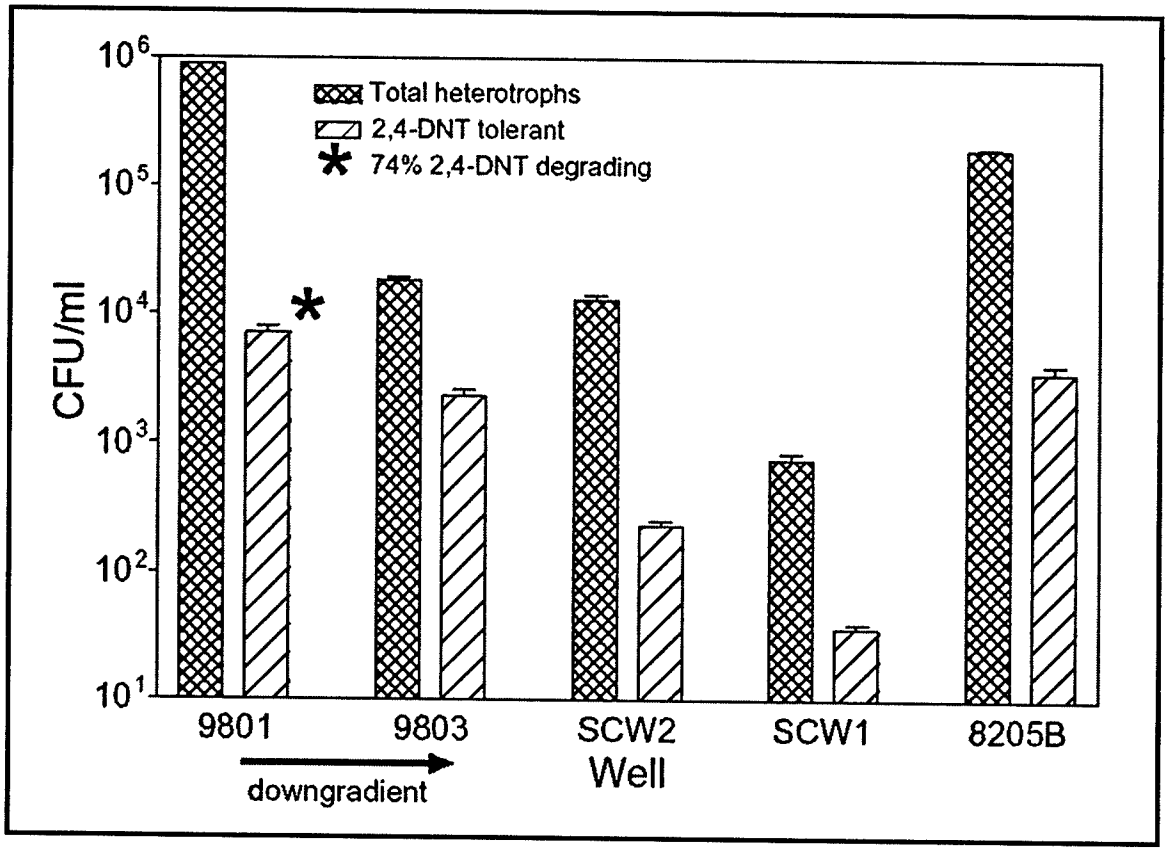

Figure 13. 2,4-DNT degrading bacteria were isolated from DNT-contaminated water at BAAP

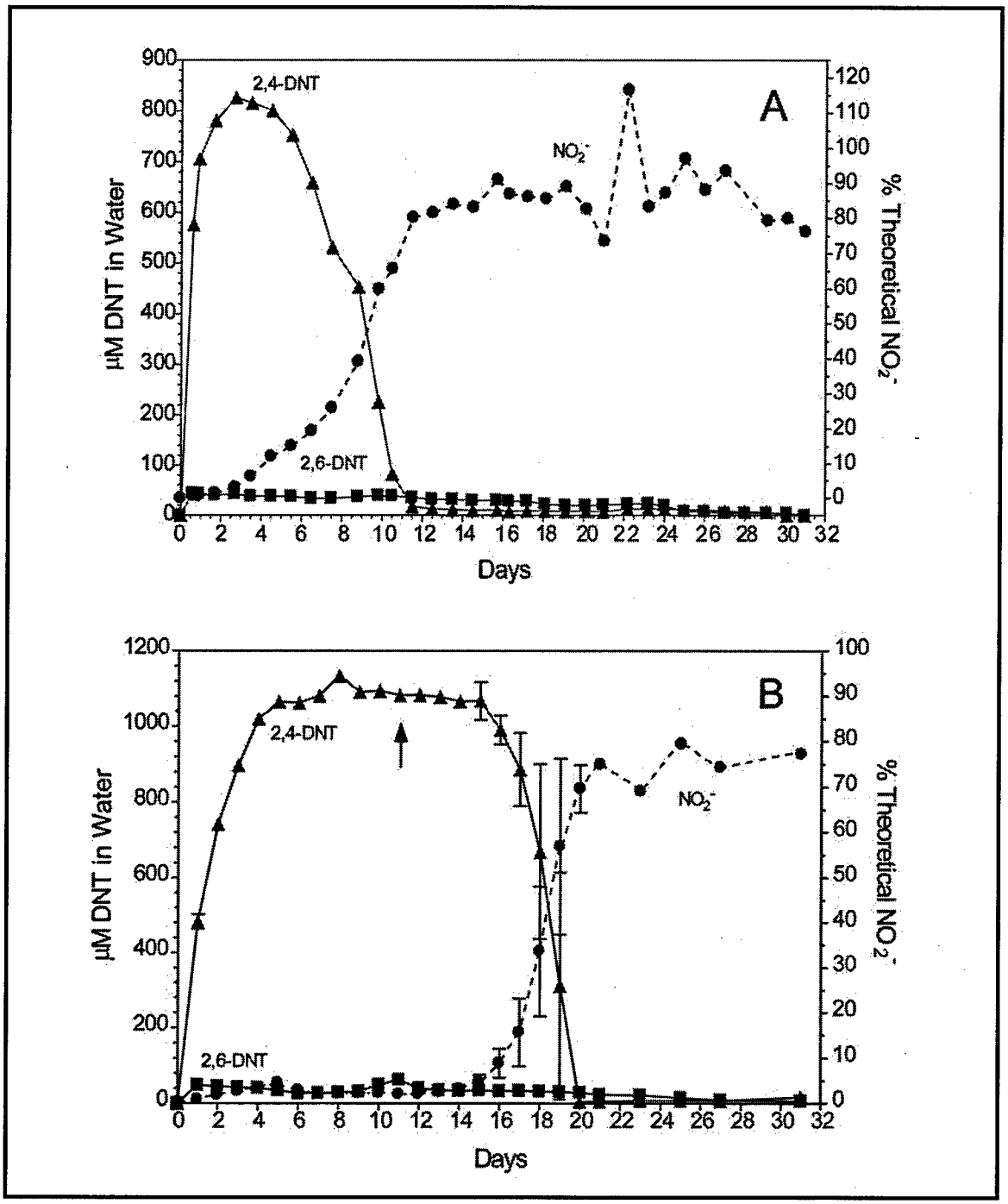

Figure 14. Degradation of DNT in soil columns with (A) 50 mM phosphate buffer, and (B) BAAP groundwater

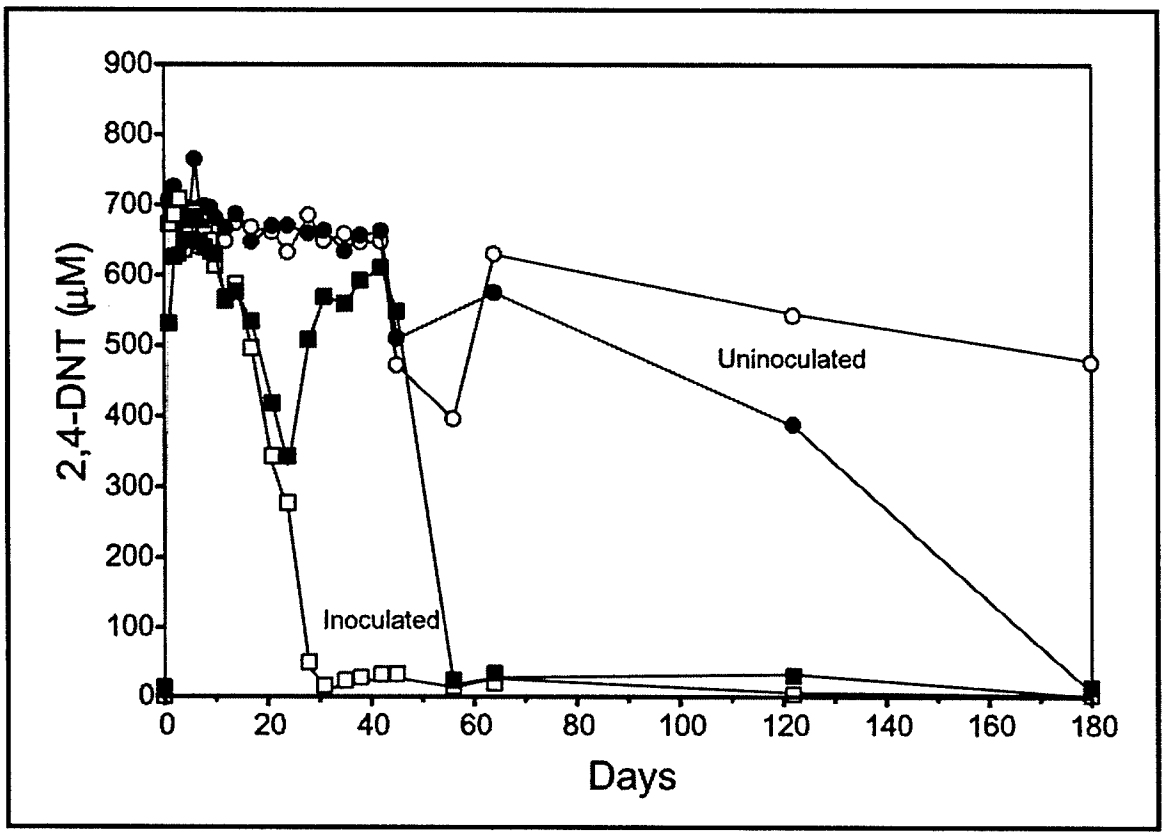

Figure 15. Degradation of 2,4-DNT in soil columns incubated at 13 °C

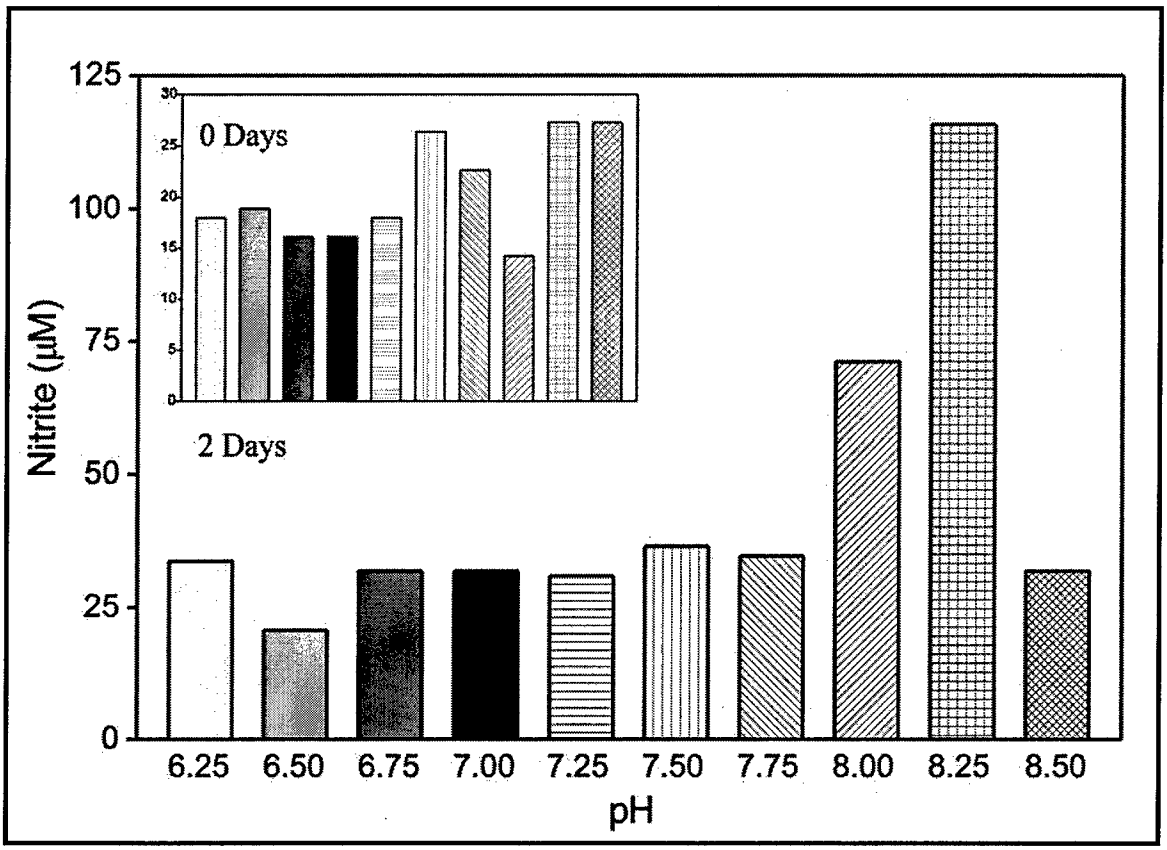

Figure 16. 2,4-DNT degradation (as measured by nitrite release) by cultures of bacteria enriched from BAAP soil and incubated over a range of pH values

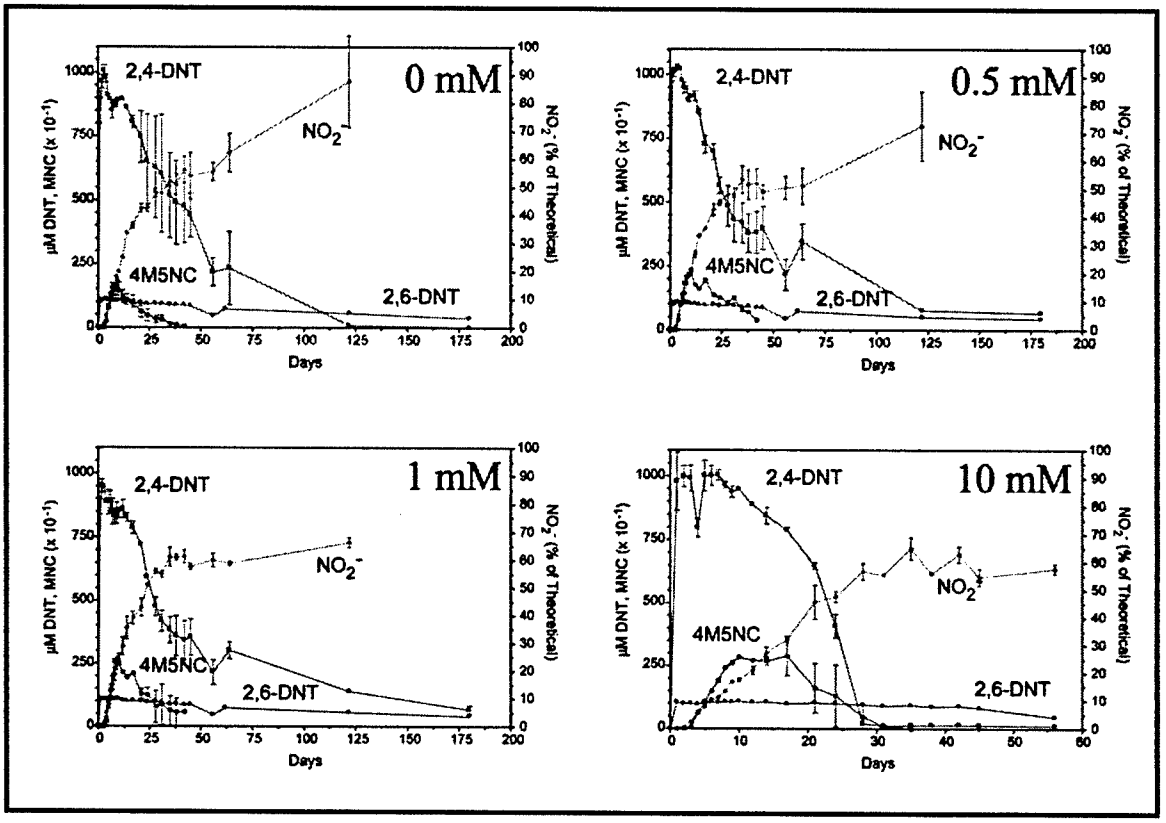

Figure 17. Effect of ortho phosphate on DNT degradation

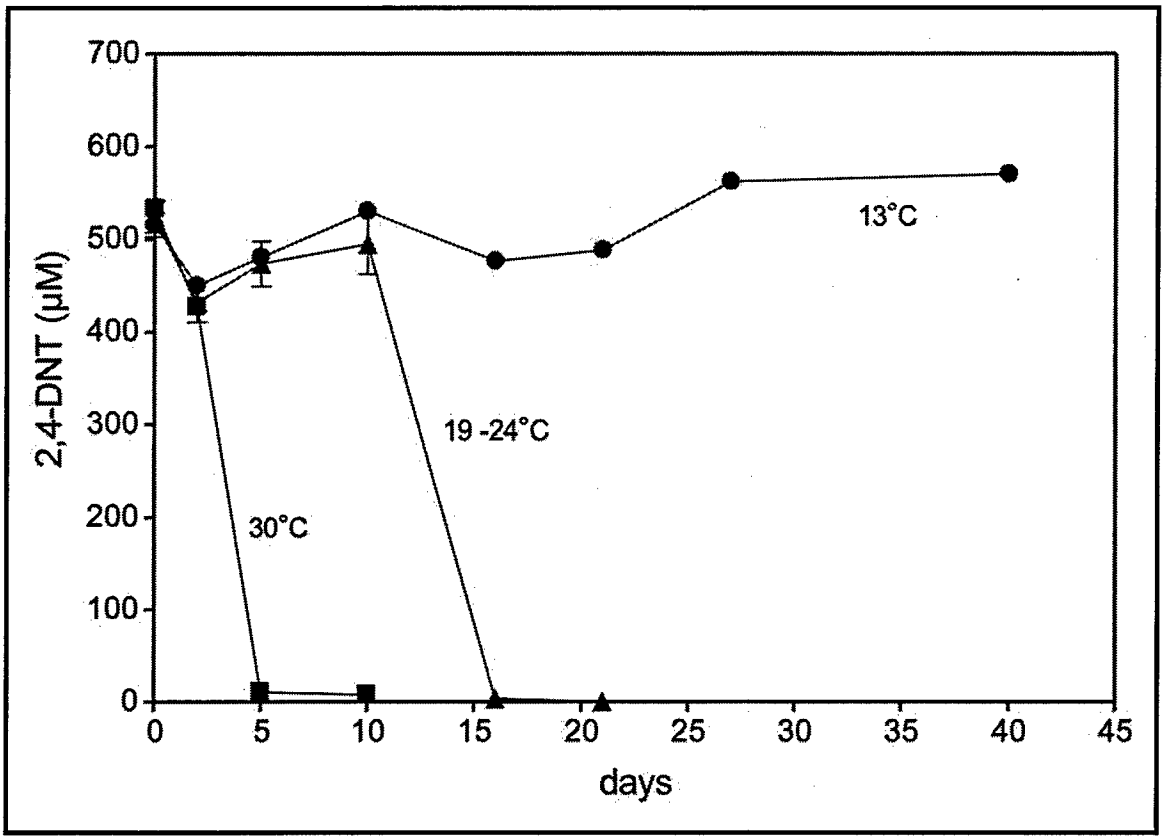

Figure 18. Effect of temperature on 2,4-DNT degradation in 10% BAAP soil slurries in ground water

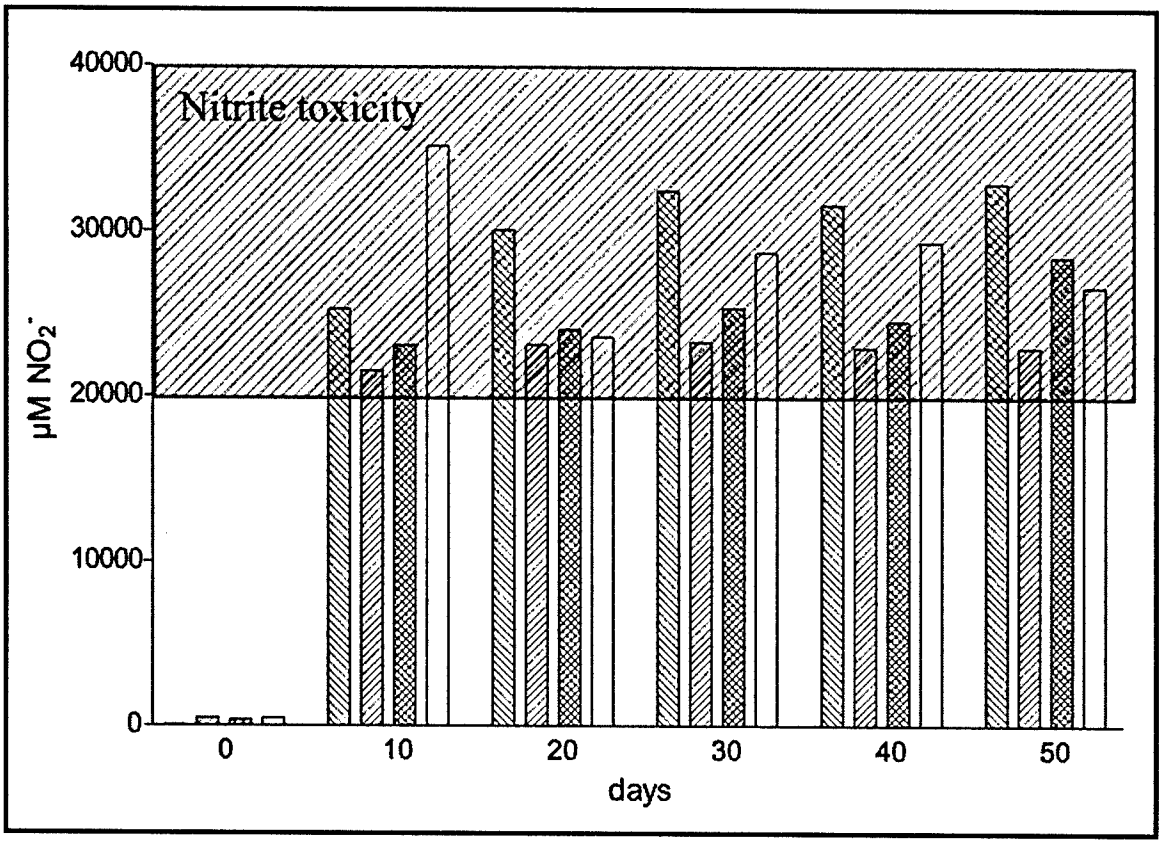

Figure 19. Microcosms constructed with BAAP soil, inoculated with DNT-degrading bacteria, and moistened with varying strength phosphate buffer released the same amount of nitrite before DNT degradation ceased

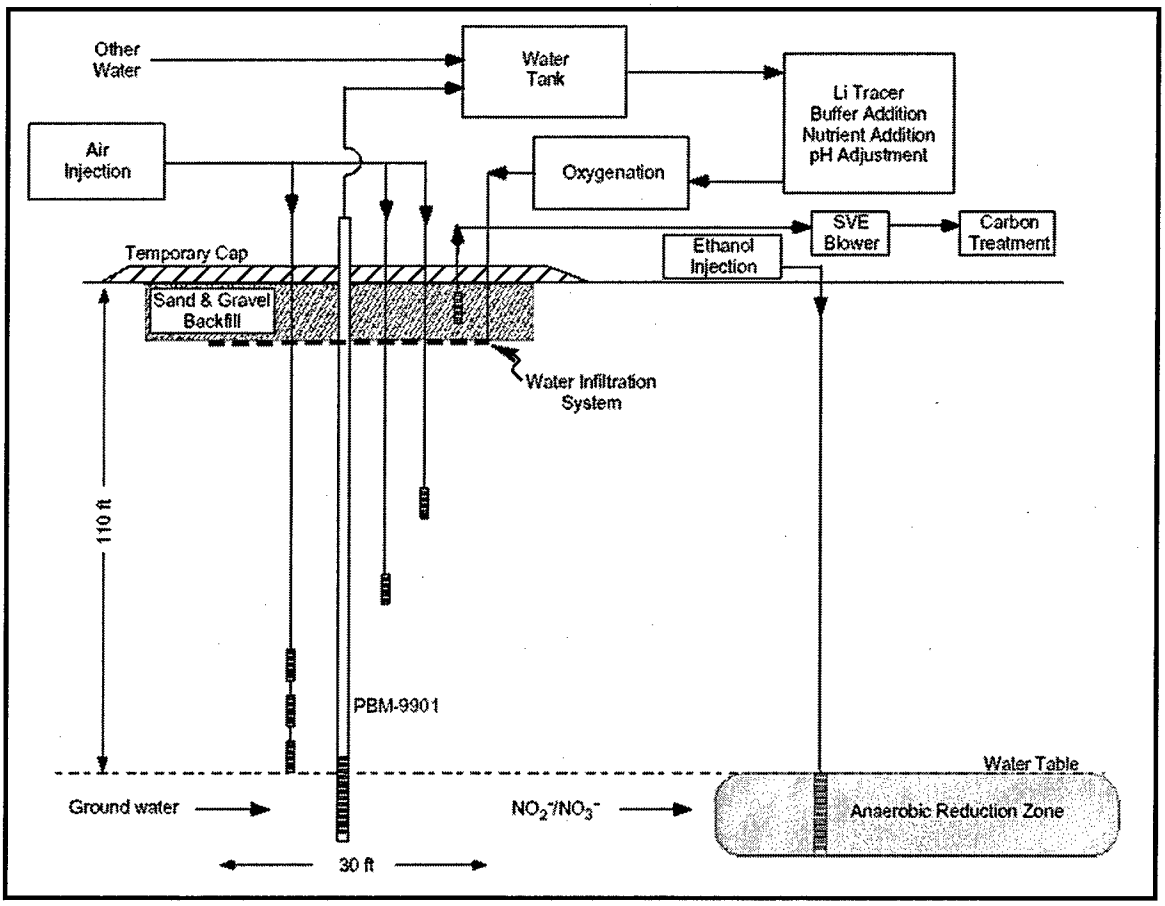

Figure 20. The pilot scale treatment system at BAAP was constructed in Waste Pit 1 at the Propellant Burning Ground

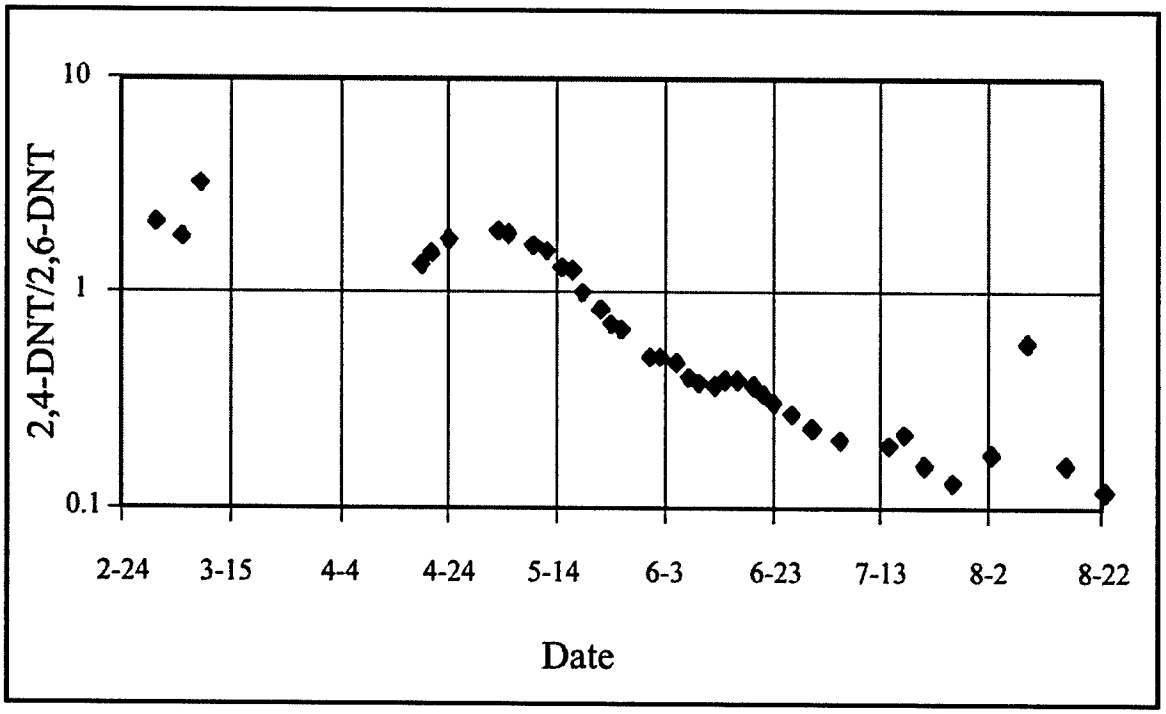

Figure 21. The ratio of 2,4-DNT/2,6-DNT dissolved in the ground water at BAAP decreased during the operation of the pilot scale treatment system

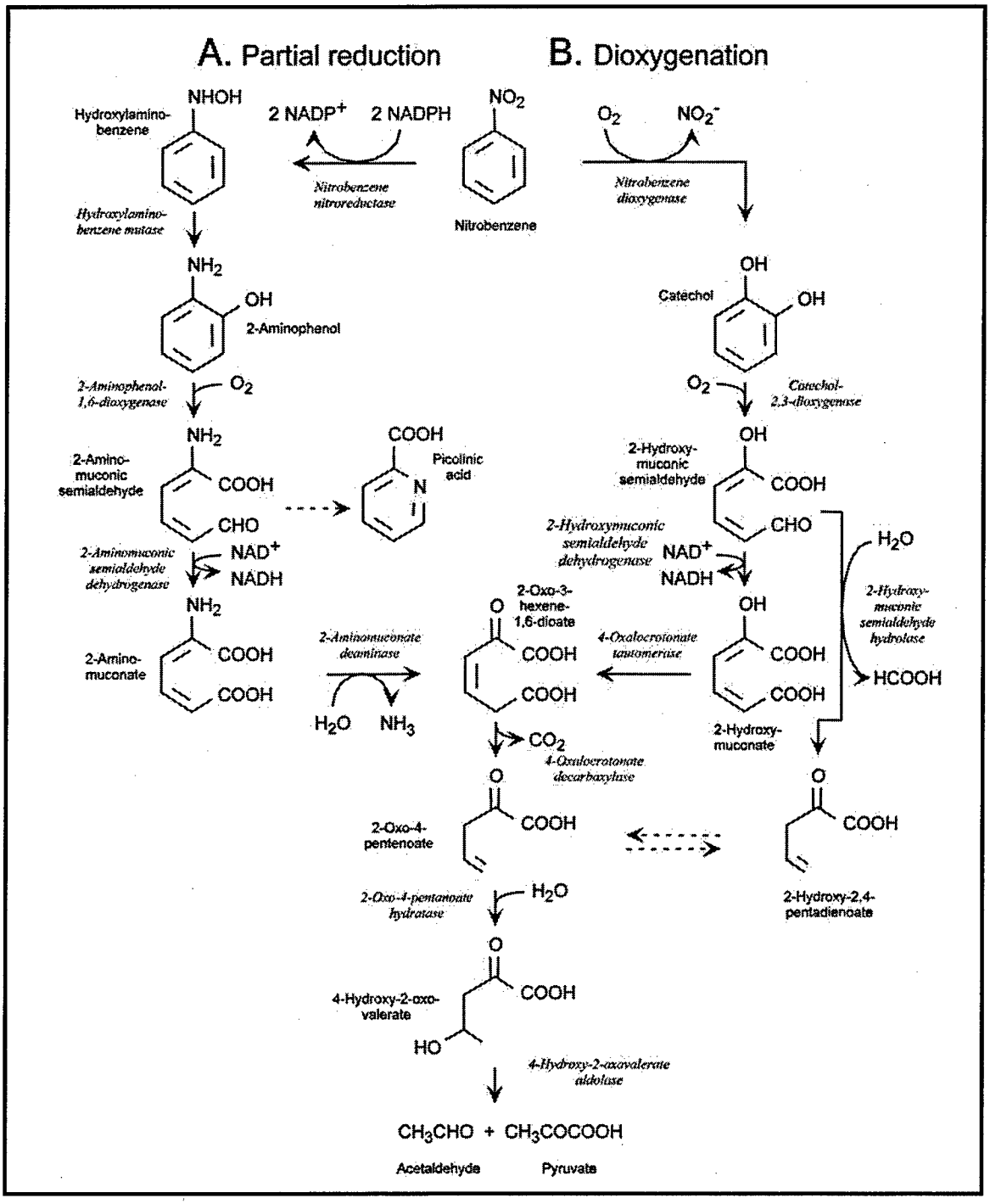

Figure 22. Pathways for biodegradation of NB (Nishino et al. 2000b)

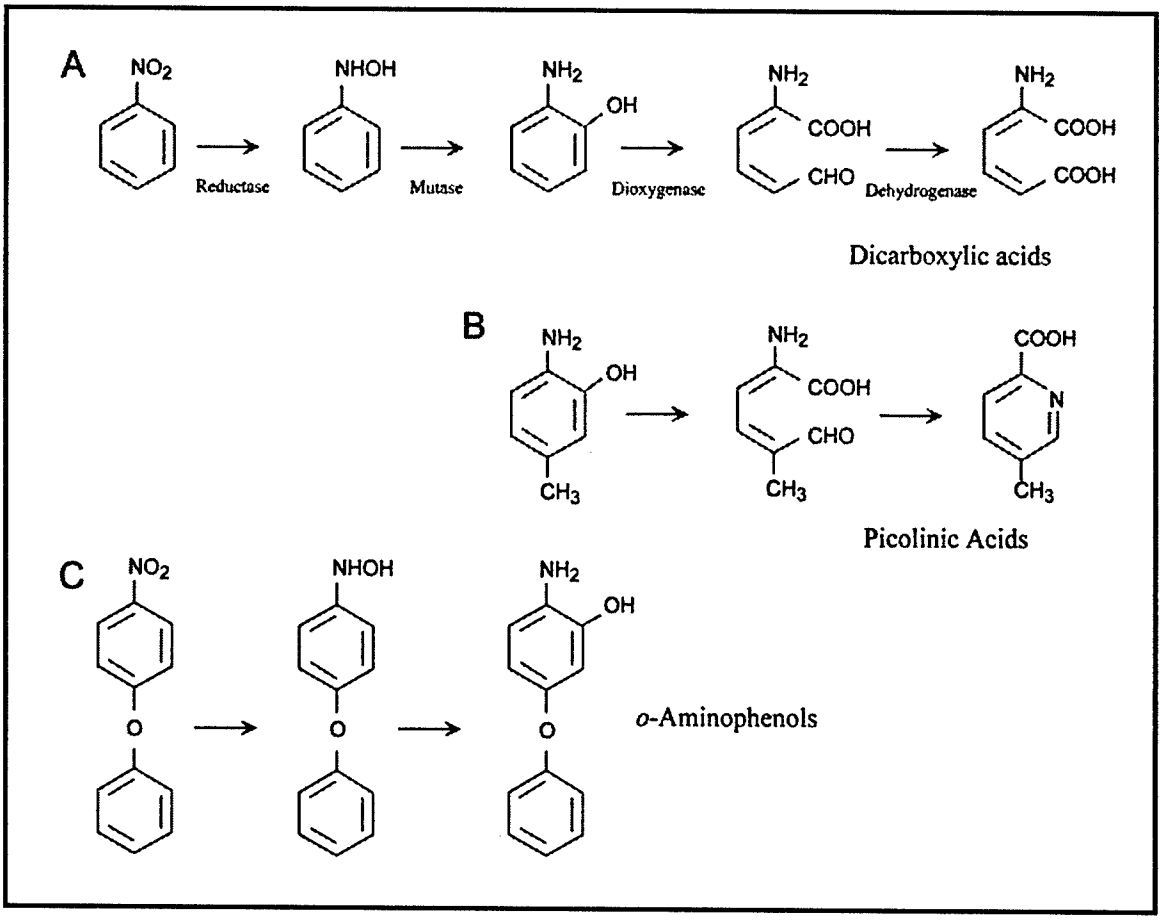

Figure 23. The enzymes of the partial reductive pathway for NB degradation can be exploited to produce novel and specialty chemicals

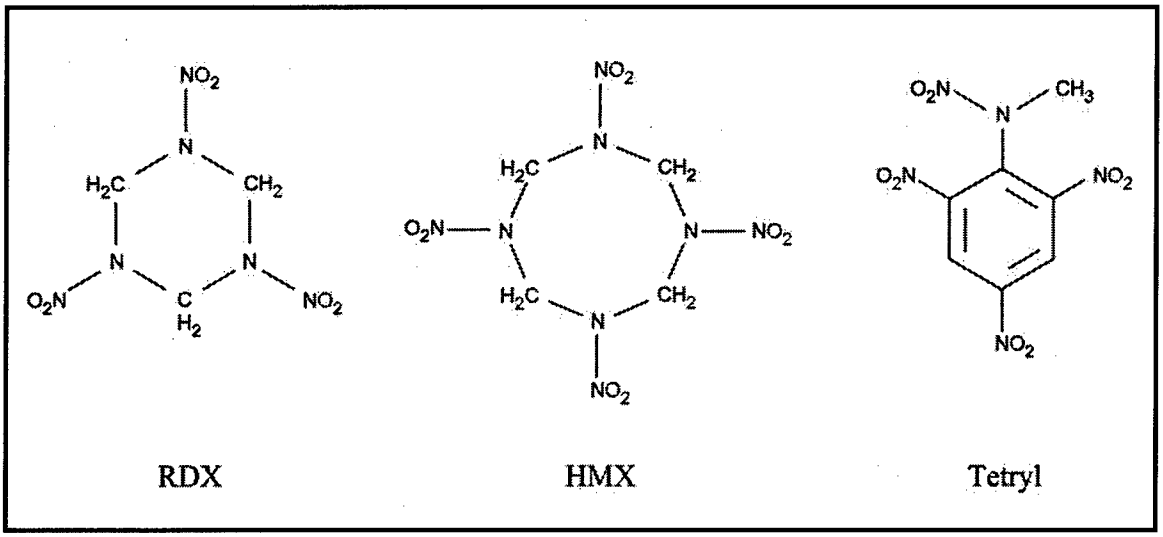

Figure 24. RDX and HMX are the two most widely used nitramine explosives

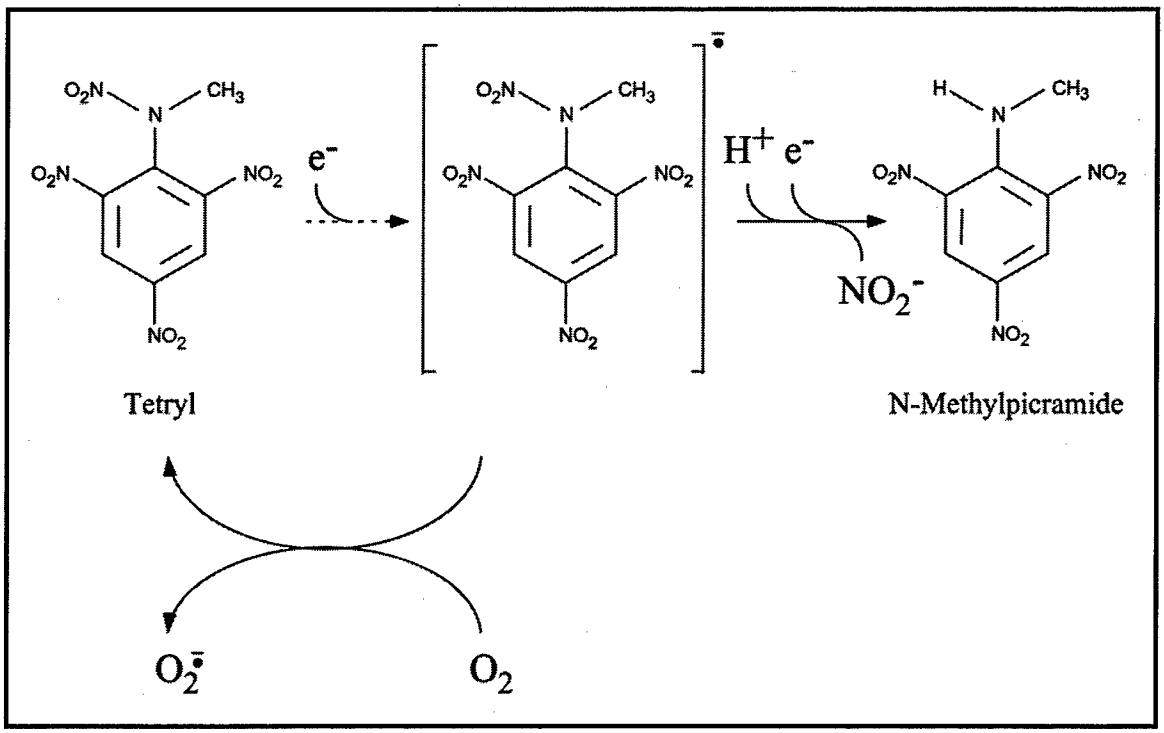

Figure 25. Proposed scheme for elimination of nitrite from tetryl by ferredoxin NADP oxidoreductase under anaerobic conditions (Shah and Spain, 1996)

Table 1
Sequence Analysis for 2,4-DNT Dioxygenase Genes

Gene	% Identity to Naphthalene Dioxygenase	Molecular Weight	Enzyme	Protein
dntA	59	37,600	$Reductase_{DNT}$	DntAa
orf2	31	48,900	Unknown	
dntAb	66	11,900	$Ferredoxin_{DNT}$	DntAb
dntAc	80	49,800	$ISP_{DNT}\alpha$	DntAc
dntAd	78	23,000	$ISP_{DNT}\beta$	DntAd

Table 2
Removal of Nitrotoluenes from Ground Water by FBR

		Influent (mg/L)				
		2,4-DNT	2,6-DNT	TNT	2-NT	4-NT
		% Removal				
HRT (min)	Duration (d)	7.13-13.2	7.4-12.5	0.9-1.3	2.2-3.7	2.2-4.2
30	24	98	58	73	100	100
60	20	100	56	67	100	100
15	14	96	34	40	95	100
10	9	86	18	33	100	100
20	52	99	39	46	100	100
30	9	100	88	54	100	100
60	11	100	94	58	100	100

Table 3
Characteristics of VAAP and BAAP Soils

Soil	pH	Density (g/cm^3)	2,4-DNT (mg/kg)	2,6-DNT (mg/kg)
VAAP, batch A	4.60	1.40	18540	1380
VAAP, batch B			10890	870
BAAP	9.35	1.69	8940	480

Table 4
DNT Degradation Rates in Eimco Airlift Reactors

	Initial Concentration (g/L)	Degradation rate (g/L/d)
2,4-DNT	0.9-2.0	0.9-1.4
	<0.4	0.2-0.3
2,6-DNT	.03-.06	0.1-0.3

Table 5
Composition of Single Base Gun Propellants

Propellant	M1	M6	M10	IMR
Component	% Composition			
Nitrocellulose	85	87	98	100
2,4-DNT	10	10	-	8*
Potassium sulfate	-	-	1	1
Dibutylphthalate	5	3	-	-
Diphenylamine	1	1	1	0.7
Graphite	-	-	0.1	-

* Used as a deterrent to coat surface of the explosive.

Table 6
Nitrobenzene-Degrading Bacteria

Isolate	Geographic Source	Type of Sample	G*	O*	C*	Pathway Used
JS45	Mississippi	Manufacturing plant	-	+	+	Partial reductive
JS761	Mississippi	Manufacturing plant	-	-	+	Partial reductive
JS762	West Virginia	Manufacturing plant	+	-	+	Partial reductive
JS763	Texas	Manufacturing plant	+	-	+	Partial reductive
JS764	Mississippi	Manufacturing plant	+	+	+	Partial reductive
JS765	New Jersey	Waste treatment plant	-	+	+	Oxidative
JS766	Mississippi	Waste treatment plant	+	-	+	Partial reductive
JS767	Mississippi	Waste treatment plant	-	+	+	Partial reductive
JS769	Florida	Waste treatment plant	-	-	+	Partial reductive

* G = Gram reaction, O = oxidase reaction, C = catalase reaction

Attachment A. Technical Publications Produced under Flask to Field

Peer-Reviewed Publications

1. An, D., D. T. Gibson, and J. C. Spain. 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42. J. Bacteriol. 176:7462-7467.
2. Davis, J. K., Z. He, C. C. Somerville, and J. C. Spain. 1999. Genetic and biochemical comparison of 2-aminophenol-1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to *meta*-cleavage dioxygenases: divergent evolution of 2-aminophenol *meta*-cleavage pathway. Arch. Microbiol. 172:330-339.
3. Davis, J. K., G. C. Paoli, Z. He, L. J. Nadeau, C. C. Somerville, and J. C. Spain. 2000. Sequence analysis and initial characterization of two isozymes of hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. Appl. Environ. Microbiol. 66(7):2965-2971.
4. Fiorella, P. D., and J. C. Spain. 1997. Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl. Environ. Microbiol. 63:2007-2015.
5. Goodall, J. L., S. M. Thomas, J. C. Spain, and S. W. Peretti. 1998. Operation of mixed-culture immobilized cell reactors for the metabolism of *meta*- and *para*-nitrobenzoate by *Comamonas* sp. JS46 and *Comamonas* sp. JS47. Biotechnol. Bioeng. 59:21-27.
6. Haigler, B. E., G. R. Johnson, W.-C. Suen, and J. C. Spain. 1999. Biochemical and genetic evidence for *meta*-ring cleavage of 2,4,5-trihydroxytoluene in *Burkholderia* sp. strain DNT. J. Bacteriol. 181:3965-3972.
7. Haigler, B. E., S. F. Nishino, and J. C. Spain. 1994. Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. J. Bacteriol. 176:3433-3437.
8. Haigler, B. E., W.-C. Suen, and J. C. Spain. 1996. Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. J. Bacteriol. 178:6019-6024.
9. Haigler, B. E., W. H. Wallace, and J. C. Spain. 1994. Biodegradation of 2-nitrotoluene by *Pseudomonas sp.* Strain JS42. Appl. Environ. Microbiol. 60:3466-3469.
10. He, Z., J. K. Davis, and J. C. Spain. 1998. Purification, characterization, and sequence analysis of 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. J. Bacteriol. 180:4591–4595.
11. He, Z., L. J. Nadeau, and J. C. Spain. 2000. Characterization of hydroxylaminobenzene mutase from pNBZ139 cloned from *Pseudomonas pseudoalcaligenes* JS45. A highly associated SDS-stable enzyme catalyzing an intramolecular transfer of hydroxy groups. Eur. J. Biochem. 267:1110-6.
12. He, Z., and J. C. Spain. 1999. Comparison of the downstream pathways for degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45 (2-aminophenol pathway) and by *Comamonas* sp. JS765 (catechol pathway). Arch. Microbiol. 171:309-316.
13. He, Z., and J. C. Spain. 1998. A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of *Pseudomonas pseudoalcaligenes* JS45. J. Bacteriol. 180:2502-2506.
14. He, Z., and J. C. Spain. 2000. One-step production of picolinic acids from 2-aminophenols catalyzed by 2-aminophenol 1,6-dioxygenase. J. Ind. Microbiol. Biotechnol. 25:25-28.
15. He, Z., and J. C. Spain. 1999. Preparation of 2-aminomuconate from 2-aminophenol by coupled enzymatic dioxygenation and dehydrogenation reactions. J. Ind. Microbiol. Biotechnol. 23:138-142.

16. He, Z., and J. C. Spain. 2000. Reactions involved in the lower pathway for degradation of 4-nitrotoluene by *Mycobacterium* strain HL 4-NT-1. Appl. Environ. Microbiol. 66(7):3010-2971.
17. He, Z., and J. C. Spain. 1997. Studies of the catabolic pathway of degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: removal of the amino group from 2-aminomuconic semialdehyde. Appl. Environ. Microbiol. 63:4839–4843.
18. Jain, R. K., J. H. Dreisbach, and J. C. Spain. 1994. Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. Appl. Environ. Microbiol. 60:3030-3032.
19. Johnson, G. R., R. K. Jain, and J. C. Spain. 2000. Properties of the trihydroxytoluene oxygenase from Burkholderia cepacia R34: an extradiol dioxygenase from the 2,4-dinitrotoluene pathway. Arch. Microbiol. 173:86-90.
20. Kadiyala, V., B. F. Smets, K. Chandran, and J. C. Spain. 1998. High affinity *p*-nitrophenol oxidation by *Bacillus sphaericus* JS905. FEMS Microbiol. Lett. 166:115-120.
21. Kadiyala, V., and J. C. Spain. 1998. A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. Appl. Environ. Microbiol. 64:2479-2484.
22. Lendenmann, U., and J. C. Spain. 1996. 2-Aminophenol 1,6-dioxygenase: a novel aromatic ring cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. J. Bacteriol. 178:6227-6232.
23. Lendenmann, U., J. C. Spain, and B. F. Smets. 1998. Simultaneous biodegradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in an aerobic fluidized-bed biofilm reactor. Environ. Sci. Technol. 32:82-87.
24. Nadeau, L. J., Z. He, and J. C. Spain. 2000. Production of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether using nitrobenzene nitroreductase and hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. J. Ind. Microbiol. Biotechnol. 24:301-305.
25. Nadeau, L. J., and J. C. Spain. 1995. Bacterial degradation of *m*-nitrobenzoic acid. Appl. Environ. Microbiol. 61:840-843.
26. Nishino, S. F., G. Paoli, and J. C. Spain. 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. 66:2139-2147.
27. Nishino, S. F., and J. C. Spain. 1995. Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765. Appl. Environ. Microbiol. 61:2308-2313.
28. Nishino, S. F., J. C. Spain, H. Lenke, and H.-J. Knackmuss. 1999. Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries. Environ. Sci. Technol. 33:1060-1064.
29. Schenzle, A., H. Lenke, J. C. Spain, and H.-J. Knackmuss. 1999. 3-Hydroxylaminophenol mutase from *Ralstonia eutropha* JMP134 catalyzes a Bamberger rearrangement. J. Bacteriol. 181:1444-1450.
30. Shah, M. M., and J. C. Spain. 1996. Elimination of nitrite from the explosive 2,4,6-trinitrophenylmethylnitramine (Tetryl) upon its reduction by ferredoxin NADP oxidoreductase from spinach. Biochem. Biophys. Res. Comm. 220:563-568.
31. Smets, B. F., R. G. Riefler, U. Lendenmann, and J. C. Spain. 1999. Kinetic analysis of simultaneous 2,4-dinitrotoluene (DNT) and 2,6-DNT biodegradation in an aerobic fluidized-bed biofilm reactor. Biotechnol. Bioeng. 63:642-653.
32. Somerville, C. C., S. F. Nishino, and J. C. Spain. 1995. Purification and characterization of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. J. Bacteriol. 177:3837-3842.
33. Spain, J. 1997. Synthetic chemicals with potential for natural attenuation. Bioremed. J. 1:1-9.
34. Spain, J. C. 1995. Biodegradation of nitroaromatic compounds. Ann. Rev. Microbiol. 49:523-555.
35. Spiess, T., F. Desiere, P. Fischer, J. C. Spain, H.-J. Knackmuss, and H. Lenke. 1998. A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain. Appl. Environ. Microbiol. 64:446–452.
36. Suen, W.-C., B. E. Haigler, and J. C. Spain. 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J. Bacteriol. 178:4926-4934.

37. Vorbeck, C., H. Lenke, P. Fischer, J. C. Spain, and H.-J. Knackmuss. 1998. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 64:246–252.
38. Zhang, C., S. F. Nishino, J. C. Spain, and J. B. Hughes. 2000. Slurry-phase biological treatment of 2,4- and 2,6-dinitrotoluene: role of bioaugmentation and effects of high dinitrotoluene concentrations. Environ. Sci. Technol. 34:2810-2816.

Technical Reports

1. Nishino, S. F., and J. C. Spain. 2001. Technology status review: bioremediation of dinitrotoluene (DNT). Internet Report. http://www.estcp.org/documents/techdocs/DNT_Report.pdf
2. Nishino, S. F., and J. C. Spain. 2001. 2,4-DNT degradation in a fluidized bed reactor fed with waste water from ICI Canada Inc. AFRL/MLQL.
3. Nishino, S. F., and J. C. Spain. 1999. 2,4-DNT degradation in soil from Badger Army Ammunition Plant: preliminary studies. AFRL/MLQR.
4. Nishino, S. F., and J. C. Spain. 2000. 2,4-DNT degradation in wastewater from ICI Canada Inc.: preliminary studies. AFRL/MLQL.
5. Nishino, S. F., and J. C. Spain. 2000. DNT degradation in groundwater from Air Products industrial sites: preliminary studies. AFRL.
6. Nishino, S.F., and J. C. Spain. 2001. DNT Degradation in Soil from Badger Army Ammunition Plant: Final Report. AFRL/MLQL.

Conference/Symposium Proceedings Papers

1. Spain, J., and S. Nishino. 1999. Oxidative pathways for biodegradation of nitroaromatic compounds. *In* M. Hofman (ed.), CD-ROM Proceedings Ninth European Congress on Biotechnology. Branche Belge de la Société de Chemie Industrielle, Brussels, Belgium.
2. Spain, J. C., S. F. Nishino, M. R. Green, J. E. Forbert, N. A. Nogalski, R. Unterman, W. M. Riznychok, S. E. Thompson, P. M. Sleeper, and M. A. Boxwell. 1999. Field demonstration of FBR for treatment of nitrotoluenes in groundwater, p. 7-14. *In* B. C. Alleman and A. Leeson (ed.), Bioremediation of Nitroaromatic and Haloaromatic Compounds. Battelle Press, Columbus, OH.
3. Nishino, S. F., and J. C. Spain. In Press. Identification of bottlenecks to the *in situ* bioremediation of dinitrotoluene. In situ and on-site bioremediation: the sixth international symposium, June 4-7, 2001.

Published Technical Abstracts

1. An, D., D.T. Gibson, W. Wallace, and J.C. Spain, 1994. Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain 2NT. Annual Meeting, American Society for Microbiology.
2. Cuffin, S.M., P.M. Lafferty, P.N. Taylor, J.C. Spain, S.F. Nishino, and K.A. Williams. 2001. Bioremediation of dinitrotoluene isomers in the unsaturated/saturated zone. Sixth International In Situ and On-Site Bioremediation Symposium
3. Davis, J.K., C.C. Somerville and J.C. Spain, 1996. Cloning of hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
4. Davis, J. K., C.C. Somerville, and J.C. Spain. 1997. *Pseudomonas pseudoalcaligenes* JS45 possesses two genes encoding hydroxylaminobenzene mutase. Annual Meeting of the American Society for Microbiology.

5. Davis, J.K., C.C. Somerville, and J.C. Spain, 1997. Cloning and characterization of the genes for 2-aminophenol 1,6-dioxygenase from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology
6. Davis, John K., C.C. Somerville, and J.C. Spain, 1997. *Pseudomonas pseudoalcaligenes* JS45 possesses two genes encoding hydroxylaminobenzene mutase. Annual Meeting American Society for Microbiology.
7. Driesbach, J., S. Nilsen, C. Shelley, and J. Spain, 1996. Biodegradation of 4-halophenols by Arthrobacter sp. Annual Meeting American Society for Microbiology.
8. Fiorella, P.D., and J.C. Spain, 1996. Transformation of TNT by Pseudomonas pseudoalcaligenes JS45. Annual Meeting American Society for Microbiology.
9. Haigler, B.E., C.C. Somerville, and J.C. Spain, 1997. Sequence analysis differences between the 2,4-dinitrotoluene degrading genes of *Burkholderia* sp. strain R34 and strain DNT. Annual Meeting American Society for Microbiology
10. Haigler, B.E., and J.C. Spain, 1995. Purification and characterization of 4-methyl-5-nitrocatechol oxygenase from *Pseudomonas* sp. strain DNT. Annual Meeting American Society for Microbiology.
11. He, Z., J.K. Davis, and J.C. Spain. 1998. Purification, characterization, and sequence analysis of 2-aminomuconic-6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
12. He, Z., L. Nadeau, and J. C. Spain. 1999. Characterization of hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
13. He, Z. and J.C. Spain, 1997. Final steps in the pathway for biodegradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
14. Johnson, G.R., B.E. Haigler, R.K. Jain, and J.C. Spain. 1998. Cloning and characterization of genes encoding enzymes for degradation of 2,4-dinitrotoluene from *Burkholderia* cepacia R34. Annual Meeting American Society for Microbiology.
15. Johnson, G. R., and J. C. Spain. 1997. Phylogenetic analysis of nitrotoluene dioxygenases. Annual Meeting American Society for Microbiology
16. Johnson, G.R. and J. C. Spain. 1999. Evolutionary origins of the pathway for 2,4-dinitrotoluene degradation. Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives.
17. Lendenmann, U., B.F. Smets, and J.C. Spain, 1997. Biodegradation of 2,4-DNT and 2,6-DNT in a fluidized bed reactor with a mixed bacterial culture. Annual Meeting American Society for Microbiology.
18. Lendenmann, U., and J.C. Spain, 1996. 2-Aminophenol 1,6-dioxygenase: a novel aromatic ring-cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
19. Nadeau, L.J., and J.C. Spain, 1994. The bacterial degradation of *m*-nitrobenzoic acid. Annual Meeting, American Society for Microbiology.
20. Nadeau, L. J., Z. He, and J. C. Spain. 1999. The biosynthesis of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether using two enzymes, a nitroreductase and hydroxylaminobenzene mutase, from *Pseudomonas pseudoalcaligenes* JS45. Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives
21. Nishino, S.F., and J.C. Spain, 1994. Oxidative pathway for the degradation of nitrobenzene by a gram-positive bacterium. Annual Meeting, American Society for Microbiology.
22. Nishino, S.F., and J.C. Spain, 1995. The effect of 2,6-dinitrotoluene on 2,4-dinitrotoluene degradation. Annual Meeting Society for Environmental Toxicology and Chemistry.

23. Nishino, S.F., and J.C. Spain, 1995. Degradation of mixtures of nitroaromatic compounds by a gram positive bacterium. Annual Meeting American Society for Microbiology.
24. Nishino, S.F., and J.C. Spain, 1996. Degradation of 2,6-dinitrotoluene by bacteria. Annual Meeting American Society for Microbiology.
25. Nishino, S.F., and J.C. Spain, 1997. Ring cleavage of 3-methyl-4-nitrocatechol by 2,6-dinitrotoluene degrading bacteria. Annual Meeting American Society for Microbiology.
26. Nishino, S.F., and J.C. Spain. 1998. Mineralization of 2,4- and 2,6-dinitrotoluene in soil slurries by pure cultures of DNT-degrading bacteria. Annual Meeting American Society for Microbiology.
27. Nishino, S.F. and J.C. Spain. 1999. 2,4-DNT degradation in soil from Badger Army Ammunition Plant. Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives.
28. Nishino, S.F. and J.C. Spain. 2000. *In situ* biodegradation of 2,4- and 2,6-dinitrotoluene. Annual Meeting American Society for Microbiology.
29. Paoli, G.C., S. F. Nishino and J. C. Spain. 1999. Metabolism of 3-methyl-4-nitrocatechol by 2,6-dinitrotoluene degrading bacteria. Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives.
30. Parales, R.E., J.V. Parales, D. An, A. Kumar, S.M. Resnick, J.C. Spain, and D.T. Gibson, 1995. 2-Nitrotoluene dioxygenase from *Pseudomonas* sp. Strain JS42: Protein purification, Molecular Biology, and Substrate Specificity. American Society for Microbiology International Symposium on Pseudomonas.
31. Schenzle, A., H. Lenke, J.C. Spain, and H.-J. Knackmuss, 1996. 3-Hydroxylaminophenol mutase from *Alcaligenes eutrophus* JMP134 catalyzes a Bamberger-type rearrangement. Annual Meeting American Society for Microbiology.
32. Shah, M.M., and J.C. Spain, 1995. Reduction of the explosive 2,4,6-trinitrophenylmethylnitramine (tetryl) catalyzed by oxygen sensitive nitro reductase enzymes. Nitration Symposium, National Meeting American Chemical Society.
33. Smets, B.F., J.C. Spain, and R.J. Mueller. 1998. Desorption and aerobic biodegradation of dinitrotoluenes in aged contaminated soils. Battelle Conference on Remediation of Chlorinated and Recalcitrant Compounds.
34. Somerville, C.C., S.F. Nishino, and J.C. Spain, 1994. Purification and characterization of nitrobenzene reductase from *Pseudomonas pseudoalcaligenes*, JS45. Annual Meeting, American Society for Microbiology.
35. Somerville, C.C., and J.C. Spain, 1996. Purification of a novel nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. Annual Meeting American Society for Microbiology.
36. Spain, J.C. 1998. Biotreatment of dinitrotoluenes from munitions manufacturing sites. US/Germany Environmental Technology Data Exchange Meeting.
37. Spain, J.C., and J.C. Cornette. 1998. Biotreatment of nitroaromatic compounds. US/Canada/UK TTCP Workshop on Environmental Aspects of Energetic Materials. Quebec, Canada.
38. Spain, J.C. S.F. Nishino, C. Zhang, and J.B. Hughes. 1999. DNT degradation in soil from Badger Army Ammunition Plant. Partners in Environmental Technology Technical Symposium and Workshop.
39. Spiess, T., F. Desiere, J. Spain, H.-J. Knackmuss, and H. Lenke, 1997. Degradation of 4-nitrotoluene by a *Mycobacterium* sp. strain. Annual Meeting of the German Association of Microbiology-Hamburg.
40. Suen, W.C., B.E. Haigler, and J.C. Spain, 1994. 2,4-Dinitrotoluene dioxygenase genes from *Pseudomonas* sp. strain DNT. Annual Meeting, American Society for Microbiology

41. Venkateswarlu, K. and J.C. Spain, 1997. Nitrophenol biodegradation by *Bacillus sphaericus* JS905 expressing a soluble monooxygenase. Annual Meeting American Society for Microbiology.
42. Vorbeck, C., H. Lenke, J.C. Spain, and H.-J. Knackmuss, 1994. Initial steps in aerobic metabolism of 2,4,6-trinitrotoluene (TNT) by a *Mycobacterium* sp. Annual Meeting, American Society for Microbiology.
43. Zhang, C., J.B. Hughes, R. Daprato, S.F. Nishino, and J.C. Spain. 2001. Stoichiometry of dinitrotoluene mineralization in a pilot-scale slurry-phase bioreactor system. In situ and on-site bioremediation: sixth international symposium.
44. Zhang, C., S. F. Nishino, J. B. Hughes, and J. C. Spain. 1999. Aerobic biodegradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene in a pilot-scale sequential slurry reactor system. Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives.

Published Text Books or Text Book Chapters

1. Nishino, S.F., and J.C. Spain. 1996. Biodegradation and transformation of nitroaromatic compounds, p. 776-783. *In* C.J. Hurst, G.R. Knudsen, M J. McInerney, L.D. Stetzenbach, and M.V. Walter (eds.), Manual of Environmental Microbiology. ASM Press, Washington, D.C.
2. Nishino, S.F., and J.C. Spain. In Press. Biodegradation, transformation and bioremediation of nitroaromatic compounds. Manual of Environmental Microbiology, 2nd Edition. ASM Press, Washington, D.C.
3. Nishino, S. F., and J. C. Spain. 1999. Strategies for aerobic biodegradation of dinitrotoluenes, p. 47-57. *In* R. Fass, Y. Flashner, and S. Reuveny (ed.), Novel Approaches for Bioremediation of Organic Pollution. Kluwer Academic/Plenum Publishers, New York.
4. Nishino, S. F., J. C. Spain, and Z. He. 2000. Strategies for aerobic degradation of nitroaromatic compounds by bacteria: process discovery to field application, p. 7-61. *In* J. C. Spain, J. B. Hughes, and H.-J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton.
5. Spain, J. C. 1995. Bacterial degradation of nitroaromatic compounds under aerobic conditions, p. 19-35. *In* J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Publishing Corp., New York..
6. Spain, J. C. (ed.). 1995. Biodegradation of nitroaromatic compounds. Plenum Publishing Corp., New York.
7. Spain, J. C., J. B. Hughes, and H.-J. Knackmuss (ed.). 2000. Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton.
8. Ward, C. H., M. Alexander, J. A. Ryan, and J. C. Spain. 1999. Transformation, p. 187-201. *In* W. C. Anderson, R. C. Loehr, and B. P. Smith (ed.), Environmental availability of chlorinated organics, explosives and metals in soils. American Academy of Environmental Engineers, Annapolis.

Patent

Spain, J. C., S. F. Nishino, and U. Lendenmann 2001. Process for the biodegradation of dinitrotoluene. United States patent 6,248,580.

Attachment B. Future Research Topics Identified at the 2nd International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives

The Second International Symposium on Biodegradation of Nitroaromatic Compounds and Explosives was held in Leesburg, Virginia on September 8-9, 1999. The meeting, sponsored by the Air Force Office of Scientific Research and the Defense Threat Reduction Agency, featured invited speakers from the US, Germany, the UK, Canada, and Switzerland. After the symposium, the invited speakers met for a half-day session to discuss the state of the art in explosives biodegradation and to propose fruitful areas for research. The meeting participants included: Jim Spain, US Air Force; Hans Knackmuss, University of Stuttgart; Joe Hughes, Rice University; Rebecca Parales, University of Iowa; Hiltrud Lenke, Fraunhofer Institutute, Stuttgart, Germany; Neil Bruce, University of Cambridge, UK; Gerben Zylstra, Rutgers University; Jacqueline Shanks, Iowa State University; Wolfgang Fritsche, Fredrich Schiller University, Germany; Jalal Hawari NRC Biotechnology Research Institute, Canada; Thomas Hofstetter, EAWAG/ETH, Switzerland; Eberhard von Low, Phillips University, Marburg, Germany; Joel Burken, University of Missouri-Rolla; Jochen Michels, Dechema, Germany; Douglas Jerger, IT Corporation, and Herb Fredrickson, USACE Waterways Experiment Station.

The consensus of the participants was that the technology for cometabolic treatment of soil in ex-situ systems including composting and slurry reactors is fairly advanced and cost effective. It seems clear that considerably less is known about in situ remediation, natural attenuation, and biotreatment of contaminated water. Therefore, the focus of the meeting and of the research areas listed below are on the latter topics. The research areas are the ones that were deemed to have a high payoff and to be achievable in the near future.

In the US, cleanup of soils heavily contaminated with explosives is being conducted primarily by excavation followed by composting and incineration. A number of sites have been cleaned up and most of the rest are in the process of cleanup. Major exceptions include Volunteer and Ravenna Army Ammunition Plants where contamination is extensive and cleanup of contaminated soil and groundwater has not started. A substantial amount of contaminated soil also remains at Badger Army Ammunition Plant. Large volumes of lightly contaminated soils would be more appropriately treated in situ if a suitable technology were available. Treatment of explosives contaminated groundwater is done almost exclusively by pump and treat with carbon sorption.

In other countries the cleanup of explosives contaminated sites is just beginning or has not been considered to date. Thus, although new strategies for ex situ bioremediation of explosives contaminated soil may not be fielded in time to help solve the problem in the US, they could be very useful in international applications. Novel in situ soil and groundwater remediation strategies could be fielded in the US in time to provide considerable cost savings.

SUGGESTED RESEARCH TOPICS

Kinetics and mechanisms of physical and chemical interactions with soil matrices (matrix engineering for enhanced binding)

Biological treatment of TNT contaminated soil by anaerobic/aerobic processes or composting gives rise to complete or partial binding of the metabolites to the soil matrix. Therefore it is of vital importance to characterize the bound residues and to determine the nature of the physical or chemical binding. Furthermore it is important to evaluate the short and long term stability of the bonds towards spontaneous or biological hydrolysis. Turnover of natural humic material might release low molecular weight compounds. Fungal enzymes can cleave C-C as well as O-C bound residues.

From current work done in many laboratories it is clear that disappearance of TNT and its metabolites during anaerobic/aerobic treatment is largely due to the reactivity of reduced intermediates or of humic acid precursors. From the broad variety of the chemical structures of the intermediates it is clear that different kinds of binding must exist. Both physical and chemical binding have been identified through the application of different derivatization techniques that break up the micelle structures of humic material. Different covalent bonds in the solid state as well as in solubilized soil material were shown using ^{15}N-NMR techniques. The "bound residues" are susceptible to further transformations, such as residual nitro group reduction and increasing humification i.e. multivalent binding to the humic matrix.

In the majority of cases the nature and the long term stability of bound immobilized metabolites is unclear. Therefore simple and reliable analytical procedures are required that allow a clear differentiation between sorption and covalent binding of metabolites during the anaerobic/aerobic treatment and particularly during the composting process. An understanding of the degree and the chemical quality of immobilization and the factors that determine the binding of the pollutants are crucial for evaluating the remediation product.

Research should be conducted to broaden the understanding of the mechanisms and kinetics of binding of reduced metabolites of TNT. High priority should be given to the nature of the additives that provide the binding matrix for the immobilization process. This requires the identification of the functional groups and the overall redox status of the sorbent and the sorbate which allow optimum and irreversible binding. Immobilization of contaminants can only be considered harmless if the chemical structures bound to soil components exhibit long term stability. Lysimeter experiments must be conducted to make sure that low molecular weight residues released by natural turnover of humic material are subject to further degradation and finally to mineralization.

Fundamentals of microbial processes

A fundamental understanding of microbial processes is needed to improve our ability to predict and enhance biodegradation of nitroaromatic compounds. Although several microbial processes have been discovered for the degradation of nitroaromatic compounds a complete understanding of the enzymatic steps in biodegradation is lacking. In addition, not much is known about effects of gene regulation on biodegradation, diversity and distribution of the organisms, effects of substrate mixtures, potential toxicity of metabolites, effects of metabolites on the degradative

pathway, transport of polar nitro compounds into the cell, and movement of bacteria in soil toward substrates.

Important research objectives to pursue are the discovery and development of new organisms with novel pathways and enzymes for the degradation of nitroaromatic compounds. Implicit in the analysis of new organisms is an understanding of the basic physiological parameters affecting biodegradation including gene regulation, substrate transport, and chemotaxis. Such understanding should also be applied in the context of predicting natural attenuation. A final potential area of study is the possibility of producing valuable products from nitroaromatic wastes. Development of biocatalytic processes that exploit relatively inexpensive nitroaromatic feedstocks for the production of commercially useful products could potentially reduce levels of waste at the source.

Metabolic engineering for mineralization of explosives

A good biochemical and genetic understanding of explosives metabolism has an important role to play in the development of processes for the bioremediation of explosives contaminated land. There is little doubt about the need to continue the elucidation of new degradative pathways, particularly if the diversity of available biocatalysts active against explosives is to increase. The selective pressure of environmental pollution is developing microorganisms that might be harnessed for explosives removal by biotechnological processes. Nevertheless, the fact that explosives persist in the environment emphasizes the failings of the existing catabolic activities in dealing with this problem. Screening natural diversity is unlikely to yield organisms that satisfy all the demands for the degradation of the most highly recalcitrant explosives, since these compounds have only been present in the environment for tens of years and microorganisms have therefore had little time to evolve enzymes suited to the task. The application of genetic engineering and biochemical techniques promises to improve and evolve natural biodegradative capabilities further. In particular, methods of directed evolution may hold the key to adapting explosives degrading enzymes for bioremediation purposes.

Metabolic engineering may also have a considerable influence on the development of bioremediation systems in the near future; while this field is still a very young one, much has been accomplished and this is beginning to have an impact on environmental biotechnology. The combination of increasing commercial interest and advances in understanding the genetic and biochemical basis of the bottlenecks and the identification of crucial metabolites exhibiting high chemical reactivities (misrouting potential) is now producing a more rational approach to engineering metabolic pathways. New genetic tools are continually being developed to make the manipulation of particular genes or chromosomes and the construction of hybrid pathways easier. Recently, attention has focused on the use of transgenic plants for phytoremediation. Innate biodegradative abilities of plants are less impressive than those of adapted bacteria and fungi, but these disadvantages are balanced by the large amounts of plant biomass that can easily be sustained in the field. This raises the exciting prospect for the possibility of combining the impressive biodegradative abilities of certain bacteria with the high biomass and stability of plants to yield an optimal system for in situ bioremediation of organic pollutants

The major research goal is to discover or engineer robust organisms that can mineralize explosives in soil. The overall goal can be subdivided into several related research topics. The

impressive biodegradative abilities of certain bacteria with the high biomass and stability of plants to yield an optimal system for in situ bioremediation of organic pollutants

The major research goal is to discover or engineer robust organisms that can mineralize explosives in soil. The overall goal can be subdivided into several related research topics. The first step is to identify enzymes or microorganisms that degrade explosives; natural diversity can be exploited as a source of the enzymes. It should then be possible to engineer the enzymes for bioremediation purposes by increasing the activity toward the more recalcitrant explosives. It must be kept in mind that a single enzyme will not degrade explosives. It will be necessary to assemble new pathways or modify existing ones. Transport mechanisms should be identified and enhanced where necessary to allow entry of the explosives into the cell. Strategies such as denitrification for detoxification of the high levels of nitrite produced by biodegradation of explosives should be considered or developed as necessary. Finally, regulatory systems should be examined and developed for process control and biosensor applications.

In Situ Treatment

Current technologies for the treatment of nitroaromatic contaminated media focus on ex situ treatment processes (i.e., composting, bioslurry reactors, and pump-and-treat with activated carbon). The development of in situ technologies for the treatment of soil contamination, or for the treatment of groundwaters would provide cost effective approaches to address current remediation concerns. Of particular interest for in situ treatment of nitroaromatic compounds are applications of phytoremediation and stabilization/mobilization technologies. Such strategies are particularly appropriate where no biocatalyst able to mineralize the explosive exists.

Recent studies on phytoremediation of explosives have conclusively demonstrated the uptake of common nitroaromatic explosives, cocontaminants, and many of the partial decomposition products of nitroaromatic metabolism by bacteria. The fate of explosives in plant tissues is less clearly understood and represents a significant bottleneck in our ability to assess the potential applications of phytoremediation. A limited number of metabolic products have been isolated from only a few plant species. Interestingly, these studies have demonstrated that plants are capable of the oxidative metabolism of TNT –a property not commonly found in microbial systems - which may allow for eventual mineralization by plant associated microbes. Furthermore, it has been shown that transgenic plants may be capable of overcoming certain toxicity concerns that currently limit applications of the technology.

The unique sorption/desorption behavior of nitroaromatic compounds may allow for in situ stabilization and/or mobilization of explosives in groundwater. The propensity of nitroaromatic compounds to sorb to certain mineral surfaces is being exploited for analytical purposes and field studies have demonstrated the potential to modify binding capacities of aquifer solids through simple and benign modifications of groundwater chemistry. The primary barrier limiting the application of in situ stabilization/mobilization of explosives is the paucity of information regarding the interaction under site specific conditions between existing groundwater and surface mineral composition.

Polynitroorganics are highly oxidized and thus are susceptible to reduction by metals. Consequently, a joint anaerobic (reductive)/aerobic (oxidative) polishing process is worth

bioprocess may lead to excellent results. For example, in biobarriers the microbial process can be optimized by the addition of zero valent metals as process initiators.

Plant metabolism of explosives, the long term-fate of metabolic products, and the ability to enhance phytoremediation or to recruit oxidative enzymes from plants into bacteria through metabolic engineering are high priorities for future research. The development and demonstration of processes that allow for an in situ sorption-barrier would curtail future needs for pump-and-treat systems and decrease exposure to contaminated waters. Additional understanding of natural attenuation of nitroaromatic compounds is badly needed. Finally, joint processes could be considered as another future research challenge for field testing and application.

Assessment of Bioremediation

The success of a soil remediation technology can be described in absolute (chemical) or in relative (toxicological) terms. Chemical analyses can be used to determine if a technology meets pre-defined and acceptable remediation targets. Such targets are often established on the basis of human health risk assessments and previously published laboratory toxicological studies. The remediation targets are seldom based on sound ecotoxicology. Chemical analyses of remediated soil samples can not detect decreases in (eco)toxicity. This can only be done using sensitive and relevant laboratory- and field-based toxicity tests. Although a reduction in soil toxicity is the desired goal, it is not always evident during the intermediate phases of an environmental bioprocess, particularly if a contaminant is being partially metabolized. Quite often, the toxicities of the metabolites are unknown. They may have toxic as well as genotoxic (and possibly carcinogenic) effects on susceptible receptors in the ecosystem (including man). For a remediation technology to be successful, complete or "acceptable" detoxification of the contaminated soil should be demonstrated.

The successful decontamination of a site involves a number of considerations, such as the physico-chemical and hydrogeological characteristics of the site, as well as the fate and transport of pollutants (toxicants) in soil and groundwater. A soil remediation technology can alter all of the above. Integrated chemical and toxicological monitoring methods will provide valuable information describing the initial state of a contaminated site, as well as the scientific evidence demonstrating the beneficial use of a soil remediation technology.

The results of an integrated chemical-ecotoxicological evaluation of the remediation success will strongly affect the social acceptance of a new site redevelopment strategy. The eventual use of the restored and "decontaminated" site is the social basis for the definition of the remediation targets. The choice of land use (residential, agricultural, commercial, or industrial) will affect the transfer paths of residual contaminants and metabolites from soil-air-groundwater to humans and other receptors. Unfortunately, the number of (bio)analytical approaches and tools capable of assessing the hazard of an explosives-contaminated site is limited.

Chemical endpoints are often used, but are based on laboratory toxicological data of explosives (and rarely their metabolites) and are then extrapolated to predict the undesired effects in humans. Although pertinent in certain cases (such as residential land use), these criteria may be too conservative if the land will be used for commercial or industrial facilities (where human exposure is minimal). Work is being carried out on the toxicological (eco-, geno-, and possible

Chemical endpoints are often used, but are based on laboratory toxicological data of explosives (and rarely their metabolites) and are then extrapolated to predict the undesired effects in humans. Although pertinent in certain cases (such as residential land use), these criteria may be too conservative if the land will be used for commercial or industrial facilities (where human exposure is minimal). Work is being carried out on the toxicological (eco-, geno-, and possible human-related toxicities) characterization of explosives on contaminated sites (soil and groundwater).

The environmental behavior of explosives and their metabolites have only been determined to a limited extent. In the absence of such information, toxicity tests of soil samples taken from "contaminated" and "remediated" sites can be carried out, but the proper interpretation of the results will depend upon chemical analyses. In order to compare the relative efficiencies of different (bio)technologies, a common set of chemical and toxicological criteria should be used. Soil quality criteria of explosives would be useful, but agreement between the different stakeholders (government, technology vendor, or technology user) is necessary. Other technical considerations include the design of the chemico-toxicological evaluation and the choice of (bio)analytical tests. Bioavailability of explosives and their transformation products are also poorly understood. As an alternative to costly site-specific risk assessments of contaminated and "restored" sites, a generic soil eco-health and quality criteria system (based on the anticipated land use) has been proposed. This system could be used internationally, since sites contaminated with explosives (including Brownfields) would be of general public interest.

Several research goals must be met to support scientifically defensible claims for soil remediation. Chemical and ecotoxicological characterization of explosives and their metabolites in environmental samples should continue. Analysis of polar metabolites and remobilized bound residues in soil and water should be undertaken. Standard compounds for new metabolites should be synthesized and made available. There is a strong need for international agreement on the choice of standardized and validated laboratory and field-based toxicity tests pertinent for eventual land use. The effects of soil types on certain risk assessment strategies should be investigated. Finally, models linking laboratory toxicity tests to the expected effects in humans should be addressed.

Appendix B
Polycyclic Aromatic Hydrocarbons (PAHs): Improved Land Treatment with Bioaugmentation

Strategic Environmental Research and Development Program

Polycyclic Aromatic Hydrocarbons (PAHs): Improved Land Treatment with Bioaugmentation

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Draft report

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Preface

Project Title

Polycyclic Aromatic Hydrocarbons (PAHs): Improved Land Treatment with Bioaugmentation.

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1 Landfarming Background

Polycyclic Aromatic Hydrocarbons

Chemical structure and source of contamination

Polycyclic aromatic hydrocarbons (PAHs) are multi-ringed, organic compounds, characteristically nonpolar, neutral and hydrophobic. PAHs have two or more fused benzene rings in a linear, stepped or cluster arrangement. While there are more than 100 known PAHs, Table 1 provides the chemical structure, abbreviated name, and molecular weight for the 16 PAHs that were analyzed in this study.

PAHs occur naturally as components of incompletely burned fossil fuels and they are also manufactured. Several of these manufactured homologues are used in medicines, dyes, and pesticides, but most are found in coal tar, roofing tar, and creosote, a commonly used wood preservative. PAHs are major chemical constituents of a wide variety of contaminants found at Department of Defense (DoD) installations. They are found in burning pits, and as spills of creosote, fungicides, heavy oils, Bunker C fuels, and other petroleum-based products. The higher molecular weight homologues are particularly recalcitrant and toxic. Some lower molecular weight PAHs are volatile, readily evaporating into the air. Others will undergo photolysis. Because they are hydrophobic and neutral in charge, PAHs are strongly adsorbed onto soil particles, especially clays. Park et al. (1990) studied the degradation of 14 PAHs in two soils. They found air phase transfer (volatilization) an important means of contaminant reduction only for naphthalene and 1-methylnaphthalene (the 2-ring compounds). Abiotic mechanisms accounted for up to 20% of the total reduction, but only involved 2-and 3-ring compounds. Biotic mechanisms were responsible for the removal of PAHs over 3-rings. The persistence of PAHs in the environment, coupled with their hydrophobicity, gives them a high potential for bioaccumulation.

Toxicity and benzo(a)pyrene toxic equivalent factors

The 16 compounds examined in this study (Table 2) are grouped together, first, because more information is available on them, and second, they are suspected to be the most harmful of the PAHs. The effects they exhibit in animal and human studies are representative of the class as a whole. In addition, these are the PAHs to which the public is most commonly exposed. Also, they are found in highest concentration on National Priority List hazardous waste sites (ATSDR 1995).

As a class of compounds, PAHs have been classified as carcinogens, mutagens and immunosuppressants. Even slight differences in PAH chemical structure and activity result in

different toxic potencies and different health effects from the individual PAHs. The importance of PAH chemical structure as an indicator of potential carciogenicity has been reviewed in Pitot and Dragan (1996). Some PAHs have been classified as carcinogens only in laboratory animals. Others, including benzo(a)pyrene (BaP) and benzo(a)anthracene, have been identified as human carcinogens. Still others are possible carcinogens or not classifiable because the testing is incomplete. Tumors usually occur at the point of entry into the body (i.e., the skin, lungs, eyes, intestines). However, metabolism of these compounds can result in an increase in their toxic potency and tumor formation in secondary organs (i.e., bladder, colon, liver). Metabolites of these compounds can also be carried into cells where they form adducts with DNA through covalent bonding. The best studied mutation is in the 12^{th} codon of the Hras codon. The PAHs elicit multiple responses from the bodys' immune system due to their effects on humoral and cell-mediated immunity as well as host resistance (Burns et al., 1996). The mechanisms of PAH immunosuppression have been reviewed by White et al. (1994). BaP is often used as an indicator for risk assessment of human exposure because it is highly carcinogenic, persistent in the environment, and is, toxicologically, well understood. This breadth of knowledge doesn't exist for most of the other PAH compounds.

Because PAHs occur as mixtures of different concentrations of different homologues, toxic equivalency factors (TEFs) were proposed, similar to those used in the risk assessment of mixtures of polychlorinated biphenyls (PCBs). The Environmental Protection Agency (EPA) took the first step in 1984 by separating PAHs into carcinogenic and non-carcinogenic compounds. All of the PAHs were rated, using BaP as a reference and giving it a value of 1.00. However, this method led to an over-estimation of exposure risk since the carcinogenicity of most of the compounds was unknown and the interactions between compounds in mixtures hadn't been determined. In an attempt to overcome this liability, Nisbet and LaGoy (1992) developed a new method based on the compounds' response while testing one, or more, PAHs concurrently with BaP in the same assay system (usually lung or skin cell carcinoma). BaP remained the reference carcinogen assigned the value of 1.00. Sixteen other PAHs were ranked in comparison to BaP carcinogenicity. This system was tested by Petry et al. (1996) who assessed the health risk of PAHs to coke plant workers. There are drawbacks to any system that uses equivalency factors. The uncertainties in this case arise primarily from dealing with inconsistent mixtures. Carcinogenic potency could be affected by differences in bioavailability, a competition for binding sites, co-carcinogenic action, or the effects of metabolism. Nevertheless, Petry et al. (1996) found that the BaP equivalents developed by Nisbet and LaGoy (1992) were valid markers for PAH health risk assessment.

Environmental risk assessment, in a slight contrast to human health risk, looks at the PAHs that usually occur in contaminated environmental systems and that have the highest TEFs (by the Nisbet and LaGoy (1992) system). The seven PAHs listed in Table 2 have the highest environmental risk: benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, and dibenzo(a,h)anthracene.

PAH bioavailability

As indicated earlier, contaminants react chemically and physically with different kinds of soil particles, which change the physical and chemical natures of both components. Biological

availability, or bioavailability, is used to describe both the amount of toxin available in soil to harm organisms (humans, other animals, plants) or, in the case of bioremediation, the amount of toxin available to be metabolized by microorganisms after these contaminant-soil interactions. *In situ* bioremediation is a managed or spontaneous process in which microbiological processes are used to degrade or transform contaminants to less toxic or nontoxic forms, thereby remedying or eliminating environmental contamination. While these microbiological processes may decrease contaminant concentrations to levels that no longer pose an unacceptable risk to the environment or human health, the contaminants that remain in treated soils still might not meet stringent regulatory levels, even if they represent site specific, environmentally acceptable endpoints (National Research Council, 1997). PAHs in soils may be biodegraded by microorganisms to a residual concentration that no longer decreases with time, or which decreases slowly over years with continued treatment (Thoma, 1994; Luthy et al., 1994; Loehr and Webster, 1997). Further reductions are limited by the availability of the PAHs to microorganisms (Bosma et al., 1997; Erickson et al., 1993). Additionally, as contaminants age they become less available compared to freshly contaminated material. The adherence and slow release of PAHs from soils is another obstacle to remediation (National Research Council, 1994; Moore et al., 1989). Because they bind with soils and suffer subsequent slow release rates, residual PAHs may be significantly less leachable by water and less toxic as measured by uptake tests (Gas Research Institute, 1995; Alexander, 1995; Kelsey et al., 1997). Generally, contaminants can only be degraded when they exist in the aqueous phase and in contact with the cell membrane of a microorganism (Fletcher, 1991). The contaminant serves as a growth substrate for the microorganism and is incorporated into the cell through membrane transport and is utilized as an energy source in the cell's principal metabolic pathways. However, physical or chemical phenomena can limit the bulk solution concentration of the contaminant and thus significantly reduce the ability of the microorganisms to assimilate the contaminant. Therefore, the availability of the contaminant can control the overall biodegradation of these compounds.

Another important factor relevant to biodegradation and bioavailability is the location and density of microorganisms. The majority of bacteria in the environment are attached to surfaces and their distribution in and on soils is very patchy. The majority of these bacteria range in size from 0.5 to 1.0 μm while micropores present in soils are far less than 1 μm. It is generally believed that bacteria are attached predominantly to the surface of soil particles and not to the interior surfaces of the micropores. It has been estimated that over 90% of the microorganisms present in geologic matrices accumulate on the surfaces of soil (Costerton et al., 1987). Therefore, the majority of contaminant-microbial interactions occur in the biofilm that develop within macropores on the surfaces of soils.

This suggests that partitioning of an organic contaminant from the solid phase of the soil to the aqueous phase in the larger pore spaces controls soil biovailability. These partitioning mechanisms may include chemical bonding, surface complex formations, electrostatic interactions, and hydrophobic effects (Schwarzenbach et al., 1993; Stumm, 1992). For hydrophobic contaminants like PAHs, sorption increases with the content of the organic matter in the soil/sediment and the degree of hydrophobicity of the specific PAH. Typically, the rate of desorption can be attributed to the mass transfer of the sorbate molecules from sorption sites on and in the soil. Active bacteria should correspond to the higher available PAH concentrations, which occurs where desorption is the most intense.

As already discussed, the partitioning of PAHs represents a significant part of the mass transfer process. However, describing this process is complicated by a combination of diffusion, dissolution, encapsulation, and adsorption phenomena. The combined effect of these factors can generally be defined as *sequestration.* Luthy et al. (1997) devised a conceptual model to illustrate the different sorption mechanisms associated with specific domains within the soil (Figure 1).

Case A represents absorption into amorphous or "soft" natural organic matter (SOM). This adsorption takes place within an organic phase in a manner similar to solvent partitioning. Another situation, Case B, represents absorption into condensed or "hard" organic polymeric matter or combustion residue (i.e., into solid-like organic matter). A Case C represents adsorption onto water-wet organic surfaces (i.e., soot). Case D represents adsorption to exposed water-wet mineral surfaces (i.e., quartz); and Case E represents adsorption into microvoids or microporous minerals (i.e., zeolites) with porous surfaces at water saturation <100% (Luthy et al., 1997).

These different domains within soils illustrate how structural and chemical heterogeneity can significantly affect how PAHs behave. Excluding entrapment due to weathering at interfaces (i.e., NAPL/water), the Case A presumably would exhibit fast kinetics with low desorption energy and high extractability, while Case B would show slow kinetics, high desorption energy, and low extractability. Case C is characterized by fast kinetics with low desorption energy and high extractability, while Case D would exhibit fast kinetics, low desorption energy, and high extractability. The final case, E, would show slow kinetics, high desorption energy, and low extractability. Assuming all other environmental parameters are consistent among the five different cases, a qualitative ranking of bioavailability is as follows: cases A, C, D > B, E (Talley et al., 2000).

Current methods for assessing sorption and sequestration of PAHs on soils do not provide a basic understanding of the bioavailability of recalcitrant PAHs. They also lack information to aid interpretation of results of ecotoxicological testing of residuals after biotreatment. Whether residual PAHs remaining after biotreatment represent an acceptable cleanup endpoint requires understanding of the mechanisms that bind contaminant PAHs within soil or sediment. Research is needed that will assess the fundamental character of the binding of PAHs in parallel with the development of biotreatment and ecotoxicity testing, to show how the nature of PAH association with soils relates to bioavailability and achievable treatment endpoints.

Problem summary

The PAHs are large, multi-ring compounds, many of which are toxic to humans and the environment. While the lighter molecular weight homologues may be removed by volatilization, the higher molecular weight compounds are increasingly more toxic and more resistant to both chemical and biological degradation. PAHs are tightly bound to the humic fraction of the soil. The binding strength increases with exposure time, making "aged soils" more difficult to remediate. Research was needed to:

- Increase the availability of the PAHs for biological degradation
- Establish remediation endpoints that maintain public health and safety and are realistically achievable.

The Flask-to-Field PAH project focused on the first of these objectives, increasing the availability of PAH compounds for biological degradation and increasing the overall biodegradation of the high molecular weight homologues.

Available Treatment Options

Treatment of PAH-contaminated soil can be performed either *ex situ* or *in situ*, and each of these has both abiotic and biotic technologies available. Of the *ex situ* treatments, the abiotic choice is a destructive technology, incineration. Biotic options include slurry bioreactors and compost reactors. The available abiotic *in situ* treatments include soil flushing and stabilization. Electrokinetic (E-K) separation is in preliminary development. Biotic treatments performed *in situ* include bioventing, phytoremediation (on soils with low concentrations) and landfarming. These options generally separate out into either of two treatment approaches; highly engineered solutions such as solid phase or slurry phase treatment, and minimally engineered *in situ* treatment. Examples of each strategy are presented in Table 3, along with a summary of the inherent benefits, and limitations associated with each technology. The difficulties associated with the use of biotreatments have been analyzed in Talley and Sleeper (1997) with reviews of pertinent technologies. Detailed information, including cost summaries and case studies can also be obtained at http://www.frtr.org.

Thrust Area: Early Studies

The approach taken during the "Flask" studies separated the objective into two broad tasks: first, isolating and characterizing a microorganism, or consortium of microorganisms, capable of degrading the higher molecular weight PAHs, and second, selecting a means of releasing the PAHs from the soil into the surrounding soil pore spaces where it would, presumably, be available to degradation. Each of these tasks was further separated into smaller research areas. In order to find an organism(s) that would degrade higher molecular weight PAHs, a new isolation method was developed. New and existing strains have been characterized and metabolic pathways have been described. It became necessary to explore the potential of co-metabolism for the degradation of these PAHs. At the same time, the effects of chemical surfactants on the soil-PAH binding were studied. Bacteria that are natural surfactant producers were isolated and the biosurfactant action compared to the chemical surfactants. When a decision for bioaugmentation had been made, a method had to be found to deliver the chosen microorganisms into the contaminated soil.

Several different technologies contributed to the "Flask" portion of the study, as illustrated in Figure 2: slurry reactors, landfarming, and composting. The project began with an examination of available treatment options. Three promising technologies were selected, a high technology

system of slurry bioreactors, and two, low technology, soil treatment systems, composting and landfarming. Research in each of these technologies provided insight into areas of PAH bioremediation useful to the final treatment selection: isolation and characterization of PAH-degrading bacteria and elucidation of the degradation pathways, surfactant chemistry, bioaugmentation, and carrier technology. This produced a final treatment with aspects of all three technologies. For field studies, landfarming with bioaugmentation and biostimulation was selected as the treatment option for PAH-contaminated soils. The treatments and the technologies that support them are discussed in the following sections.

Solid-phase treatments

Solid phase treatments, commonly known as landfarming and composting, are two of the most commonly applied technologies for the remediation of PAH-contaminated soil (Gray et al., 2000; Harmsen, 1991; Mueller et al., 1991 a,b; Mueller-Hurtig et al., 1993; Yare, 1991). Most PAH-contaminated soils contain a significant number of PAH degraders that have been enriched because of the presence of the PAHs but they are often constrained in their degradation capability because of some limiting factor. Common limiting factors include inadequate aeration, poor contact of the microorganisms with the PAHs due to the adherence of the PAHs to surfaces and NAPL phase materials, and the absence of sufficient nitrogen to sustain extensive mineralization of the contaminant carbon. Any engineering activity that reduces these limitations brings the native degraders into action.

Advantages of solid phase treatment are that large quantities of contaminated soil can be treated at the same time, and operation and maintenance activities (costs) are minimal. In general, contaminated soil is placed in aboveground treatment areas that are designed for proper effluent collection and then the soil is handled in specific ways to enhance indigenous microbial activity. Composting usually involves the addition of readily degradable organic matter (bulking), and fertilizer. Bulking refers to the addition of inexpensive, readily available materials (straw, manure, sewage sludge, wood chips, rice hulls, etc.) that enhance aeration of the soil and improve soil texture. The bulking agents also dilute the soil contaminant, reducing the concentration of toxic chemicals. The resulting mineralization of the added organic matter also aids in the degradation of PAHs. Some control of aeration and temperature is usually required (Potter et al., 1997). Kastner et al. (1999) found that the addition of compost enhanced the degradation of PAHs in soil due to the presence of the organic solids in the compost. Composting was not selected as our treatment option because the loss of PAHs was due to soil binding which did not reduce the toxicity of the soil (Johnson, 1998).

Landfarming remediation of PAH-contaminated soils, based on the degradative activities of natural microbial communities, is a well-used and generally reliable technology. It has been applied to a variety of soils and contaminant types (i.e., creosote, coal tar, petroleum) and is generally preferred over non-biological approaches such as stabilization, chemical oxidation and incineration (Mueller et al., 1995). The focus of landfarming is to stimulate the degradation capabilities of natural microbial communities by providing oxygen and nitrogen, and to use physical mixing of the soil to distribute the contaminants over a greater surface area and bring them into contact with the microorganisms. Traditional agricultural procedures are used to provide mixing (tilling, bulking), moisture (irrigation), and nitrogen (fertilizer). Fertilizer can be

applied as commercial formulations or as organic waste material (commonly manure). Landfarming is often, but not necessarily, limited to the treatment of 6-12 in. of soil at a time (normal depth of tilling), but as one layer (lift) is successfully treated, successive lifts can be applied on top. The disadvantages of traditional landfarming are the time required, the operation and maintenance costs, and the space required for the lifts.

The degradation that results from solid phase remediation often follows a two-stage process involving an initial rapid phase with extensive PAH degradation (a few months) and a second slower phase of degradation (months to years) with relatively little further change in PAH concentration (Brown et al., 1995; Cornelissen et al., 1998; Mueller et al., 1998; Pollard et al., 1994). Differences in desorption rate from soil particles into the interstitial water is frequently cited as the cause of the two-stage process. Initially, rapid desorption occurs and degradation is limited by microbial factors. At some point, desorption slows and degradation rates decrease accordingly. The transition from the fast to the slow phase is often critical. If it occurs before clean up criteria are met, treatment times are greatly extended and costs increase substantially. Clean up criteria are often based on the concentration of benzo(a) pyrene equivalents. If concentrations can't be reduced below a previously established clean up value, then the soil is not considered "clean" and it must either be disposed of as a hazardous waste or further "engineered" with another treatment technique such as chemical oxidation, the addition of surfactants, and/or physical restructuring, in an attempt to encourage more degradation. Treatment times are greatly extended and costs increase substantially. The treatment options then become: (1) disposal, (2) short-term treatment, or, (3) impoundment. Impounding the treated soils and allowing natural degradation processes to eventually degrade the high molecular weight (HMW) PAHs can be considered. With the impoundment option, leachable hydrocarbons, mainly the low molecular weight PAHs, are not likely to cause environmental problems since they have been largely removed during the initial degradation phase. In essence, these soils can be considered as "biostablized" (Luthy et al., 1997; Mueller et al., 1999; Talley et al., 2000), that is, the very slow leaching of the residual HMW PAHs is counterbalanced by the degradation capabilities of the indigenous microbial communities. However, this requires that the impounded soil be carefully managed and monitored over periods of years; a task that can add considerable long-term cost.

Therefore, the problem became one of identifying the cause(s) of the rapid to slow transition of degradation and learning what could be done to control it.

Slurry-phase treatment

Slurry phase treatment involves the processing of contaminated soil using a contained system, or bioreactor, in which the contaminated soil receives the maximum amount of mixing and aeration. A variety of reactors can be used; fixed film, plug flow, and slurry reactors. Degradation rates in a bioreactor are usually considerably faster than than solid phase systems (Pinelli et al., 1997). This is because intimate contact between the PAHs and the microorganisms is provided and optimal conditions for microbial growth and degradation are maintained with considerable uniformity. Because of the contained nature of the system, inoculation with selected organisms (bioaugmentation), or the addition of surfactants, is reasonable. However, slurry phase treatment is expensive to set up and to operate and maintain, especially as only relatively small quantities

of soil can be treated at a time. As the size of the reactor increases, optimal conditions will be compromised due to the physical nature of the systems. Costs can often can be reduced by using existing facilities, such as a lined lagoons and basins, for the treatment.

Performance comparison

From a comparison of the performance of slurry-phase and solid-phase treatments, it can be seen that solid-phase treatment is slower than slurry-phase treatment (Figure 3 modified from Mueller et al., 1991a, b). Mueller et al. (1991a,b) divided PAHs into three groups based on number of rings and added the mixtures to soil in both slurry and solid treatments. Group 1 consisted of 2-ring PAHs (naphthylene, methyl and dimethyl naphthalenes, and biphenyl). Group 2 consisted of 3-ring PAHs (acenaphthylene, acenaphthene, phenanthrene, anthracene, methyl-anthracene). Group 3 consisted of various 4-, 5-, and 6-ring PAHs (fluoranthene, pyrene, benzo(b)fluorene, chrysene, benzo(a)pyrene, benzo(a)anthracene, indenopyrene, benzo(b,k)fluoranthene). For the Group 3 PAHs 40% removal occurred in 30 days in the slurry reactor and in 12 weeks in the solid phase reactors. However, degradation of these higher molecular weight PAHs appeared to plateau in the slurry reactor, whereas degradation was still occurring at the end of the solid phase reactor experiment. Thus, the added time of treatment may be rewarded by a greater extent of degradation.

The Flask-to-Field selected treatment option (Landfarming)

In order to avoid the possibility of long-term passive treatment or the use of additional active treatment technologies, the initial, rapid, phase of PAH degradation must be expanded and the second, slower, phase enhanced. This will provide a greater and more predictable degradation of HMW PAHs.

Improvements in the degradation of HMW PAHs result from the enhancement of the metabolic capabilities of natural microbial communities, and, the increased availability of the PAHs to the microorganisms. These two considerations appear to have the biggest effect on the second phase of degradation. Enhancing metabolic capabilities of microbial communities will require an understanding of where the deficiencies originate, including an appreciation for the metabolic pathways used by these organisms. Enhancing bioavailability will require knowledge of the interactions between the degrading microorganisms and the availability of the PAHs from the bound state. To achieve these enhancements, chemicals and microorganisms can be added to soil. Any additions of amendments must generate enough cost savings in the end to pay for the additions, an aspect that was considered carefully in this work. Each of these enhancement strategies is presented in detail.

Microbiological studies

Isolation/characterization of PAH-degrading bacteria. Bacterial isolates that have been enriched for their ability to grow on low molecular PAHs (i.e., naphthalene, phenanthrene, fluorene, and to some extent, indan, acenaphthene, and anthracene) were studied. The common bacterial genera encountered include *Pseudomonas*, *Alicaligenes*, *Mycobacterium*, *Rhodococcus*, *Comamonas*, and *Sphingomonas*. This is a relatively small range of genera considering the

prevalence of PAHs in the environment, yet it does show that the ability to degrade low molecular weight PAHs is common.

Recent studies have emphasized the potential importance of *Mycobacterium* and *Sphingomonas* species (Bastiaens et al., 2000; Bouchez et al., 1995; Fredrickson et al., 1995; Kastner et al., 1994; Meyer et al., 1999), with some indication that *Sphingomonas* strains are more likely to degrade aqueous phase PAHs and *Mycobacterium* are more likely to degrade solid PAHs (crystals) because of their hydrophobic cell surfaces (Bastiaens et al., 2000). Quantitative polymerase chain reaction (PCR) has been used to identify microorganisms able to degrade PAHs in soil. PCR is a technique that increases the number of copies of a specific region of DNA. From a single microorganism, enough DNA can be produced to allow precise identification of that species. In some soil samples where PAH degradation is actively occurring, the responsible organism appears to be a *Bulkholderia*-type organism, which is difficult to grow in the laboratory (Laurie and Lloyd-Jones, 2000). Again, it must be emphasized that these studies find bacteria that seem to grow well on phenanthrene and fluorene, but with a limited ability to grow on or co-metabolize HMW PAHs.

A number of studies, have shown that bacteria are able to grow on the four-ring PAHs, specifically, fluoranthene (Boldrin et al., 1993; Dagher et al., 1996; Kastner et al., 1994; Lloyd-Jones and Hunter, 1997; Mueller et al., 1990; Weissenfels et al., 1991) and pyrene (Boldrin et al., 1993; Churchill et al., 1999; Dean-Ross and Cerniglia, 1996; Fritzsche, 1994; Grosser et al., 1991; Heitkamp et al., 1988a,b; Jimenez and Bartha, 1996; Kastner et al., 1994; Lloyd-Jones and Hunter, 1997; Rehmann et al., 1998; Schneider et al., 1996). *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, and *Sphingomonas* are the genera commonly encountered. Results from a phylogenic study with a variety of *Pseudomonas* and *Sphingomonas* strains isolated from PAH contaminated soils shows that these two groups can be separated, to some extent, based on their physiological characteristics (Biolog™ Identification System) and their fatty acid composition. The Biolog™ results are shown in Figure 4, modified from Mueller et al. (1997). The symbols represent different soil samples used for isolation and the numbers refers to strain designations. The cluster in the upper left (7,8,9,10, 15,16, 17) consisted entirely of phenanthrene degraders (predominantly *Pseudomonas* species). The cluster in the lower right (1,4,5,6,13,24,25,26,29) was fluoranthene degraders, predominantly *Sphingomonas* species. These results suggest that certain catabolic characteristics are associated with specific genera. That is, *Sphingomonas* and *Mycobacterium* could be the primary genera that are able to attack HMW PAHs. This may be related to characteristics of their cell membranes that allow these PAHs to diffuse inside the cells or it may be related to the presence of key membrane-associated dioxygenases. Interestingly, most of the pure cultures that have been isolated for their ability to grow on pyrene have been *Mycobacterium*. We are aware of only one case where a Gram-negative microorganism, a *Pseudomonas* strain, was able to grow on pyrene (Thibault et al., 1996).

Numerous fungal species are also able to partially degrade PAHs, both low and high molecular weight (Baldrian et al., 2000; Boonchan et al., 2000; Mueller et al., 1997). The initial reactions of PAH degradation by the fungi are usually ascribed to their extracellular liginolytic enzymes (usually the laccases and perioxidases) and these organisms may be involved in PAH turnover in unpolluted soils. The effectiveness of a co-culture of a fungus (*Penicillium)* with the bacterium *Stenotrophomonas* and a mixed bacterial population in degrading five-ring PAHs suggests that

the initial oxidation products produced by the fungi are then further degraded by the bacteria. There was no indication that this co-culture affects the bioavailability of the HMW PAHs. It may not be a particularly useful technique for bioremediation but it is interesting in terms of the natural way in which PAHs might be degraded in the environment.

The most important observation is that there are a number of PAH degraders that have relatively broad co-metabolic capabilities, especially for HMW PAHs (Dagher et al., 1996; Mahaffey et al., 1988; Mueller et al., 1997a; Schneider et al., 1996; Schocken and Gibson, 1984). The term "*co-metabolism*" as used in this study, is the ability to transform (oxidize) a particular PAH but without growth on that PAH. Presumably, partial degradation products are generated, which cannot be further metabolized to produce carbon and energy. There have been several reports of PAH-co-metabolizing *Sphingomonas* species (Dagher et al., 1996; Fredrickson et al., 1995; Kastner et al., 1994; Mueller et al., 1990). *Sphingomonas paucimobilis* strain EPA 505 has been shown to have a substantial cometabolic capability for HMW PAHs (Mueller et al., 1990; Ye et al., 1996). *Sphingomonas* strain B1 (formally *Beijerinckia* B1), isolated originally for its ability to grow on biphenyl, has been shown to co-metabolize benzo(a)anthracene to acid metabolites (Mahaffey et al., 1988). Whether sphingomonads are commonly associated with PAH degradation is yet to be assessed, but a study examining the diversity of bacteria able to degrade PAHs (Ye et al., 1996) showed that *Sphingomonas* species tended to be the isolates capable of degrading fluoranthene, whereas the bacteria able to degrade phenanthrene where more commonly associated with *Pseudomonas* strains. Dagher et al. (1996) compared three PAH-degrading *Pseudomonas* sp. with an *Sphingomonas* sp. and found the latter the most efficient PAH degrader, with the ability to possibly grow on FLA.

With these previous studies as a basis, other fluoranthene degraders were isolated and evaluated with respect to their taxonomic and metabolic characteristics. Little is known concerning the bacterial genera that may be responsible for microbial degradation of HMW PAHs in PAH-contaminated sites, however, the results reported here suggest that *Sphingomonas* spp. are commonly isolated from PAH-contaminated soils and their metabolic diversity may vary considerably. Therefore, our efforts focused on sphingomonads and on *S. paucimobilis*, in particular.

Co-metabolism to enhance metabolic capabilities. The co-metabolic capabilities of *S. paucimobilis* strain EPA 505 are shown Table 4 and Figure 5. After a 28 day incubation of a PAH mixture with EPA 505 and a chemical surfactant there was considerable reduction in the higher molecular weight PAHs.

Ye et al. (1996), using resting cell suspensions of EPA 505 and individual PAHs, in place of a mixture, showed a similar pattern of degradation of HMW PAHs, but to a greater extent. This is shown in Figure 5. Error bars for the standard deviation of triplicates are not shown in this figure, but error was approximately ± 10%. The PAHs in this experiment were not growth substrates for EPA 505.

This co-metabolic capability is not typical of all PAH degrading strains studied. Table 4 shows the effectiveness of several isolates in their ability to degrade different PAH fractions of creosote. The CRE strains were isolated for their ability to grow on phenanthrene, the UNP/NP

strains for their ability to grow on fluoranthene, and the PYR1 strain for its ability to grow on pyrene. It is clear that, of the strains studied, EPA 505 was the most effective in terms of removing the 4,5, and 6-ring PAHs (67% removal compared to 28 % removal for next best strain, *Mycobacterium* strain PYR-1; Heitkamp et al., 1988 a, b) and in producing the greatest removal of PAHs overall (91% removal). Thus, just because a strain grows well on particular PAH, does not mean that it will have broad co-metabolic capabilities. The next question investigated was whether this metabolic capability could be expected in most soils.

Metabolic characteristics. In preliminary mechanistic studies performed with pyrene, a non-growth PAH for EPA 505, it appears that at least two different partial degradation products of pyrene were produced. Both degradation products represent the opening of an aromatic ring and both indicate an inability to cleave carbon moieties to use for growth. The proposed pathway for the co-metabolism of pyrene by strain EPA 505 is shown in Figure 6 (from Ho et al., 2000). The pathway represents possible attacks on the pyrene rings based on products detected by, and inferred from, GC-MS analysis.

The products suggest that pyrene is dihydroxylated at both the 2, 3-position and the 9, 10-position. There was no evidence to suggest two separate dioxygenase systems, and thus it is assumed that a single enzyme system is able to recognize both positions. Clearly both of the oxidized rings are then opened, one by *meta* cleavage and one by *ortho* cleavage. This is consistent with the mechanism by which the strain attacks fluoranthene (see below). It is also consistent with the reported 1,2-dioxygenation of pyrene and subsequent *meta* cleavage (Heitkamp et al., 1988a,b; Walter et al., 1991) and the 4, 5–dioxygenation followed by *ortho* cleavage (Rehmann et al. 1998), both seen with *Mycobacterium* and *Rhodococcus* sp. that are able to grow on pyrene. In the *Sphingomonas* species, however, the specificity of the enzymes involved in the cleavage of either a 3-carbon fragment (*meta* cleavage) or a 2-carbon fragment (*ortho* cleavage) are apparently too narrow to allow further metabolism of the pyrene products.

It is interesting to speculate that this may be true of *Sphingomonas* species in general since, at the present time, there are no known *Sphingomonas* species that are able to grow on pyrene. *Mycobacterium* strains on the other hand can be readily isolated for their ability to grow on pyrene. In preliminary studies, we have observed transient pyrene degradation products from selected *Mycobacterium* strains that have the same spectral characteristics as the substituted perinaphthalene product produced by EPA 505. If this is the case, then initial degradation of pyrene may be similar in both *Mycobacterium* and *Sphingomonas* species. Recent evidence demonstrates that *nah*-like genes can be found in *Mycobacterium* and *Rhodococcus* species (Hamann et al., 1999). However, the *Mycobacterium* stains clearly have the ability to further metabolize the intermediates produced from a naphthalene-like attack on pyrene. This was verified by the work of Rehmann et al. (1998) and Heitkamp et al. (1988a,b), who observed further degradation products. Why *Sphingomonas* strains have not acquired the necessary enzymes for this transformation is unclear. Perhaps it is related to the organic matter that each species normally utilizes in nature. It is assumed that EPA 505 will hydroxylate other HMW PAHs in the same manner and produce ring opened intermediates that cannot be further metabolized. Thus, this strain appears to have a remarkable breadth of co-metabolic

capability due to very loose specificity of the dioxygenase enzymes that are responsible for metabolizing phenanthrene and fluoranthene.

Biodegradation pathways. As microorganisms in the environment are confronted with polycyclic aromatic hydrocarbons (PAHs) of increasing numbers of aromatic rings, the biochemical strategy for removing carbon fragments that can be oxidized through the intermediary metabolism of the organisms, becomes more complex. In many cases, the activity of PAH-degrading enzymes is constitutive, yet the presence of certain growth PAHs seems to increase enzyme activity (Aitken et al., 1998). Stringfellow et al. (1995a,b) have shown that the growth substrate phenanthrene (and salicylate) was able to induce the co-metabolism of fluoranthene and pyrene, both of which were not growth substrates, in a *Pseudomonas saccharophilia* strain P-15, an isolate from PAH-contaminated soil. Studies with the same organism also revealed that chrysene and benzo(a)anthracene could be mineralized and the mineralization was stimulated by pre-growth on phenanthrene (Chen and Aitken, 1999). Chrysene itself was not a metabolic inducer. Benzo(a)pyrene was not mineralized significantly regardless of whether cells were pre-grown on phenanthrene or not. In soils, it is possible that an inducer such as salicylate could be added directly to the soil to induce greater PAH degradation activity, but it is probably impractical in the final analysis (Chen and Aitken, 1999). The more likely scenario is to make the HMW PAHs as available as possible during the time when the inducing PAHs, most likely phenanthrene, are actively being degraded.

The potential of sequential degradation of the PAHs complicates issues of co-metabolic induction. Phenanthrene is usually in high concentration in PAH-contaminated soil. If it is competing with the co-metabolized PAH for the same active site in an enzyme, co-metabolism is likely to be slowed by phenanthrene, assuming it is the preferred substrate for the enzymes. In studies examining the effect of PAH mixtures on co-metabolism, Luning Prak and Pritchard (personal communication, 2001) have shown that phenanthrene does inhibit the co-metabolism of pyrene in strain EPA 505. These experiments were carried out in the presence of the chemical surfactant Tween 80 in order to produce concentrations of PAHs that could be readily followed by HPLC analysis. In these studies, the rate of phenanthrene degradation is the same in the presence and absence of pyrene, suggesting that if only one enzyme system is responsible for the initial PAH metabolism, then phenanthrene is clearly the preferred PAH substrate. The presence of phenanthrene does not totally preclude pyrene co-metabolism (i.e., there is some decay over time), but pyrene metabolism is much slower than it is in the absence of phenanthrene. However, once the phenanthrene was degraded, pyrene metabolism commenced at a faster rate than that seen with no pre-exposure to phenanthrene.

These results have important implications for landfarming treatment. If the bioavailability of the PAHs can be enhanced by the addition of surfactants, then once the readily degradable PAHs are removed, one can assume that, for a short period, the microbial communities will be fully induced and this will be the time in which maximum co-metabolism can be expected. Thus, if organisms with broad co-metabolic capabilities are to be added to soil during landfarming treatment, timing is important. They must be added during a time early enough in treatment where they will be induced by growth on low molecular weight PAHs and yet not so late they are out-competed for these low molecular weight PAHs by the indigenous microbial communities.

Surfactants to enhance bioavailability.

a. *Chemical surfactants.* One limitation to degradation of HMW PAHs is their low solubility in water and high affinity for surfaces. Bioavailability of HMW PAHs in soil has been extensively studied and it is clear that slow desorption of the PAHs from soil particles plays a key role in the ability of bacteria to degrade these PAHs. As a contaminated soil ages, PAHs tend to move into the deeper recesses of soil particles, soil aggregates, and the organic matter sorbed to soil particle surfaces (Pignatello and Xing, 1996; Zhang et al., 1998; Cornelissen et al., 1998; Jixin et al., 1998). Consequently, desorption is usually described as a rapid initial release of PAHs that are close to the surface and a very slow release of PAHs that are more deeply sorbed. Although considerable amounts of sorbed PAHs will eventually leach out over years, this time frame is usually too long for shorter-term remediation techniques, such as landfarm treatment (months), to be effective. If strategic modifications of bioremediation techniques can be made to increase desorption rates over the shorter treatment term, then the added amount of degradation may mean meeting cleanup goals in a reasonable time.

Since most microorganisms take in PAHs from the aqueous phase, their degradation rate is often limited by the mass transfer from the sorbed or non-aqueous liquid phase into the aqueous phase. One method to enhance PAH transfer rates into the aqueous phase is to add surfactants to increase micellar solubilization of the PAHs. Surfactants have been found to enhance the degradation rates of individual PAHs in pure and mixed cultures (Grimberg et al., 1996; Guha and Jaffe, 1996 and 1998; Liu et al., 1995; Madsen and Kristensen, 1997; Tiehm, 1994; Volkering et al., 1995; Willumsen, et al., 1998). Success is controlled by the type and concentration of surfactant utilized and the type of organisms tested. Many surfactants can be toxic to the microorganisms used. In the work of Willumsen et al. (1998), although Tween 80 (0.24 mM) had a stimulatory effect on the mineralization of fluoranthene by both *Sphingomonas* and *Mycobacterium* sp., Triton X-100 (0.48 mM) was quite toxic to most PAH degraders, as determined by their ability to mineralize glucose (Figure 7, from Willumsen et al., 1998). Fan 9 and VF1 are both *Mycobacterium* strains. EPA 505 and FLA 10-1 are both *Sphingomonas* strains. Interestingly, one of the strains tested in these experiments was able to recover from the effects of Triton X-100 after extended incubation. This suggests that the surfactant interacts with the cell membrane, perhaps allowing for greater transport of PAHs inside, and that the extent of this interaction determines the eventual toxicity.

Pritchard et al. (1995a) showed that at high biomass concentrations (10^{10} cells/ml), Triton X-100 was generally stimulatory to fluoranthene mineralization by EPA 505 in minimal salts medium. However, at high concentrations of Triton, the total mineralization of fluoranthene was decreased. An examination of the mass balances, expressed as percent of total ^{14}C added, in Table 5, showed that the difference was in the amount of soluble product produced.

Triton apparently caused the release of fluoranthene degradation products from the cell into the medium (P. Pritchard, personal communication). This corresponded to the production of a bright red color, which we believe is the recyclized hemiacetal of the

initial ring-opening product of fluoranthene. If biomass is decreased, mineralization of fluoranthene is inhibited initially but seems to recover after a period of days (Pritchard et al., 1995). Mineralization extent was less in the cases where recovery occurred, which was again due to the release of degradation products into the medium that are not fully metabolized. Exposure of the strain EPA 505 to Triton apparently results in the lysing of many cells, and those that do survive are very sensitive to being washed (L. Fredrickson and P. Pritchard, personal communication). Having survived exposure to Triton, the cells become more tolerant to the surfactant, but this acclimation was an unstable characteristic since cells quickly reverted back to high sensitivity when cultured in the absence of surfactant (L. Fredrickson and P. Pritchard, personal communication). These results are, in part, due to the limiting calcium effect. Surfactant effects on degradation and viability are clearly strain specific (Willumsen et al., 1998). Figure 8 (L. Fredrikson and P. H. Pritchard, unpublished data), shows the effect of Triton on four different fluoranthene degraders. The differences in fluoranthene mineralization were probably due to the susceptibility of the membranes to dissolution by Triton. These results suggest that surfactants interact with the cell membrane, perhaps allowing for greater transport of PAHs inside, but at the same time, rendering the cells more sensitive to environmental situations. In the case of strain EPA 505, Tween 80 worked aas well as Triton in stimulating fluoranthene degradation and it was less toxic.

Only a few studies have examined the effectiveness of surfactants in enhancing the degradation of PAH mixtures (Tiehm, 1994; Guha and Jaffe, 1998), especially where it involves co-metabolism (Boonchan et al., 2000). There is currently no framework upon which to predict surfactant effects in PAH-contaminated soils. In the presence of surfactant, Boonchan et al. (2000) found that *Stenotrophomonas maltophilia* VUN 1,0010s degraded mixtures of PAHs faster in the presence of surfactant than in the absence of surfactant, and the maximum specific degradation rate of each component from the mixture was smaller than when it was degraded alone. Similar decreases in the degradation rate of individual PAHs due to the presence of other PAHs have been found in the absence of surfactants for mixed cultures (Guha et al., 1999) and for pseudomonads (Stringfellow and Aitken, 1995a; Bouchez et al., 1995). Further work in this area is necessary to provide a greater understanding of how surfactants influence the microbial degradation of mixtures and to make the application of surfactants a standard engineering tool in biological soil remediation (Volkering et al., 1998). To this end, we investigated the degradation of mixtures of PAHs by *Sphingomonas paucimobilis* EPA 505 in the presence of the surfactant Tween 80. The degradation of phenanthrene, fluoranthene and pyrene together in a mixture in the presence of Tween 80, has shown that, despite their increased availability to the microorganisms, a succession of degradation occurs (Luning Prak and Pritchard, personal communication). The succession is probably a function of the specificity of the initial dioxygenase system in the cells, which, for strain EPA 505, was a preference of phenanthrene over fluoranthene and fluoranthene over pyrene (Luning Prak and Pritchard, personal communication).

b. Biosurfactants. Microbes produce surface-active agents,biosurfactants, when grown on insoluble or immiscible compounds (Desai and Banat, 1997; Lin, 1996; Neu, 1996). The

use of biosurfactants has been proposed as an alternative to chemical surfactants to enhance the bioavailability of hydrophobic contaminants (Hunt et al., 1994; Oberbremer et al., 1990). Biosurfactants are a structurally diverse group of surface-active agents. They are amphipathic molecules consisting of a hydrophilic portion and a hydrophobic moiety, usually in the form of bound fatty acids. They exhibit low interfacial tension (IFT) and low critical micelle concentration (CMC) values.

The CMC is the amount of surfactant needed to achieve the lowest possible surface tension. Alternatively, the CMC can be defined as the surfactant concentration at which the addition of free monomer will form micellar structures. In the process of micelle formation, the aggregating surfactant molecules have the ability to surround slightly soluble compounds, which disperses them into the aqueous phase (Singer and Finnerty, 1984). Biosurfactants have about a 10- to 40-fold lower CMC than chemical surfactants (Desai and Banat, 1997). Distilled water has a surface tension of 73 dyne/cm and an effective biosurfactant can lower this value to <30 dyne/cm (Lang and Wagner, 1987). Rhamnolipids lower the surface tension of water from 72 mN m^{-1} to 25-30 mN m^{-1} at concentrations of 10-200 mg l^{-1} (Lang and Wullbrandt, 1999).

Biosurfactants can be divided into groups based on their overall structure: glycolipids, lipopeptides and high molecular weight biopolymers. Glycolipids contain various sugar moieties (i.e., rhamnose, sophorose, trehalose) attached to long chain fatty acids. Lipopeptides consist of a short polypeptide (3-12 amino acids) attached to a lipid moiety. High molecular weight biopolymers are lipoprotein, lipopolysaccharide-protein complexes and polysaccharide-protein-fatty acid complexes. Biosurfactants are produced by a variety of bacteria, including *Pseudomonas* sp., *Bacillus* sp., and *Mycobacterium* (Cooper et al., 1989; Patel and Desai, 1997, Zhang and Miller, 1995). They are also produced by yeasts (Cooper and Paddock, 1984; Hommel et al., 1987) and fungi (Fautz et al., 1986).

Biosurfactants have several advantages over synthetic surfactants such as a lower toxicity for most biosurfactants (Lang and Wagner, 1993), higher biodegradability (Desai and Banat, 1997), more "environmentally friendly" (Georgiou et al., 1990) and good specific activity at pH, temperature and salinity extremes (Kretschmer et al., 1982). Rhamnolipid biosurfactant biodegradability was demonstrated in the Organization for Economic Cooperation and Development (OECD) 301D "Ready Biodegradability" Test and it showed no toxicity to activated sludge in the OECD 209 Test (Maslin and Maier, 2000).

Hydrocarbon biodegradation by naturally occurring microbial populations is the primary mechanism for hydrocarbon contaminant removal from the environment. The addition of biosurfactant stimulated indigenous bacteria to degrade hydrocarbons at higher rates than those achieved by nutrient addition alone (Atlas, 1993). Contradictory results are found in the literature concerning the effects of

biosurfactants on PAH biodegradation (Banat, 1995; Churchill et al., 1995; Deschenes et al., 1996; Finnerty, 1994; Guha et al., 1998; Miller, 1995; Providenti et al., 1995; Rocha and Infante, 1997; Volkering et al., 1998; Zhang and Miller, 1995). Many reports indicate that surfactants can enhance hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrates (Zhang and Miller, 1994; Zhang et al., 1997). The bioavailability of organic contaminants in soil decreases significantly during aging (Hatzinger and Alexander, 1995), therefore, the effect of biosurfactants on aged pollutants may be particularly significant and make them potentially useful for bioremediation.

c. *Solubilization of PAHs.* Rhamnolipid (monorhamnolipid and dirhamnolipid), lichenysin B and Triton X-100 have CMCs of 40, 10 and 106 mg/L (Edwards et al., 1991; Lin et al., 1994; Zhang and Miller, 1992). Mass solubility ratios (MSR) for several PAHS are shown in Table 6 for *P. aeruginosa* strain 64 biosurfactant (rhamnolipid) and Triton X-100 (nonionic synthetic surfactant). The MSR of phenanthrene for *B. licheniformis* ATCC 39307 biosurfactant (lichenysen, a cyclic lipopeptide) is also shown in Table 6. In general, the MSR declines with increasing molecular weight of the PAH. Both rhamnolipid and lichenysin solubilized more phenanthrene than Triton X-100. Rhamnolipid solubilized more pyrene and fluoanthene than Triton X-100. The MSRs for benzo(a)pyrene and chrysene could not be calculated for Triton X-100 because the solubilization of these compounds was too low for detection even at the highest surfactant concentration tested (30X CMC).

The solubilization of phenanthrene, fluoranthene, pyrene, chrysene and benzo(a)pyrene by rhamnolipid and Triton X-100 is shown in Figure 9. The difference in effectiveness between rhamnolipid and Triton X-100 for phenanthrene became less obvious when the other PAHs were examined. The ability of each surfactant to solubilize each PAH is directly correlated with the size of the PAH. The 4-and 5-ring PAHs were poorly solubilized with either surfactant.

2 Objectives

Landfarming of hydrocarbon-contaminated soils may provide a cost-effective alternative to other methods of contaminant removal. However, the HMW PAHs have been found resistant to biodegradation in these systems. Sites that contain high levels of these compounds are especially challenging. Data presented in this report indicate that by carefully augmenting the metabolic capabilities of the existing soil bacterial communities, normally insoluble compounds can be rendered more available to these communities for degradation. These initial steps can lead to a cost-effective means for removing these dangerous chemicals from the environment.

Our objective was to demonstrate improved bioremediation of the more toxic, higher molecular weight PAHs through landfarming with bioaugmentation and biostimulation. The use of known surfactant-producing bacteria should increase the availability of PAHs in the soil to the degrading bacteria. The use of biostimulation with bulking agents and slow-release nitrogen will sustain microbial growth.

Our hypothesis was that the gradual release of PAHs from soil particles, as accomplished by a biosurfactant, would promote the degradation of higher molecular weight PAHs. In addition, we proposed that the process would be assisted by transfer of the bacteria in a medium that would serve as a refuge for the bacteria, protecting them from predation.

3 Technical Approach

The approach used in this multi-year study was, first, to validate the choice of technology at bench scale through a microcosm study using contaminated site soil. Preliminary flask studies were carried out in order to determine if our approach would lead to consistently greater reductions of the high molecular weight PAHs in soil. We conducted microcosm experiments using bioaugmentation (cells on a vermiculite carrier) and bioavailability-enhancing approaches. Additional parameters necessary for follow-on pilot projects were established (ie., nutrient supplementation and appropriate bulking agent). The bench-scale project was followed by two pilot-scale studies simulating field conditions. These projects validated the flask results and examined the engineering parameters necessary to take this project to the field.

Site Description

The site soil for the flask and field studies was obtained from the POPILE, Inc. Superfund site, a former wood-treatment facility located in El Dorado, Arkansas. This soil was currently in use in a landfarming treatment pilot study at the ERDC Environmental Laboratory in Vicksburg, MS. It was selected for use because it was available, fully characterized, a large amount of background landfarming data was available, and a complementary project was on-going.

Wood treatment operations ended at the El Dorado site in July, 1982 and the site was purchased by POPILE, Inc. In 1984, POPILE consolidated three impoundment ponds into one. This closure activity was administered by the Arkansas Department of Pollution Control and Ecology. In 1988 and 1989, an Environmental Protection Agency (EPA) field investigation revealed contaminated soils, sludges and groundwater at the site. The primary contaminants found at the site include pentachlorophenol (PCP) and creosote compounds associated with wood treatment, including PAHs. The EPA determined that an emergency removal action was necessary. This was conducted from September 1990 to August 1991. The emergency action consisted of modifying the site drainage, placing and seeding topsoil, and solidifying and placing sludges into an on-site, soil-holding cell.

The EPA's design contractor, Camp, Dresser and McKee/Federal Programs, was tasked with the development of the Remedial Investigation/Feasibility Study for the POPILE site. The remedy that was approved involved the excavation and treatment of approximately 165,000 cubic yards of contaminated soils and sludges in onsite land treatment units (LTUs). Indigenous microorganisms were expected to break down the target contaminants to less harmful and less mobile constituents.

Two types of impacted soils exist at the site. The first is the soil from a holding cell that has been stabilized with rice hulls and fly ash (pH approximately 10) as part of earlier emergency remedial activities. The second is soil from a process area contaminated by spills, leaks and open air-drying during wood treatment activities. The current evaluation was conducted on contaminated material only from the process area. The process area soils had unusually high concentrations of PAHs (13,000 mg/kg, avg.) and PCP (1500 mg/kg, avg.). Even with the high contaminant levels, the soil maintained microbiological activity.

Soil Characterization

The metal and mineral content and physical characterization of the soil from the POPILE site, El Dorado, AK, was determined prior to the initiation of the first pilot study. A complete presentation of the soil characterization is reported in Hansen et al. (1999). Pertinent portions are summarized in Table 7 to supply an overview of the study conditions.

The initial chemical characterization included total Kjeldahl nitrogen as well as nitrate, nitrite and ammonia, total organic carbon, and total phosphate. Chemical and physical properties examined included pH, soil buffer capacity, cation exchange capacity and field moisture capacity.

The field moisture capacity (FMC), as defined by the USDA – Natural Resources Conservation Service, is the moisture content of the soil, expressed as a percentage of the oven dry weight, after the gravitational, or free, water has drained away. More simply, this is the moisture content 2 to 3 days after a soaking rain. It is also known as the normal field capacity, the normal moisture capacity, or the capillary capacity. The original POPILE soil delivered to the pilot facility had an FMC of 23%. Landfarming protocols (U.S. Army Corps of Engineers, 1996) state that the moisture content be maintained between 30 and 90% of the FMC in order to sustain microbial growth. For POPILE soil, this correlates to 6.9 to 20.7% moisture.

Soil pH affects the contaminant chemistry and interactions with the soil particles. The initial pH of POPILE soil was 9, but this decreased rapidly with treatment and was maintained throughout the project at an average 7.7.

Flask (Bench-scale) Experimental Design

Building on research begun with composting systems and bioslurries, we isolated and characterized from various PAH-contaminated site soils, bacteria that could produce biosurfactant and/or degrade PAHs, directly or co-metabolically. Biosurfactant production by our chosen microorganism, *Pseudomonas aeruginosa* strain 64, was examined for toxicity to PAH-degrading bacteria and its effectiveness in increasing the extractability of PAHs in water. A relatively low-cost, solid, carrier system (vermiculite) was developed for delivery and maintenance of the bacteria in the contaminated soil environment. The effect on PAH removal of various nutrient sources and bulking agents was evaluated. An 11-month microcosm study

was conducted using contaminated site soil to evaluate PAH removal under biostimulation and bioaugmentation conditions.

Flask (Bench-scale) Materials

Bacteria

Many soils contaminated with different toxicants (i.e., hydrocarbons, TNT, PAHs, PCBs etc.), were screened for biosurfactant and bioemulsifier producers. *Pseudomonas aeruginosa* strain 64 was originally isolated in enrichment cultures on TNT by Dr. Doug Gunnison of the USACE-ERDC in Vicksburg, Mississippi. *P. aeruginosa* strain 64 was notable in its production of both surfactant and a biomarker pigment, pyocyanin. This microorganism was also found to be able to survive and grow in PAH-contaminated soils. *P. aeruginosa* strain 64 was grown in Luria-Bertani broth (Bacto) supplemented with 0.3% (v/v) glycerol at room temperature. The glycerol is an energy source and promotes pyocyanin pigment (blue-green color) production. Broth cultures were shaken at 150 rpm on gyratory shaker at room temperature.

Amendments

The ground rice hulls used as the bulking agent were purchased from Rice Hull Specialty Products, Inc. (Stuttgart, AK). The rice hulls were purchased with no oil treatment. They have a seive range of 30/80, which corresponds to a particle size of 180 – 600 μm, and a bulk density of 19-24 lb/ft^3. Nitrogen, in the form of pellitized, dried blood fertilizer (Nitrogen-phosphate-potassium, 12-0-0) was purchased from Dragon Corporation (Roanoke, VA). The nitrogen pellets have a bulk density of 8-10 lb/ft^3. The carrier for microorganisms was a vermiculite, AF2 (medium), obtained from Strong-Lite Products, Corp. (Pine Bluffs, AK).

Flask (Bench-scale) Methods

Isolation and characterization of PAH-degrading bacteria

Isolates that were able to grow on fluoranthene were obtained by incubating samples of PAH-contaminated soil in dilute complex medium for 24 hours and then plating dilutions on agar containing low concentrations of complex medium. Once colonies formed, the plates were sprayed with fluoranthene to create an opaque film of the PAH over the surface of the agar (Kiyohara et al., 1982). Colonies that showed clearing around the colony were picked and further characterized for their ability to degrade other PAH, as determined by clearing around colonies when sprayed with the appropriate PAH. Where clearing was observed, individual isolates were tested for growth on the PAH. None of the isolates grew on the HMW PAHs (pyrene and above) and so clearing around the colonies suggested that co-metabolism was occurring. Most of these isolates were also able to degrade low molecular weight PAHs.

A variety of PAH degraders that can utilize fluoranthene or pyrene as growth substrates have been studied using a modified enrichment procedure and a plate screening technique (Ho et al., 2000). Many of these isolates were also able to transform other non-growth PAHs. With this enrichment technique, both fast-growing and slower-growing bacterial strains had an equal chance of being isolated since no serial transfer or dilution was done. By using a dilute complex medium, supplemental growth substrates are provided along with the PAH. This helps provide energy reserves that are required for the initiation of PAH oxidation. Most of the isolates, in fact, degraded PAHs faster when grown in the presence of the supplemental carbon sources (Ho et al., 2000). Table 8 (modified from Ho et al., 2000) compares the co-metabolic capability of 30 different fluoranthene degraders randomly isolated from 15 different contaminated soils. Phospholipid fatty acid (PLFA) analysis of the microorganisms from these soils showed that *Sphingomonas* and *Mycobacterium* species were well represented among the fluoranthene (FLA) and pyrene (PYR) degraders.

Because PAH-degrading microorganisms most likely associate with hydrophobic mixtures of PAHs and hydrocarbons in the environment, a somewhat different method of isolating bacteria was tested (Bastiaens et al., 2000). Hydrophobic membranes containing sorbed PAHs were incubated in PAH-contaminated soil suspensions for 3-4 weeks and then the membranes were washed and incubated on nutrient agar plates until colonies appeared. Only bacteria with hydrophobic cell surfaces would stick to the membranes. Most of the isolates were *Mycobacterium* species. Samples of the same soils incubated with PAH crystals in suspension (no membrane) produced mainly less hydrophobic *Sphingomonas* species. However, very few of the *Mycobacterium* isolates were able to grow on, or co-metabolize, fluoranthene or pyrene. Most were phenanthrene degraders. Thus, PAH metabolic diversity appears to be quite variable. Hydrophobic bacterial cell surfaces are, apparently, not a prerequisite for metabolic diversity.

A modified enrichment procedure and plate screening technique used in this study to isolate PAH degraders from contaminated soils and sediments was the method employed by Kiyohara et al. (1982). However, we used PYGT medium to provide supplemental growth substrates along with the PAH to provide energy reserves for PAH oxidation (Ho et al., 2000). It readily provided bacterial strains that could not only utilize FLA or PYR as growth substrates, but many were also able to transform other non-growth PAHs. As stated earlier, one of the advantages of this method is that both fast and slow growing bacterial strains had an equal chance of being isolated since no serial transfer or dilution was performed. This spray plate method is generally used with mineral salts medium and the PAH as the only carbon and energy source. Most of our isolates, in fact, degraded PAHs faster when grown on PYGT, suggesting that PAH degradation capability was not affected by alternative growth substrates. Several of the FLA degraders were unable to grow, or grew very poorly, in liquid culture with FLA, although they did consistently produce clearing zones with FLA on PYGT plates. Since five of these isolates were phenanthrene degraders, we can argue that our enrichment procedure was capable of detecting FLA-co-metabolizing, PHE-degrading strains. The low incidence of FLA-co-metabolizing/PHE degrader strains was not surprising given that there are no reports of PHE degraders in pure culture with the ability to co-metabolize FLA. *Sphingomonas* and *Mycobacterium* species are well represented among the FLA degraders.

Only two or three isolates were able to grow on or co-metabolize PAHs as well as EPA 505. Most strains were unable to co-metabolize pyrene, chrysene, benzo(b)anthracene, and benzo(a)pyrene in the absence of phenanthrene (a growth substrate for all isolates) although a somewhat greater number were able to do so in the presence of phenanthrene. These results show that good co-metabolic capability in indigeneous microbial communities is unlikely in most PAH-contaminated soils. In a landfarming context, it is doubtful that HMW PAHs will be effectively reduced to regulatory significant levels by this method. The value of bioaugmentation to landfarming of PAH-contaminated soil becomes apparent as a method of extending the initial rapid phase of removal and increasing the likelihood of attaining regulatory closure levels.

Biosurfactant production

Surfactant production by indigeneous bacteria. The supernatant of a mixed culture grown from PAH- and PCP-contaminated site soil showed no significant decrease in surface tension compared to un-inoculated media. By contrast, supernatant from a culture prepared from soil that received an inoculum of *P. aeruginosa* strain 64 showed significant reduction in surface tension. This suggests that although many species of soil bacteria are capable of producing surface-active agents, the current autochthonous community did not express this ability under these conditions. Since surfactants can increase the aqueous solubility of many target contaminants, augmenting the soils with bacteria, which can produce surfactants, is warranted.

Surfactant production by added bacteria *(P. aeruginosa* strain 64). When rhamnolipid is used in short-term extractions of phenanthrene or fluoranthene from sand versus sand with illite clay, desorption levels are different. However, there was little difference in the amount desorbed from the two substrates after 24 h. Rhamnolipid biosurfactant was much more effective in extracting phenanthrene and fluoranthene than lichenysin biosurfactant. Therefore, *P. aeruginosa* strain 64 rhamnolipid biosurfactant was selected for testing on PAH-contaminated soils.

To evaluate the potential of the *P. aeruginosa* strain 64 biosurfactant for desorption of PAHs, a preliminary test was performed using three contaminated soils, Southern Maryland Wood Treatment (SMWT) site, soil directly from the POPILE Wood Treatment Site in Arkansas, and POPILE soil stored at the U.S. Army Waterways Experiment Station in Vicksburg, MS (Table 9). *P. aeruginosa* strain 64 was grown in broth containing glycerol (an energy source that also helps promote pyocyanin pigment production). After removing cells, the supernatant at different concentrations was used to extract the PAH-contaminated soil.

Bioaugmentation

Past success of bioaugmentation at field scale for enhanced bioremediation has been mixed, but there are examples of its effective use (Vogel, 1996). Thibault et al. (1996) showed enhanced degradation of pyrene in soils following inoculation with pyrene-degrading *Pseudomonas* species, but the use of the surfactant Witconol SN70 inhibited the introduced organism. The introduction of *Mycobacterium* strain PYR-1, a pyrene degrader, enhanced the removal of pyrene from contaminated soil. Eriksson et al. (2000) had some success in the degradation of PAHs

following inoculation with a mixed culture enriched for PAH degradation capability. They attributed some of the effect to the possible production of biosurfactants by the inoculum. Interestingly, they found that certain degradation products, specifically 4-hydroxy-9H-fluorenone, increased substantially during the biological treatment. This metabolite could be a degradation product of fluorene or fluoranthene. Kastner et al. (1998) showed that inoculation with a fluoranthene-degrading *Sphingomonas* strain and a pyrene-degrading *Gordona* strain resulted in no enhanced degradation of these PAHs, yet the strains survived well in the contaminated soil. Weir et al. (1995) showed similar results with inoculation of a phenanthrene-degrading *Pseudomonas* strain that was encapsulated.

To treat hydrocarbon contamination, it is often fruitless to compete with the degradative capabilities of the indigenous microbial communities, especially where mixing, oxygenation and nitrogen supply have been optimized. Large biomasses are required and, although initial degradation rates may be enhanced, the time saving effect may not balance the cost of adding the organisms. In addition, a rapid die-off of introduced organisms invariably occurs in the field and therefore, one should not expect to impact degradation processes weeks after treatment initiation. Reinoculation is possible, but the cost benefit ratio is likely to be unreasonable. Bioaugmentation works best if nature is supplemented as an event, rather that trying to replace nature with a long-term management approach. That is, it is best to use bioaugmentation as a boost to something that nature is slow to generate or unable to produce consistently.

Our goals for bioaugmentation in the landfarming treatment of PAH-contaminated soils were:

- use an inoculation process that is designed to supplement and accelerate the activities of natural microbial communities rather than replace or ignore them,
- increase the metabolic capability to promote co-metabolism of HMW PAHs,
- enhance bioavailability using biosurfactants introduced via bioaugmentation,
- use vermiculite as an inexpensive carrier for the microorganisms added into the soil.

Carrier technology development

Producing large quantities of biosurfactant to apply during a bioremediation process is currently cost prohibitive (Lang and Wullbrandt, 1999) and logistically complex. Alternatively, it is possible to add a microbe that produces a biosurfactant, particularly if the microbe will grow on one of the contaminants or degradation metabolites in the soil to be treated (Arino et al., 1998). The effect of biosurfactants present at concentrations below their CMC on the degradation of PAHs has received little attention. Different hydrocarbon-degrading bacterial strains that produce biosurfactants were examined. Our attention has focused on *Pseudomonas aeruginosa* strain 64, which produces rhamnolipid biosurfactant on water-soluble and water-immiscible growth substrates. In this study, biosurfactant application involved whole-cell cultures because of economic considerations for biosurfactant addition.

It is known that introducing organisms into contaminated soil is mechanically difficult and often results in poor survival if the organisms are added from liquid culture (O'Reilly and Crawford, 1989). If bioaugmentation protocols are to be successful, it is critical to establish a protocol for

the inclusion of augmenting bacteria into established soil communities in a way that maintains their viability. One approach for improving the effectiveness of bioaugmentation is to introduce the inoculum on an inert carrier. Immobilization or encapsulation has been shown to enhance survival and activity of the inoculum in bioremediation scenarios (Lin and Wang, 1991; Middledrop et al., 1990; Oh et al., 2000; O'Reilly and Crawford, 1989; Rhee et al., 1996; Weir et al., 1995). These studies used a variety of carriers including alginate, polyurethane foam and inert granular formulations. We have had considerable success using vermiculite, an inorganic material that is environmentally safe. Since dry vermiculite adsorbs several times its weight in liquid, it is possible to add complex microbial growth medium to sterilized vermiculite and have it soak into the interstitial spaces. Inoculation with organisms then allows the cells to grow "into" the vermiculite where they are shielded from predation and direct competition with indigenous organisms in the soil and where they can slowly grow and "shed" cells into the surrounding soil. This prevents the introduced bacteria from being out-competed initially and increases the survival rate. Previous studies in the laboratory have demonstrated that vermiculite carried *S. paucimobilis* strain EPA505 cells remained viable in soils for months after inoculation (Pritchard et al., 1995; Pritchard et al., 1999). In addition, studies with EPA 505 have shown that its ability to mineralize fluoranthene in soil is not effected by immobilization on vermiculite (Lin et al., 1995; Pritchard et al., 1995).

Benefits of this procedure include:

- increased shelf life of microbial cultures so that they are available when needed,
- increased microbial survival in the soil,
- increased aeration of the soil by the inert carrier material, and
- a possible means for slowly releasing added nutrients to the soil system.

Thus, the vermiculite appears to be an excellent biocarrier, assuring some degree of staying power for the inoculum.

To determine the effectiveness of bioaugmentation with vermiculite-carried *P. aeruginosa* strain 64 and strain EPA505, PAH-contaminated soil was spiked with 2% (by weight) vermiculite treated in two different ways. In one case, the vermiculite was spiked with Luria-Bertani (LB) broth, a common growth medium for bacteria (control). In the second case, vermiculite was treated with LB broth and a mixture of *P. aeruginosa* strain 64 and strain EPA 505 (approximately $1x10^6$ cells/ml for each strain). Total bacterial numbers present in the soil over time were compared.

The ability of the vermiculite-carried bacteria to survive in the highly contaminated soil from the POPILE site was validated using microcosms incubated at room temperature for 110 days.

Biostimulation

Biostimulation was tested using a variety of carbon and nitrogen sources. These are listed in Table 10. Commercially available dried blood fertilizer performed well, is readily available, and cost-effective. The dried blood is a pelleted, slow-release, nitrogen fertilizer, normally used in garden applications.

In microcosm tests, contaminated soils were amended with 2% dried blood fertilizer and 2% vermiculite containing approximately 10^{10} *P. aeruginosa* strain 64 cells / gram vermiculite. The presence of dried blood provided an excellent source of degradable carbon and nitrogen for *P. aeruginosa* strain 64, which resulted in production of rhamnolipid biosurfactant in liquid cultures. Soil incubated with *P. aeruginosa* strain 64 and dried blood for 5-15 days produced a similar surfactant production. To determine the optimal concentration of dried blood to add to the soil, varying concentrations of dried blood were added to SMWT soil inoculated with *P. aeruginosa* strain 64. These were plated on LB agar with 0.3% glycerol to promote pyocyanin pigment production from the *P. aeruginosa*.

The flask studies indicated that cultures immobilized on vermiculite:

- maintain a high degree of viability when stored at room temperature.
- survive well in the presence of the chemical contaminants (i.e., PAHs and PCP) when introduced into soil.

Trials were next done to determine the viability of vermiculite-immobilized *P. aeruginosa* strain 64 in the soils with intact autochthonous communities, and comparing survival of immobilized cultures to that of cultures added directly to the soil.

Microcosm preparation

Microcosms were established in triplicate in sterile plastic specimen cups with tight-fitting, snap lids. PAH-contaminated soil (100 g) from the POPILE site was added to each cup. The distribution of amendments to the contaminated soil is shown in Table 11. The soil was mixed with an equal volume of ground rice hulls as a bulking agent. The dried blood was added at a rate of 2% of the wet weight of the soil. Vermiculite inoculated with *P. aeruginosa* strain 64 or with *S. paucimobilis* strain EPA 505 was added at 2% by weight of soil. Bacteria in broth were added to 2 g of vermiculite and allowed to soak for approximately 20 minutes. Deionized water was added to all microcosms as needed to maintain a water content of 30-40% by weight, and cup contents were stirred thoroughly each week to simulate tilling of land treatment units.

Microbial analysis

The microcosms were sampled by adding 1 g soil to 10 ml saline (0.75% NaCl). The tubes were shaken vigorously. Appropriate dilutions were made and aliquots were plated on LB agar with 0.3% glycerol. The plates were incubated 24-72 h at 30°C and colony-forming units counted.

Chemical analysis

PAHs were extracted from soil microcosm samples by mixing 10 g soil with 5 g diatomaceous earth, which had been baked at 450°C for 4 h to remove moisture. Diatomaceous earth was used to be sure the soil sample was completely dry before solvent extraction, per manufacturers recommendations. This mixture was allowed to dry on the bench overnight, packed into an extraction cell and extracted with a methylene chloride:acetone (50:50) mixture in an ASE 200 (Dionex) solvent extractor at 1500 psi and 100°C. The volume of these extracts, usually about 45

ml, was reduced to approximately 5 ml under a stream of dry nitrogen. The extracts were then processed through a Supelclean LC-Si (Supleco) clean-up column. The extract volumes were then reduced to 1.0 ml under dry nitrogen and the extracts were sealed in crimp-top amber vials. Some soil extracts contained so much material that a final volume of 2 ml was used. For all POPILE soil samples, two dilutions (1:50 and 1:200) of each sample were analyzed to keep the measured PAH concentrations within the range of the standard curve. The internal standard, P-terphenyl d14, was added to each sample vial to give a final concentration of 50-ng/µl.

All GC-MS analyses were performed on a Hewlett Packard 6890 gas chromatograph with a 5973 mass selective (MS) detector or a flame ionization detector (FID). The column used was a DB 5 (30 m long) column. Splitless injector temperature was 275°C. The column temperature was ramped from 80 to 280°C at a rate of 8° per minute, followed by a rise to 300°C at 4° per minute and a plateau at 300° for 5 minutes. This gave a run time of about 37 minutes per sample. Standard curves were prepared by injecting a mixture of 16 PAHs (Fisher Scientific PAH standards) at 10, 20, 40, 80, and 120-ng/µl. The mixture contained naphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz [a] anthracene, chrysene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [a] pyrene, indeno [1,2,3 – cd] pyrene, dibenz [a,h] anthracene, and benzo [ghi] perylene. Standard curves were checked for linearity over the range listed above.

Pilot Studies - Experimental Design

Two pilot-scale studies on landfarming bioremediation of PAHs were conducted concurrently. These studies are compared in Table 12. One study placed the contaminated soil in simulated land treatment units (LTUs). The objective of the first six months of the LTU study was to simulate landfarming of PAH-contaminated soil with and without cultivation (Phase1). These LTUs were exposed to the natural variety of climate conditions and required extensive maintenance to adjust soil moisture to suggested optimal landfarming conditions. The following 24-month LTU study eliminated maintenance and decreased the cultivation frequency. While this study required 24-months, it wasn't personnel intensive. The advantage gained was the extensive degradation database and the comparison of natural attenuation to minimal landfarming treatments. The second pilot-scale project, the trough study, used the same highly contaminated POPILE site soil as the LTUs. The troughs were operated in the open, under cover, where they were exposed to changes in temperature but didn't receive direct sunlight or precipitation. The trough study was designed to study changes in PAH degradation patterns between bioaugmentation and biostimulation. The two pilot-scale projects allowed comparison between six field treatment variations using the same soil. Table 12 also describes the amendments used for biostimulation and bioaugmentation.

LTU objectives

The LTU study was designed in two phases, to evaluate several cultivation and management strategies for the landfarming of PAHs. Cultivation was accomplished using a rear-tine rotary cultivator. In the initial phase, LTU 1 was cultivated at startup, to homogenize the soil, and on

day 71, to observe the effect of tilling on microbial respiration. LTU 1 represented remediation by natural attenuation. LTU 2, representing landfarming with tilling, was cultivated at 2-week intervals. The soil moisture in both LTUs was maintained at 50-80% of the soil field moisture capacity (FMC). Nitrogen was added to the LTUs twice. Liquid and solid forms of fertilizer were used to establish both the optimal delivery method and the effect of nutrient addition on microbial respiration. Nutrients were added prior to tilling, in conjunction with moisture addition. Soil samples were taken every two weeks. The parameters included in bimonthly soil analysis were contaminant concentration, nutrient concentration (total Kjeldahl nitrogen (TKN), total phosphate (TP)), total organic carbon (TOC), pH, and moisture content. Microbial biomass was evaluated initially and at various times during the study. Microbial respiration, measured by soil gas analysis, was monitored twice each week to follow changes in the oxygen and carbon dioxide concentrations in the soil.

Trough study objectives

The experiment was designed to confirm, at pilot-scale, the improved bioremediation of HMW PAHs using both biostimulation and bioaugmentation as observed with flask trials. The efficiency and effectiveness of these processes for landfarming of PAHs would be compared. Nutrients, and bulking agent and bacterial carrier were those used in the flask experiments. Bioaugmentation was performed with *P. aeruginosa* strain 64, a biosurfactant-producer known to survive in the POPILE soil.

Land Treatment Units - Assembly

The pilot-scale LTUs were built to simulate actual land treatment systems, and consisted of a bottom impermeable liner, a sand-bed leachate collection system, and hard standing walls to withstand impact from cultivation. A secondary containment cell was constructed, similar in concept to landfill liner (ASTM D-1973-91, modified) for added environmental security (Figure 10). Each completed LTU, as seen in Figure 11, was approximately 18 inches deep, 4 feet wide and 20 feet long (0.5 x 1 x 6 m). Complete construction details are provided in Hansen et al. (1999).

Trough Study - Assembly

Three galvanized steel water troughs, 10 feet long x 3 feet wide x 2 feet deep, (3 x 1 x 0.6 m) were used as troughs to contain the contaminated soil and the various amendments. The bottom of each trough was reinforced with 1/8-inch aluminum sheeting, cut to size. Three front-tine rototillers (12" depth) were used to mix and aerate the soil and the amendments. When not in use, the rototillers were mounted inside each trough in order to contain the contaminated soil and reduce the possibility of bacterial cross-contamination. Construction details are provided in Nestler et al. (2001a). The assembly of the trough pilot units is illustrated in Figure 12. Panel A shows the color and

consistency of the contaminated soil added to each trough. In Panel B, Rice hulls (light color) and dried blood (darker color) were added to Troughs 2 and 3 and mixed into the soil by rototiller. Gas probes were inserted into all troughs to monitor microbial respiration (Panel C).

The test soil was placed in Troughs 2 and 3 to a depth of 6 inches, and treated with amendments as designated in the experimental design, yielding a final depth of 12 in. These troughs were managed in a manner to simulate landfarming applications. Trough 1, which received no amendments, was filled with 12 in of contaminated soil. The rice hulls used in Troughs 2 and 3 were applied at 1:1 volume with the soil. The changes in the soil texture following addition of the rice hulls can be seen in Figure 13.

The nitrogen was applied at a rate of 2% of the soil (w:w). Soil and amendments were thoroughly mixed, the moisture level was checked, and brought up to 80% of the FMC. The soil was mixed again to distribute the added water. The vermiculite-culture was added to Trough 3, also at 2% w/w concentration with the soil, and again thoroughly mixed. The soil was sampled (t=0), raked to fill the sampling holes, and the drywells for microbial respiration inserted. Initial soil respiration samples were obtained following placement in the troughs.

Pilot-Scale Materials

Trough amendments

The amendments used in the trough study , the ground rice hulls, dried blood, and vermiculite, were obtained from the same suppliers to the same specifications as those used in the flask study. Bioaugmentation consisted of *P. aeruginosa* strain 64 on vermiculite. Cell density at the time of application to the vermiculite was 10^6 cells/ml.

Soil gas probes

Microorganisms in the soil consume soil pore oxygen and release carbon dioxide (CO_2) as they grow. This process, respiration, is a segment of the short-term organic carbon cycle in which carbon is converted to CO_2 (mineralization). Several ecosystem-level effects can be seen when a soil microbial community is stressed by the presence of a toxin. First, the total community respiration level decreases as the metabolic activity decreases. Then, the respiration to biomass ratio increases. As exposure time to the toxin increases, the nutrient turnover and the rate of organism turnover increases. Microbial community succession demonstrates a decline in species diversity and an overall decrease in growth and respiration with each succession. The most sensitive biomarkers of both soil degradation and repair are those associated with microbial metabolism, such as respiration, biomass, enzyme activity, and/or nitrogen mineralization. While CO_2 production is not a completely accurate assessment of biodegradation of the contaminant, respiration remains the most widely used indirect method to estimate biodegradation in contaminated soils (Li, 1998; Zibilske, 1994).

Drywells for soil respiration analysis were designed at ERDC (Figure 14) and built from parts supplied by Plastic Supply, Inc. (Brandon, MS). The probes were constructed from a 6-inch upper ring and cap of PVC superimposed on a 12-inch vertical dry well made of standard 2-inch slotted PVC. The cap was equipped with a 3-way plastic stopcock purchased from Cole-Parmer®. Tubing attached to a portable, infrared gas analyzer has a fitting for the stopcock valve. The pump of the gas analyzer withdraws and analyzes an air sample. The probe remains in the ground between sample events. Construction details are supplied in Nestler et al. (2001b).

Gas analysis was performed using an LMSx Multigas Analyzer® equipped with an infrared detector and oxygen cell (Columbus Instruments, Columbus, OH). Oxygen, carbon dioxide and methane concentrations in the soil were monitored. This unit operates effectively across a temperature range of –10 to 40° C. The CO_2 detection range is from 0 to 40% with an accuracy of ± 0.1%. The O_2 detection range is 0 to 25% with an accuracy of ±0.5%. The aspiration rate is 100 ml/min. The response time is 20 sec. and 30 sec. for CO_2 and O_2, respectively.

Pilot-Scale Methods

Sampling design

Each LTU was subdivided into 20 equal sections (Figure 15). A sampling grid sized to fit the sections was constructed from Plexiglas and drilled with 36 equidistant holes for the soil corer. The grid for LTU 1 was designed to fit around the soil gas probes so they didn't have to be removed. At each sampling interval, five randomly-selected cores were collected from each of the 20 sections. The five soil cores for each single grid were combined in a 950 cc amber jar and manually homogenized into a single sample. A random number generating computer program selected seven of these 20 grids for analysis. The remaining 13 samples were archived at 4°C in their original collection jar. The sampling holes in LTU 1 were filled by raking lightly across the soil surface. Water was added, if necessary. LTU 2 was tilled, and the gas probes were re-inserted. The random sampling process developed during the pilot study was instrumental in decreasing the error inherent in soil contaminant analysis due to contaminant heterogeneity.

Soil samples were obtained from the troughs in a similar manner to the LTUs. Replicate samples were obtained from each of the five sampling zones as a composite of five randomly- selected sub-samples. The sub-samples were collected as cores, combined in a 950cc, amber glass sample jar, and manually homogenized. The grid and soil corer used to obtain samples are shown in Figure 16. Samples were collected from the troughs weekly following gas analysis. Chemical and microbial analysis was performed as scheduled, and the remainder of each sample was archived at 4° C. The troughs were tilled after each sampling event by a rototiller with a maximum depth of 12 in. When water was added to the troughs, it was added before tilling.

Physical analysis

Atterberg limit analysis and particle size distribution (PSD) were used to evaluate the physical structure of both the untreated and treated soils. The Atterberg limit test was performed by the

Geotechnical Laboratory at the WES-USAERDC (Corps of Engineers Laboratory Testing Manual, EM-1110-2-1906, Appendix 3, III: Liquid and Plastic Limits). Particle size distribution was measured on a Coulter LS100Q particle counter according to instrument protocol. Soil moisture was analyzed on a Denver Instrument IR-100 moisture analyzer and validated by oven-drying at 105°C for 24 h.

Chemical analysis

Contaminant concentration, metals, nitrogen, phophate and total organic carbon analyses were performed by the Environmental Chemistry Branch of the Waterways Experiment Station on both treated and untreated soil. PAH and PCP concentrations were determined using SW846 EPA Method 8270c for GC/MS after extraction by Method 3540c. Total organic carbon samples were analyzed on a Zellweger Analytic TOC analyzer, according to instrument specifications. The nitrogen and phosphate analysis was performed using the Lachat 8000 Flow Injection Analyzer (FIA). The preparation methods were modified versions of EPA-600/4-79-020 (1983 revision), 365.1 and 351.2, respectively. Metals and total volatile solids were determined according to standard methods (SW 846). Soil pH was determined for a soil-distilled water slurry (1:1, wt/vol) using a Cole-Parmer® pH meter.

Microbial analysis

Microbial biomass and community composition of the indigeneous microbiota (and, in the case of Trough 3, the bioaugmented soil) were determined initially and then intermittently throughout the study. Two grams (wet weight) of the soil sample were subjected to a modified Bligh-Dyer organic solvent extraction to quantitatively recover bacterial membrane lipid biomarkers (ester-linked phospholipid fatty acids, PLFA) as outlined in White and Ringelberg (1998). Similarities between single PLFA profiles were evaluated by application of a hierarchical cluster analysis. Biomass was estimated from the total concentration of membrane lipids and ester-linked PLFA (Balkwill et al., 1988).

Metabolic analysis

Metabolic analysis was performed on the LTUs (Phase 1 only) and the troughs in order to follow respiration of soil microbial communities. Soil gas samples analyzed changes in O_2 and CO_2 concentrations over time and in response to various stimuli. Respiration was normally monitored twice each week in the LTUs. A soil gas probe was placed in the center of each of the 20 sampling sections of the LTU. The results from the twenty cells were averaged for each LTU to yield a single sample value. Soil sampling was performed after gas analysis. The gas probes of LTU 2 were removed to allow tilling of the soil and then re-inserted. The probes of LTU 1 remained in the ground.

Each trough was divided into five sample collection zones. A gas probe was placed in the center of each zone. At a minimum, the troughs were monitored weekly for the first 6 months and daily thereafter. Individual experiments ocaisionally required modifications of this sampling schedule. Results of the five samples were averaged for each trough to yield a single sample value. Following gas analysis, the probes were lifted, soil samples taken, the troughs tilled and the

probes reinserted. Further details of the gas analysis procedure and the soil probe are found in Nestler et al. (2001b).

Statistical analysis

The chemical analytical data were reduced to develop average sums of the concentrations of total PAH, and individual PAH compounds. BaP Toxic Equivalent values were calculated by multiplying the average concentration of the specific homologue by its TEQ factor. To calculate the magnitude of reduction and the rate of degradation of these contaminants, the initial and final concentration values were used. Zero order (concentration independent) removal rates were assumed due to the high concentrations of the contaminants. Statistical significance of all chemical and respiration data was established at a 95% confidence interval using n=7 (LTUs) or n=5 (troughs). Statistical differences between LTU 1 and 2 and between Troughs 1, 2, and 3 were determined using the two-tail critical t-test.

The microbiological data of the LTUs was subjected to a Tukey HSD to determine if there was significance to the differences between the data for the two LTUs, taking into account that more than two samples were taken (Ringelberg et al., 1989). An evaluation of similarities between single PLFA profiles was accomplished by application of a hierarchial cluster analysis (Wards Method). PLFA concentrations, expressed as a molar percentage, were arcsin transformed prior to the analysis. Correlations between PLFA and other study variables were assessed by Spearman rank order correlation statistics. Both cluster and correlation analyses were performed using the Statistica software package, v.5.0 (Statsoft Inc., Tulsa, OK)

4 Accomplishments

Flask Studies

PAH removal

Figure 17 shows the gas chromatographs of solvent extracts of POPILE soil after the 11-month microcosm study. Panel A illustrates the untreated soil. Some degradation was observed for some of the PAHs when the soil was amended with bulking agent (ground rice hulls) and dried blood fertilizer (panel B). There was a dramatic decrease for many of the PAHs in the microcosms amended with bulking agent, dried blood fertilizer and *P. aeruginosa* strain 64 on the vermiculite carrier (panel C). Although *S. paucimobilis* strain EPA 505 was previously shown able to degrade HMW PAHs in pure culture, it did not increase PAH removal in the study reported here. The high concentrations of PCP (approximately 2,300 mg/kg) did not inhibit PAH biodegradation in this soil. Table 13 shows the percent decrease relative to initial concentrations for PAHs in the POPILE microcosms. The decrease in concentration is calculated as ½ absorbance from October 1999 measurements to the 11-month measurements. Amendments with bulking agent (used in 1:1 volume of soil to bulking agent) diluted the contaminants compared to the controls (no treatment). Therefore, we measured the PAHs in the controls (no treatment), took ½ of those values and are reporting the % decrease observed. The biostimulated microcosm showed a 34% decrease in total PAHs and a 59% decrease in total BaP equivalents. The bioaugmented microcosm showed an 87% decrease in total PAHs and a 67% decrease in total BaP equivalents.

The concentrations of several PAHs in the amended soil inoculated with *P. aeruginosa* strain 64 declined significantly over the 11-month treatment period. Although various pseudomonads have been reported capable of catabolizing some low molecular weight PAHs, *P. aeruginosa* strain 64 did not degrade phenanthrene or HMW PAHs. Therefore, the enhancement of PAH degradation after the addition of this microorganism suggests that the autochthonous degrading community contains some co-metabolic activity that was stimulated by the biosurfactant-producing *P. aeruginosa* strain 64. One of the triplicates in this treatment failed to show significant degradation of the higher molecular weight PAHs, presumably due to the failure of the autochthonous population in that microcosm to develop as it did in the other microcosms.

The mechanism by which *P. aeruginosa* strain 64 is able to stimulate the autochthonous degrading community is not clear. Notable changes in the soil occurred in the bioaugmented microcosms that were not observed in the control (no amendment) or the biostimulation treatments. After a period of 3-4 weeks, the color of the PAH-contaminated soil was much

darker in microcosms that received *P. aeruginosa* strain 64, which could be the result of pyocyanin pigment production. Production of pyocyanin is coordinately regulated with rhamnolipid (biosurfactant) production in *P. aeruginosa*. When soil from the amendments inoculated with *P. aeruginosa* strain 64 was suspended in saline, fines remained suspended in the tubes for hours to days compared to controls (no amendments) where the soil settled immediately (Figure 20). Suspensions of soil amended with bulking agent and fertilizer settled in less than an hour. The data suggests that, while *P. aeruginosa* strain 64 did produce biosurfactant *in situ*, the concentration of the rhamnolipid did not reach or exceed the CMC of this biosurfactant. Therefore, the increased PAH biodegradation may not be due solely to the presence of biosurfactant micelles in the soil aqueous phase. It is possible that in micro-niches, the concentration of rhamnolipid did achieve sufficient concentration to mobilize sorbed PAHs into the aqueous phase via micelle formation.

There are other postulated mechanisms not dependant on micelle formation by which *in situ* biosurfactant production by *P. aeruginosa* strain 64 might increase the bioavailability of PAHs sorbed to soil particles. For example, rhamnolipid may form or integrate into an organic matrix sorbed onto soil particles. This matrix could either assist in the association of bacteria with soil-sorbed PAHs or partially desorb the PAHs from the particles making them bioavailable to naturally occurring PAH-degraders. Surfactants may sorb onto soil particles and form structures such as hemimicelles and admicelles even when present at concentrations well below the CMC, thus sorbed surfactants may play a role in facilitating transport of sorbed substrates (Volkering, 1998). Alternatively, the rhamnolipid could be acting on the autochthonous bacterial directly, by modifying the cell surfaces of the bacteria, rendering them more hydrophobic and thus encouraging bacteria-PAH interactions. Rhamnolipids produced by *P. aeruginosa* can modify the cell-surface hydrophobicity of other bacteria and effect biodegradation rates (enhancing for some bacteria and inhibiting for other bacteria; Zhang and Miller, 1994). Zhang and Miller (1994) also reported that the bioavailability of octadecane in the presence of rhamnolipid biosurfactant was controlled by both aqueous dispersion of octadecane and cell hydrophobicity.

Biosurfactant production

The results of the PAH desorption from two different contaminated site soils under water extraction are presented in Figure 18 and Figure 19. The biosurfactant caused increases in the amount of PAHs that could be extracted from soil with water, indicating that they would consequently be more available to degrading microorganisms. Similar results were obtained for *P. aeruginosa* strain 64 supernatant used to extract the PAH-contaminated soil from the POPILE site (Figure 19).

These increases generally correlate well with the amount of surfactant added, particularly for the lighter PAHs. Results for the higher molecular weight PAHs suggest that a threshold concentration of the surfactant may be required to increase the solubility of these compounds in the aqueous phase of the system. Soils are complex habitats and a wide variety of physical and chemical factors affect the bioavailability of hydrophobic compounds. Results from aqueous extracts of the soil indicate that the surfactant compounds produced by *P. aeruginosa* strain 64 can increase the concentration of at least some of the components of creosote in the aqueous phase of the system. Increases in aqueous concentrations were generally in direct proportion to

the amount of surfactant present. These results suggest that inclusion of *P. aeruginosa* strain 64 surfactant in the landfarm operation could dramatically increase the accessibility of PAHs to soil bacteria.

Nutrient amendment

The response of the added bacteria to the presence of the dried blood nutrients was both subjectively and objectively apparent. *P. aeruginosa* strain 64 "blooms", coloring the soil a dull greenish color (presumably because of the production of pyocyanin, which is a fluorescent pigment), producing a characteristic odor, and, in essence, emulsifying the soil (soil suspensions in water remain in suspension without mixing). This is illustrated in the photograph in Figure 20, where the left tube is the control, the middle tube is the soil tretaed with dried blood fertilizer and vermiculite; and the right hand tube is the sample treated with dried blood fertilizer and *P. aeruginosa* strain 64 on vermiculite carrier. Soil suspensions following treatment with *P. aeruginosa* strain 64 and the dried blood have reduced surface tension. To determine the effect of biosurfactant production *in situ*, we extracted subsamples of the soil with water and measured the PAHs that could be washed from the soil in the aqueous phase. Data is not shown, but the results were similar to those shown previously for *P. aeruginosa* strain 64 growing in PAH-contaminated soil (Figure 18). Washwater released 5-6 times more HMW PAHs when *P. aeruingosa* strain 64 was present in soil samples versus no *P. aeruginosa* strain 64 amendment of the soil.

The amount of PAHs released exceeded their aqueous solubility, indicating the solubilizing effect of the biosurfactant. From the experiments, the amount of biomass used in the inoculation of *P. aeruingosa* strain 64 seemed to have little effect, therefore inoculating with the 10^6 cells/g of vermiculite (added to soil at 2% by wt) appears to yield the optimal effect. Figure 21 shows the effect of different concentrations of dried blood fertilizer on bacterial growth in PAH-contaminated soil from SMWT site. Samples were plated on LB agar with 0.3% glycerol to promote pigment production. The addition of 2% dried blood fertilizer worked as well as the 4% concentration, which makes the lower concentration fertilizer addition more reasonable for field application. In addition, experiments with EPA 505 have shown that the presence of the dried blood did not affect the ability of the organisms to degrade PAHs.

Vermiculite carrier technology

The survival of *P. aeruginosa* strain 64 on the vermiculite carrier (versus vermiculite with no added bacteria) when introduced into contaminated soil is shown in Figure 22. Total counts of bacteria increased by three orders of magnitude in the soil supplemented with the microbe-vermiculite, suggesting, first, that addition of LB broth had a stimulating effect on the background microbial population and, second, that these organisms were a major part of the population. Their dominance lasted for at least 30 days. *P. aeruginosa* strain 64, which is a fluorescent pseudomonad, can be identified by the green pigment released into the agar medium from colonies. Pigment producing colonies were observed up to about day 25, further suggesting

bioaugmentation on vermiculite. Strain EPA 505 was not detected during this experiment. In conclusion, it appears that high concentrations of bacterial cells can be effectively introduced into soil when carried on vermiculite. Inoculated vermiculite can, therefore, be prepared and stored in large quantities prior to use in a landfarming remediation, a far simpler task than producing naked cells all at once just prior to inoculation into the soil.

Figure 23 shows the ability of the added bacteria on vermiculite to adapt to the POPILE soil during a 110 day microcosm inncubation. Biostimulation conditions (bulking agent, BA, and dried blood fertilizer, DB) produced an increase in viable bacterial colonies from the indigineous populations, but the greatest bacterial numbers were produced under bioaugmentation conditions (Pa64), demonstrating the ability of the added bacteria to survive in the highly contaminated soil.

LTU Pilot Project

Chemical characteristics

The initial nutrient composition of the soil is provided in Table 14. Compared to the optimal C: N: P of 100:10:1 for landfarming, and as anticipated for a highly contaminated soil, the initial nitrogen content in the soil was found to be an order of magnitude below optimum. During the first six months, a commercially available soluble fertilizer, as NH_4, was applied at approximately a 0.2% rate to increase the nitrogen concentration in the system to the target ratio (100:10:1). While the target concentration could be reached temporarily, it could not be maintained due to the high concentrations of hydrophobic organics bound to the soil matrix that impeded the soils ability to absorb moisture and the associated aqueous phase nutrients. A solid nitrogen addition (0.2% application rate), again a commercially available brand, was attempted with more success. The nitrogen concentration was only maintained, however, until the next rain event, at which time it was leached out of the soil. Clearly, a specially formulated slow-release form of nitrogen would be required to maintain nutrient levels in the soil without continual re-application. Following each nitrogen addition, an increase in CO_2 production, indicative of respiration, was observed. Phosphate was not limiting in this system and heavy metals were not present in concentrations that would inhibit microbial activity.

The initial soil pH in the LTUs was 9. Treatment resulted in a decrease to pH 7.4 at 3 months and 4.5 months in LTUs 1 and 2, respectively. The divergence in pH trends corresponded directly with a divergence in microbial community structure as demonstrated by the increase in gram-negative microorganisms. By 6 months, the pH in both LTUs had returned to 7.8. Continuing analysis of the biological community, did not indicate a convergence of the community structure. The soil pH in both LTUs averaged 7.7 for the duration of Phase 2.

PAH removal

The average total PAH concentration at the end of Phase 2, (30 months) in LTUs 1 and 2 was 3,860 and 4,102 mg/kg, respectively. The initial total PAH concentration of the POPILE soil

was 13,000 mg/kg (Figure 24). The reduction in total PAH in both LTU 1 and 2 was 69%. The error bars indicate the 95% confidence limit. The final PAH concentrations are not significantly different between LTU 1 and LTU 2 (p=0.05). Degradation in both LTUs followed an identical first-order decay curve with a kinetic coefficient of 0.04749 ppm/month ($r^2 = 0.96209$). The half-life of PAHs in the LTUs was 14.7 months.

Removal of PAHs from the LTUs during the study was not due to volatilization from tilling because no effect was seen when the cultivation frequency was changed after the first six months. The conclusion is that tilling is not a driving factor in PAH degradation. A plateau period in contaminant removal was observed in both LTUs that coincided with colder winter weather in the second year of operation. However, during the first winter, there was no decrease in the removal rate, which would seem to indicate the involvement of another variable. PAH concentration continued to decline in mid-May through mid-August of 2000. There was unusually extended high temperatures and drought during this time. High temperatures and low soil moisture also do not appear to adversely affect PAH degradation. Reduction in the concentration of the BaP toxic equivalent homologues over time is shown in Figure 25 and Figure 26 for LTU 1 and 2, respectively.

The extent of reduction of PAH homologues varied depending on the compound (i.e., number of rings and molecular weight) and the soil treatment, as shown in Table 15. The compounds acenaphthylene and benzo(g,h,i)perylene, and the BaP toxic equivalent homologues indeno(1,2,3-c,d)pyrene and dibenzo(a,h)anthracene, are not shown in Table 15 because, although they were present, the concentrations were below the machine detection limits and too low for estimation. The risk associated with the soil toxicity is based on the Toxic Equivalency Factors (TEF) for the seven most carcinogenic PAH homologues (Table 2). These compounds have been indicated by a * in Table 15. Molecular weight appears to determine the sequence of degradation (low molecular weight to high). LTU 1, which showed the greater biomass and the higher proportion of *Pseudomonas* bacteria, also showed a slight, but insignificantly, greater PAH reduction. LTU 2 appears to have achieved the concentration plateau sooner than LTU 1, suggesting a higher rate of degradation. In the final month of analysis, both LTUs had detectable concentrations of the toxic, 5-ring PAH, dibenzo(a,h)anthracene. We hypothesize that the natural biosurfactant production by indigeneous pseudomonads in the LTUs became great enough to desorb this PAH from the soil particles.

Microbial characterization

The calculations for microbial biomass are based on the assumption that 1 pmole of phospholipid fatty acid (PLFA) = 2.54×10^4 cells (White and Ringelberg, 1998). Although total microbial biomass increased in both LTUs during the initial phase (Figure 27), LTU 2 showed the greatest increase. During the second phase, LTU 2 showed a decline in cell numbers while LTU 1 remained constant. At 22 months, LTU 1 had greater biomass than LTU 2 (p=0.008). Tilling did have a significant impact on biomass, rapidly increasing the cell numbers. However, over the extended time, the control (untilled) populations also increased and maintained cell numbers effectively.

Within the microbial biomass, there was no correlation to PAH concentration for Gram-positive bacteria but there was for Gram-negative bacteria. As total PAH concentrations declined, Gram-negative bacterial percentages increased. This observation was consistent for both LTUs (r of -0.86 and -0.66 for LTU 1 and 2, respectively). The LTU microbiota showed signs of divergence, identified by hierarchical cluster analysis, from 3 months onwards. The evolution continued during Phase 2. By 22 months, the LTUs demonstrated different community structures (Figure 28). A Gram-negative community of mostly *Pseudomonas* sp. and a Gram-positive community primarily of *Bacillus* sp. predominated in both LTUs at 22 months. Several PLFA were found to differ significantly between the two LTUs. Within the Gram-negative classification, several PLFA were identified that are indicative of *Pseudomonas* sp. of bacteria. Two of the distinctive PLFA were *trans* acids which have been shown to increase in prevalence inside the bacterial membrane in response to toxic exposures (Heipieper et al., 1995). The ratio of *trans* to *cis* (product to parent) suggests an increasing bacterial response by the indigenous bacteria to the presence of the xenobiotics in the soil. As discussed previously, many of the pseudomonads are capable of producing surfactants that may desorb PAHs from the soil particles making them available for biodegradation as well as increasing their chemical extractability. An increase in the bioavailability of the toxicant would also induce an increase in the *trans/cis* ratio, suggestive of surfactant production. If PAH desorption from the soil exceeded the biodegradative capacity of the microbial population, the contaminant transformation could be slowed, or stopped, an event which could explain the plateau in PAH removal and the lower degradation rate once the process resumed.

In summary, none of the PLFA within the Gram-positive classification differed significantly between the LTUs. The Gram-positive input to the functioning of the LTUs would appear to be negligible. However, the Gram-negative input was found to be highly significant. The consistent tilling of LTU 2 appears to have stimulated not just growth, but development of a physiologically and taxonomically distinct microbial community. Both LTU 1 and LTU 2 demonstrated unique and different biological populations apparently capable of degrading PAHs. The population shift could be due to the different management strategies or to production of the surfactant itself (Colores, 2000).

Soil respiration

The respiration patterns observed in LTU 1 and 2 during the initial six months have been discussed in detail in Hansen et al. (1999, 2000). In summary, peaks of carbon dioxide production in the LTUs corresponded to valleys of oxygen consumption (Figure 29). LTU 2 showed a trend, during the final two months of Phase 1, towards an increase in carbon dioxide production and a corresponding decrease in the soil oxygen concentration. This data corresponds to microbial biomass estimates which showed greater cell numbers (per gram) in LTU 2 from 4 months on. Cultivation and nitrogen addition both appear to have a positive effect on microbial respiration.

Trough Pilot Project

Chemical characterization

The initial characterization of the trough study soil included total Kjeldahl nitrogen as well as nitrate, nitrite and ammonia, total organic carbon, and total phosphate. Chemical and physical properties examined included pH, soil buffer capacity, cation exchange capacity and field moisture capacity. The results are presented in Table 16.

PAH removal

The initial concentrations of PAH homologues in each trough are shown in Table 17 along with total PAH and total BaP TEF values. The initial total soil PAH concentration was 10,500 mg/kg for Trough 1 (control), 7300 mg/kg for Trough 2 and 7200 mg/kg for Trough 3. Trough 1 (control) had no amendments. Trough 2 (biostimulation) received bulking agent and fertilizer, while Trough 3 (bioaugmentation) received the bulking agent, fertilizer, and bacteria on vermiculite carrier.

The final total soil PAH concentration (16 months) in Troughs 1, 2 and 3 was 9000, 900, and 900 mg/kg, respectively (Figure 30). The final concentrations were not significantly different between Troughs 2 and 3, but the difference was significant between Trough 1 and the other troughs (p=0.05).

An increase apparent initially in the concentration of soil PAH lasted 5 months in Trough 1 and 4 months in Troughs 2 and 3. The increase is probably a result of the weekly tilling, however, experimental treatments could have affected the duration of this lag. Microbiological and enzymatic data from this period suggests that the microbial communities in Troughs 2 and 3, as well as increasing biomass, were undergoing extensive modifications of gene expression that would allow them to use both the newly available nitrogen source and the PAH compounds as carbon sources (Perkin et al. 2001). The degradation of the individual BaP toxic equivalent homologues in each of the three troughs is shown in Figure 31, where panel A represents the control, panel B, the biostimulation treatment, and panel C, the biostimulated and bioaugmented scenario.

Using normalized concentrations (Figure 32) to take into account the addition of the bulking material, a total PAH reduction of 12%, 86%, and 87% was observed in Troughs 1,2, and 3, respectively after 16 months of treatment. An initial rapid decrease in PAH concentration was observed in Trough 3, which achieved 50% degradation at 6 months. Trough 2 followed, achieving the 50% level at 8 months. No significant reduction was observed in Trough 1 after 16 months of treatment. The PAH degradation during months 4-16 in Troughs 2 and 3 followed a first-order decay curve. The Trough 2 kinetic coefficient was –5.08e-1 (r^2 = 1.322). The Trough 3 kinetic coefficient was 3.464e-1 (r^2 = 0.616).

The PAH reduction is shown for individual homologues over time in Table 18. Trough 3 is shown as being representative of both Trough 2 and 3 reductions. The degradation of PAHs appears to proceed in a pattern similar to that observed in the LTUs, with lower molecular weight

homologues being degraded first, followed by consecutively larger molecules. When a methyl group is attached to a structure, the degradation is delayed, as in naphthalene and 2-methylnaphthalene. These observations are supported by the work of Leblond et al. (2001) on PAH structure-activity relationships. It was also observed that degradation into the next higher ring number didn't require that the lower ring compounds be completely degraded. Work is in progress to attempt a correlation between structure and degradation.

Microbial characterization

The dried blood used in the troughs to provide a slow-release source of nitrogen was a complicating factor in phospholipid analysis, which was overcome by looking for lipopolysaccharide fatty acids unique to *P. aeruginosa* strain 64. These are 3OH10:0 and 3OH12:0. They were found in the initial samples and at month 3. The change in biomass over the course of the study is shown in Figure 33. The first month of the study saw large increases in biomass in Troughs 2 and 3, but particularily in Trough 3. This was, presumably, due to the bioaugmentation. The addition of dried blood enhanced microbial abundance by a factor of 4-15 times. However, the significant early differences had disappeared by month 3. Trough 3 then demonstrated a rapid recovery until 10 months when biomass values were again similar between Trough 2 and Trough 3.

The early increases in microbial abundance in Trough 3 were associated with a clearly defined shift in microbial community composition from that apparent in Trough 1 (control) but not Trough 2 (biostimulation). The relative abundance of PLFA detected in Troughs 2 and 3 are clearly distinct from those detected in Trough 1, regardless of the time sampled (Figure 34). In Trough 1, Gram-positive PLFA biomarkers average between 15-25% throughout the 41-week time trial. In Troughs 2 and 3, the relative abundance never exceeded 9%, and averaged 6%.

The low relative abundance of Gram-positive bacterial biomarkers in Troughs 2 and 3 was offset by increases in the relative abundances of Gram-negative bacterial biomarkers. The PLFA pattern detected in both of these troughs strongly suggests the enhancement of a Gram-negative pseudomonad community.

Metabolic analysis - Trough soil respiration

The O_2-CO_2 decrease/increase pattern seen in the LTUs was also evident in the troughs (Figure 35). Trough 2 is shown, being representative of the respiration response in both Troughs 2 and 3. The respiration response of the control soil to tilling (Trough 1) was minimal to none. Respiration and removal data from the first 10 months showed good correlation with the viable biomass data from the three troughs. Respiration continued in the treated troughs during the winter months (study months 5-8) when the soil temperature averaged 12°C. Microbial contaminant degradation also continued at high levels during this time. The PAH-degrading bacteria appear to remain active over a wide range of temperatures. The increase in CO_2 production after tilling is evident in Troughs 2 and 3 within 2 h (Figure 36).

Comparison of LTUs and Troughs

Using normalized concentrations to account for addition of the bulking agent, the results from landfarming PAHs are shown in Figure 37. Both biostimulation and bioaugmentation improved the rate of PAH degradation over that of traditional landfarming and natural attenuation. Bioaugmentation was the most rapid process initially, eliminating much of the bacterial acclimation time. Efforts should be made to determine how to sustain this initial rapid phase. A re-application of the slow release nitrogen and/or the carrier-bacteria element, are two parameters that require further investigation. Table 19 summarizes the results of the pilot study conditions in terms of overall removal, the speed of the process and the microbial community produced. Both supplementation and bioaugmentation used alone or in conjunction with other treatments (chemical, physical or biological), show promise in the remediation of highly contaminated soils that have previously been considered unsuitable for landfarming.

5 Conclusions on Utility in Remediation

Conclusions from Flask Studies

Biosurfactant from *P. aeruginosa* strain 64 can extract PAHs from sand, illite clay and creosote-contaminated soil. *P. aeruginosa* strain 64 (the biosurfactant-producer used to enhance the bioavailability of soil PAHs) and *S. paucimobilis* EPA 505 (providing co-metabolic degradation of PAHs) were both successfully immobilized on vermiculite. These microbes were able to be stored in the laboratory at room temperature on the biocarrier for two months without significant loss in viability and metabolic activity.

Bioaugmentation of microcosms (POPILE soil with PAHs at 13,000 mg/kg, avg., and PCP at 1,500 mg/kg, avg.) was successful for *P. aeruginosa* strain 64. After 11 months of treatment, the control (no treatment) showed a 23% decrease in total PAH and a 48% decrease in total BaP toxic equivalents. The biostimulation microcosm (bulking agent and dried blood) showed a 34% decrease in total PAH and a 57% decrease in total BaP toxic equivalents. The bioaugmentation microcosm (bulking agent, dried blood and P. aeruginosa strain 64 on vermiculite) showed an 87% decrease in total PAH and a 67% decrease in total BaP toxic equivalents.

The addition of microorganisms to improve metabolic capability was more problematic due to their sensitivity to soil conditions (i.e., toxic creosote components, competition with indigenous microbial communities, etc.). No improved decrease in PAHs was observed in soil microcosms after microorganism addition. We were not able to detect these bacteria in the POPILE soil after a couple of days. Clearly more research is required to make it a useful and practical tool to increase co-metabolic degradation of HMW PAHs.

Conclusions from LTUs

Respiration analysis, coupled with reduction in heavy PAHs (4, 5, and 6 ring), demonstrated significant biological activity in the pilot-scale units even at the very high contaminant concentrations found in the POPILE soil. Microbiological characterization, using PLFA analysis, showed an increase in biomass and a divergence of community composition between the LTUs corresponding to the different treatments. LTU 2, specifically, showed an increase in the relative percentage of Gram-negative bacteria. However, the microbial communities of both LTUs were able to degrade PAHs into the 4-ring homologues, including BaP toxic equivalent compounds. Therefore, while it appears that limited cultivation had a positive impact on the microbial biomass, in the final analysis, cultivation had minimal impact on PAH removal, with both pilot units achieving 69% reduction in total PAH.

One of the parameters under study at pilot-scale before field evaluation was the addition of water and nutrients to the soil. The high concentration of hydrophobic contaminants in the soil inhibited aqueous phase nutrient additions and results indicated that use of slow-release nutrients applied in a solid form would be a more effective method of maintaining an appropriate C: N: P ratio.

Conclusions from Trough Study

Weekly tilling actually decreased the capacity of the indigeneous, unsupplemented microbial community to degrade PAHs, even when accompanied by soil moisture control. The control trough showed a 12% decrease in PAH contamination compared to the control LTU, which showed >50% decrease in the same amount of time (16 months). However, loss of low molecular weight PAHs through photolytic degradation from the LTUs could account for some of the difference as the LTUs were exposed to direct sunlight while the troughs had only indirect, natural light.

Supplementation (Trough 2) with a bulking agent, to increase aeration, water holding capacity, and soil condition, and slow release nutrients, greatly increased the capacity of the indigeneous communities to degrade PAHs. Bioaugmentation plus supplementation (Trough 3) was able to increase the initial rate of PAH removal over that of supplementation alone. Troughs 2 and 3 showed an 86 and 87% removal, respectively.

Troughs 2 and 3 developed similar microbial communities due to the weekly tilling and the supplementation. While these communities were different from those in Trough 1, (no supplementation), they were also different from the microbial communites established in either of the LTUs (limited tilling, no supplementation).

Utility to Remediation of Highly Contaminated Soil

The pilot scale evaluation provided several elements of useful information for remediation of PAH-contaminated DoD facilities, and other sites where landfarming is a potential remediation technology. It is traditionally believed that, for landfarming treatment, cultivation of the contaminated soil through intensive tilling is required for successful bioremediation. This study demonstrated that this may not always be the case. While tilling appears to enhance the biodegradation of PAHs initially, primarily by increasing the microbial biomass, overall, landfarming of PAHs doesn't appear to be driven by soil mixing. Soil moisture content also doesn't seem to be as important as previously thought. Therefore, preliminary, pilot-scale studies should be done in order to optimize conditions favorable to the full-scale effort. High contaminant concentration usually eliminates landfarming immediately as a remediation option. Again, this study demonstrated that this might not be necessary, especially if time is not a consideration. Even at the high PAH concentrations associated with the POPILE soils, successful bioremediation is possible.

The use of genetically-engineered microorganisms (GEMs), a technology developed to pilot-scale by the PCB bioremediation thrust area, also holds promise for treatment of PAH contaminated soil. The vermiculite biocarrier developed during this project has already been proven successful within the PCB thrust area and has great potential for use in bioremediation of contaminated soil. Biosurfactant-producing microorgansisms, such as were isolated and characterized during this study, being a "green technology" are more acceptable in public opinion than commercial chemical additives. The use of the very slow-release nitrogen fertilizer in the pilot-scale units also hasgreat potential to improve landfarming successof any soil contaminant and, in addition, decrease operation and maintenance costs.

When setup costs and operation and maintenance (O&M) costs are calculated, overall remediation expenditures could be significantly reduced if a PAH landfarming site were treated quarterly instead of weekly (Rast, 2001). As an example, based on 1999 RACER cost estimates for the Northeast, USA, traditional landfarming of this soil would require a minimum $3.5 million for a single year of treatment. One alternative, 6 months of intensive treatment followed by 6 months of minimal treatment, would require approximately $1.6 million. A second alternative, 12 months of minimal treatment, would require less than a $0.5 million, primarily due to decreased expenditures for tilling and watering. Therefore, choices in landfarming options could provide significant savings at sites where time is not a factor. As well as decreasing costs, by limiting the intervention at a site the potential for human exposure to the contaminant is decreased, the production of fugitive dust (air particulates) is decreased, and the possibility of accidental groundwater contamination is decreased.

The significance of these positive results is further enhanced in light of the excessive PCP co-contamination, commonly found in PAH contaminated sites, but rarely studied in conjunction with PAH degradation in the laboratory. Biological degradation of seemingly high concentrations of PAHs has not been successfully demonstrated in previous efforts. As this study showed, microbiological analysis coupled with contaminant degradation analysis, demonstrated biological degradation of the recalcitrant compounds. Biological degradation of PAHs with previously reported inhibitory PCP concentrations is possible, and appropriate for sites where time is available for long-term active bioremediation.

6 Recommendations for Transitional Research

PAH Availability in Soil and Regulatory Clean-Up Levels

In theory, soils contaminated with PAHs may be treated utilizing various clean-up strategies. However, many proposed strategies have significant economic and feasibility problems. What is needed is an effective technology that supports the economics of disposal, eliminates adverse contaminant impacts, and supports the reuse of treated contaminated soils. Regardless of whether the biotreatment system is passive (natural attenuation) or engineered (in-place treatment), a pragmatic solution is to focus active biotreatment on the available contaminant fraction. Research is needed to identify the factors affecting the bioavailability of PAHs on soil and how this affects treatment rates and acceptable toxicological endpoints. Research is then needed to use the bioavailability information to develop a technical base for enhancing natural recovery processes involved in *in situ* biotreatment of PAH-contaminated soils. Such research could result in providing guidelines for the assessment and prediction of the bioavailability of PAHs for *in situ* biotreatment.

The natural recovery process could be enhanced through the use of genetically engineered microorganisms (GEMs) as indicated in the accompanying SERDP report on PCB bioremediation. In this repor,t the use of GEMs was successful in the biodegradation of chlorinated organics in the laboratory. Bioaugmentation in this study was accomplished with the use of vermiculite as a biocarrier, a technology developed by the PAH thrust area. GEMS would be a logical next step in investigating PAH biodegradation.

Beyond availability, the issue of residual contamination is still unresolved in the case of biological treatment of PAHs where an understanding of the complex interactions between hydrophobic organic contaminants and soil is key to establishing realistic risk assessment criteria. Cleanup criteria based only on the chemical properties of the contaminant leads to an over-prediction of the risks associated with the contamination. However, leaving an immobile, bound residue in the soil after cleanup needs to be justified scientifically. On the other hand, if residuals are released, the rate should be sufficiently slow to allow consumption by the microbial community. Thus, an alternative environmental endpoint (clean-up level) may be appropriate, rather than basing decisions solely on total concentration of contaminant in the soil or sediment. Thermal programmed desorption (TPD) is one new technology available to study the first of these issues, the mechanism of PAH binding to the soil, the process that affects their bioavailability in the environment.

Phytoremediation of PAHs

Phytoremediation is defined as the use of green plants to remove, contain or decrease the toxicity from environmental contaminants. As a final polishing step to landfarming phytoremediation should be further studied. It is a cost-effective technology with many advantages over highly engineered solutions, including public acceptance. It is only in the last decade that research has been conducted on phytoremediation of PAHs. Recent studies have shown that plants are capable of removing, not just the smaller molecular weight PAHs, such as anthracene, but also HMW compounds, such as chrysene and benzo(g,h,i)pyrene. In contrast to phytoremediation of metals, which is an extraction process, large organic compounds like the PAHs are degraded in the rhizosphere, root zone, of the plant. The rhizosphere comprises the top three to six feet of soil. Various elements of the rhizosphere appear to play a role in the degradation. First, the soil around the roots is very different in chemistry and physical structure from the bulk soil. The root system of the plant is a moist, aerobic environment, which promotes microbial activity. Secondly, an exudate is released from the roots, into the surrounding soil. The exudate is composed of simple sugars, amino acids, enzymes, aliphatic and aromatic compounds, and vitamins, and has been shown to increase the dissipation of soil-bound PAHs when applied to soil as a single treatment. Third, there are active microbial and fungal communities associated with the root system. These are enhanced by the root exudate in ways that are not completely understood. Growth, and the development of biomass, is certainly one response, but in some plant species the exudate appears to favor selection of microorganisms that degrade the contaminant.

Plants can be incorporated into current treatment strategies in several ways. The most recent development is an alternative agricultural technique, plant-based biotechnology. The goal of this "molecular farming" is to produce large quantities of root exudate to be used as a soil amendment. As these exudates vary between species and soil conditions, a systematic study needs to be undertaken before this technology is feasible.

The most promising method of incorporating phytoremediation into soil PAH remediation is to develop a new paradigm of treatment that utilizes rhizospheric degradation as part of a treatment train. Once a contaminated soil has been treated by landfarming, for example, a plant cover would be established. The advantages are:

- plant cover reduces both wind and water erosion from the treated soil,
- the improved soil structure allows continued bioremediation
- naturally occurring, nitrogen-fixing microorganisms reduce the need for chemical fertilizers, reducing costs,
- the roots establish an optimal environment and furnish energy for growth of microorganisms,
- the exudate contains oxidative enzymes that contribute to PAH degradation.

Current research indicates that the establishment of plants on contaminated sites may be an economic, effective, low maintenance approach to complete soil PAH remediation.

7 Technology Transfer

Technologies for bioremediation and natural attenuation of organic compounds have developed dramatically over the past 15 years due to extensive programs carried out in the US and in Europe. The possible breadth of application of bioremediation continues to be inhibited by significant data gaps in our ability to apply the principles of biotechnology to chemical contamination. There is a considerable amount of field experience available, and protocols have been developed, for the bioremediation of easily degradable compounds such as petroleum hydrocarbons and chlorinated aliphatic solvents. However, until now, the development of similar state-of-the-art protocols for bioremediation of the more recalcitrant polycyclic aromatic hydrocarbons (particularly at high concentrations) has continued to lag. This demonstration and its publication of improved bioremediation of these more toxic, high molecular weight PAHs through bioaugmentation and biostimulation will enhance the potential for more frequent and successful use of biotreatment as a viable options for PAH cleanup.

The combination of the use of known surfactant-producing bacteria in conjunction with vermiculite carried bioaugmentation, enhanced nutrient delivery, and appropriate bulking agent application provides the basis for a variety of field applications on higher concentrations of toxic PAHs not previously thought possible. The advances described herein allow the vast body of knowledge and experience in bioremediation and natural attenuation to be extended to hydrophobic organic compounds previously thought recalcitrant, such as the polychlorinated biphenyls (PCBs) and pesticides. The accompanying SERDP report on PCB bioremediation, for example, describes the use of the vermiculite carrier developed in the PAH project in the delivery of GEMS for chlorinated biphenyl biodegradation.

We have documented a succinct progression of bench- and pilot-scale demonstrations that applied sound scientific principles and assessed practical engineering issues in the development of enhanced parameters for contaminant remediation. The major payoff of the work will come in the future when successful bioremediation based on the success described herein is used routinely to enhance cleanup of sites contaminated with PAHs and other appropriate hydrophobic compounds.

As listed in the attachments, the work funded under the SERDP program portrayed here has been showcased in many venues, including national and international conferences focusing on recalcitrant compounds, bioremediation, and microbiology, peer review journals, and technical reports. A book is planned in the near future written for practitioners in the field of bioremediation. A device to monitor microbial respiration during bioremediation and natural attenuation was designed, tested and submitted for patent during this study. This device is a low-cost, low technology, means to decrease the costs associated with the operation and maintenance of landfarming facilities without sacrificing human health and safety.

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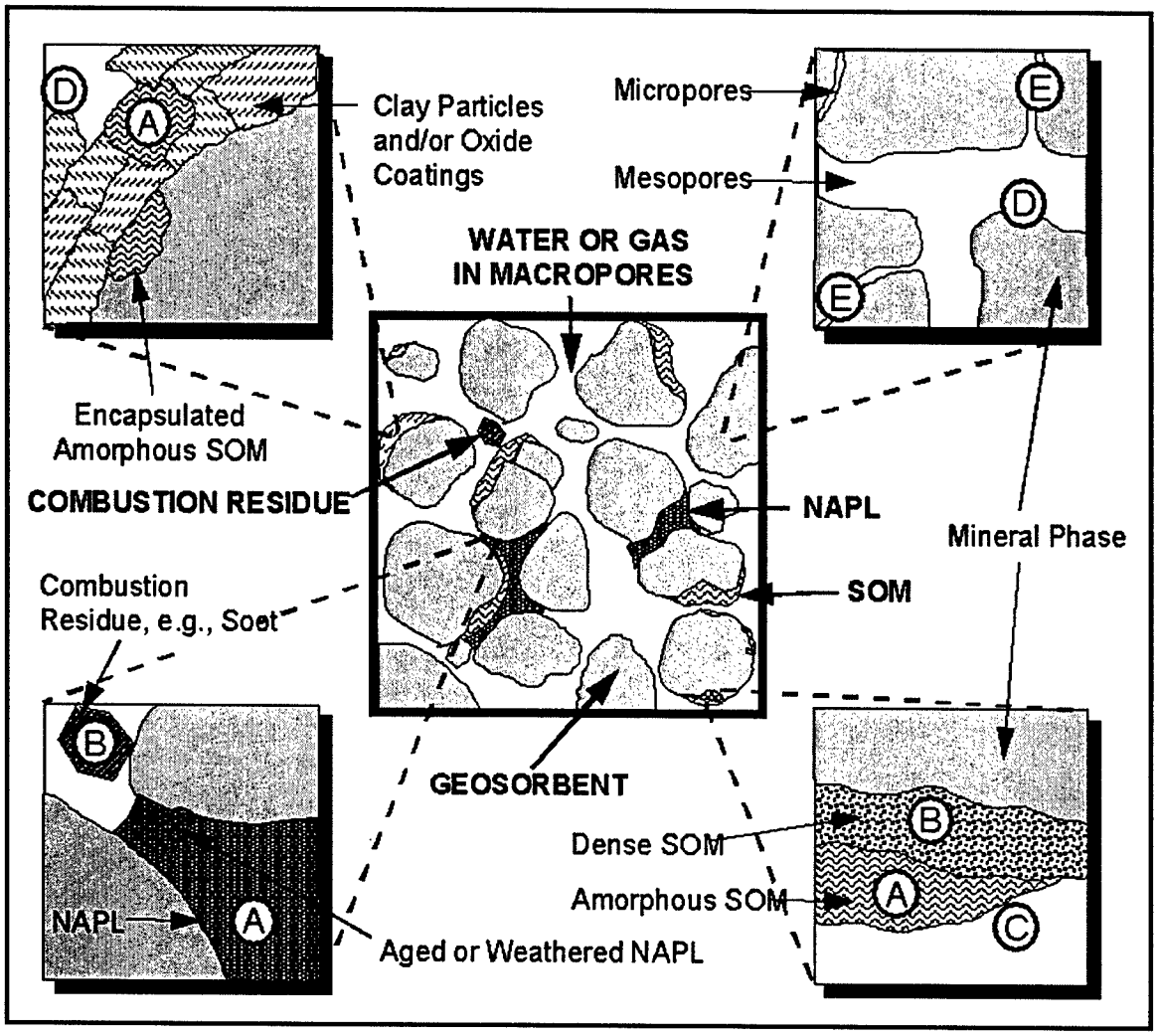


Figure 1. Model of PAH residence in soil

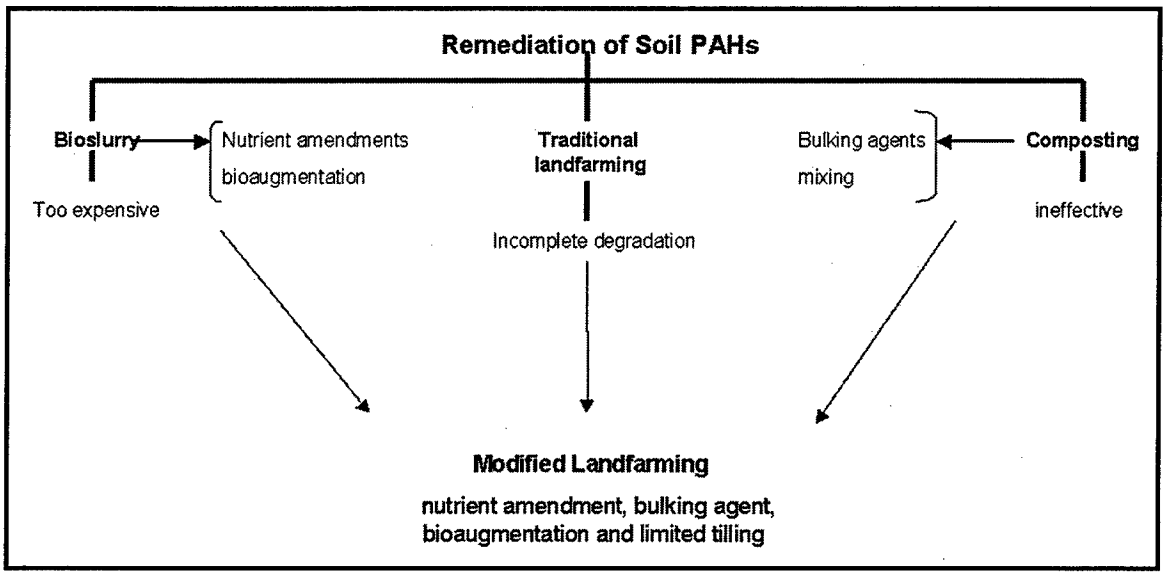


Figure 2. Project history leading to the selection of landfarming as a PAH treatment technology

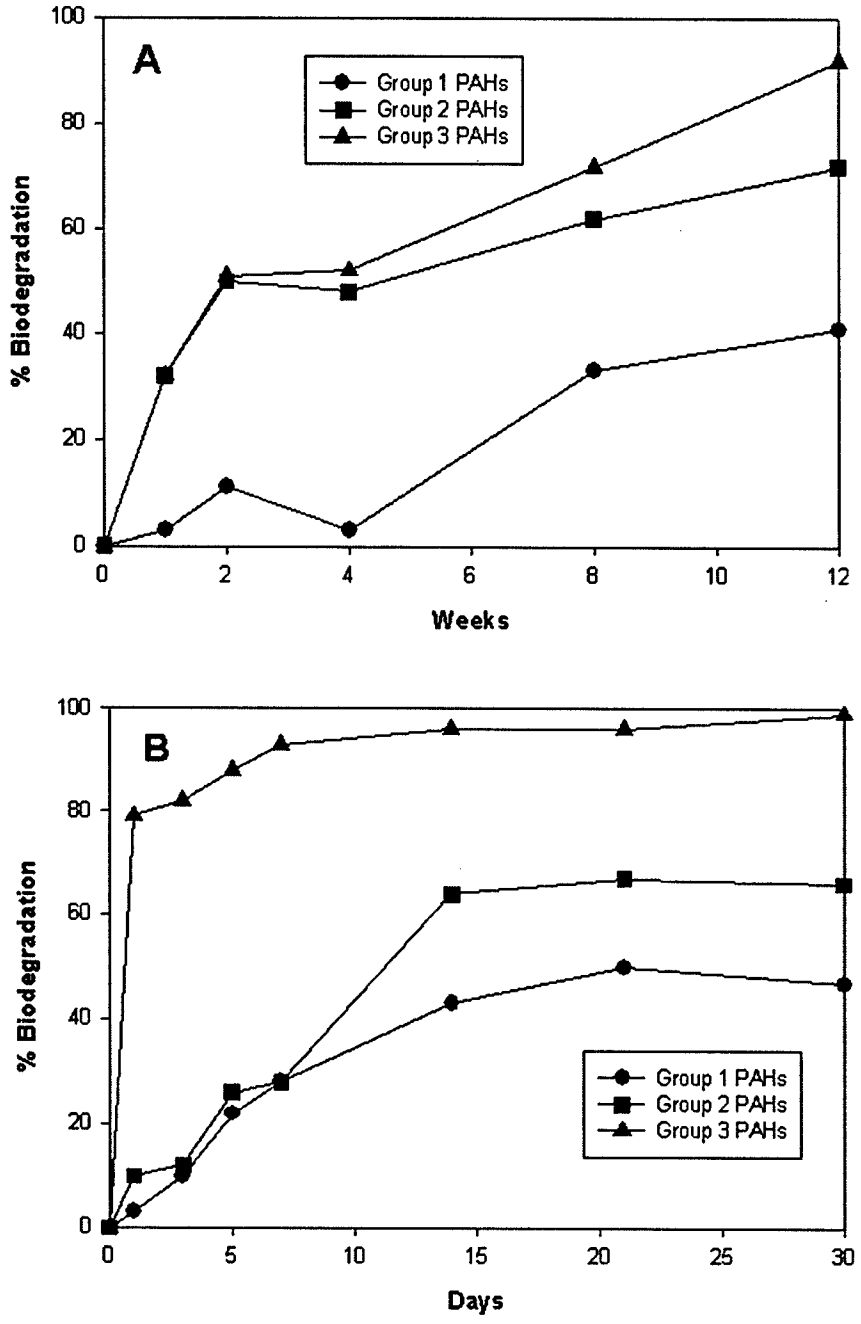


Figure 3. Biodegradation of PAHs during solid-phase (A) and slurry-phase (B) treatment of contaminated surface soils amended with nutrients

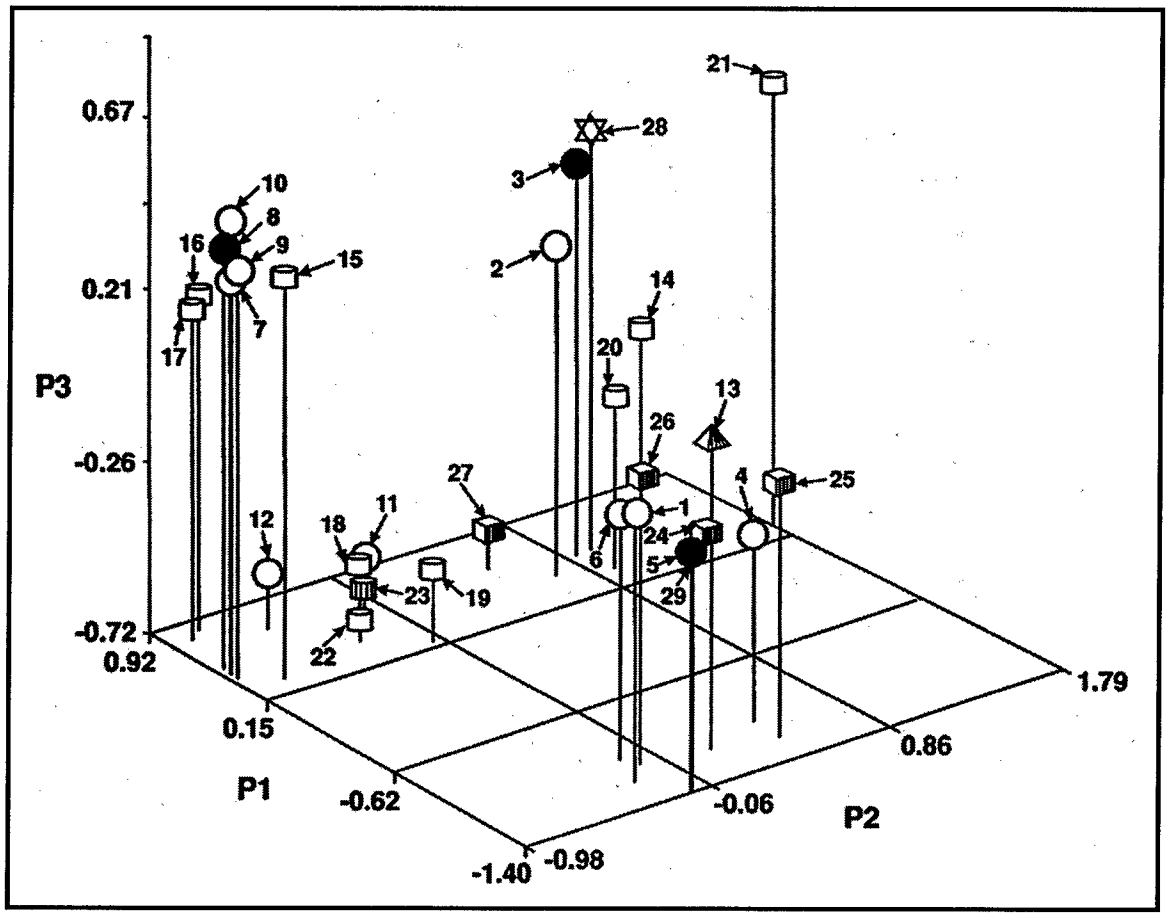


Figure 4. Balloon plot of principal components analysis of Biolog® responses from PAH-degrading isolates

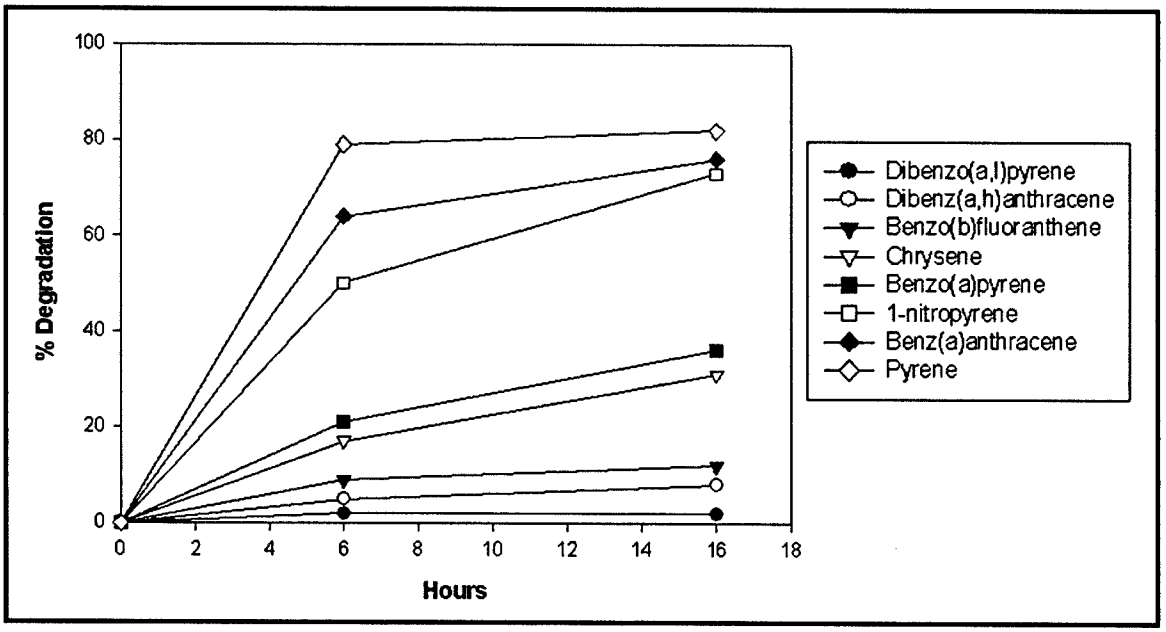


Figure 5. Co-metabolism of PAHs by EPA 505

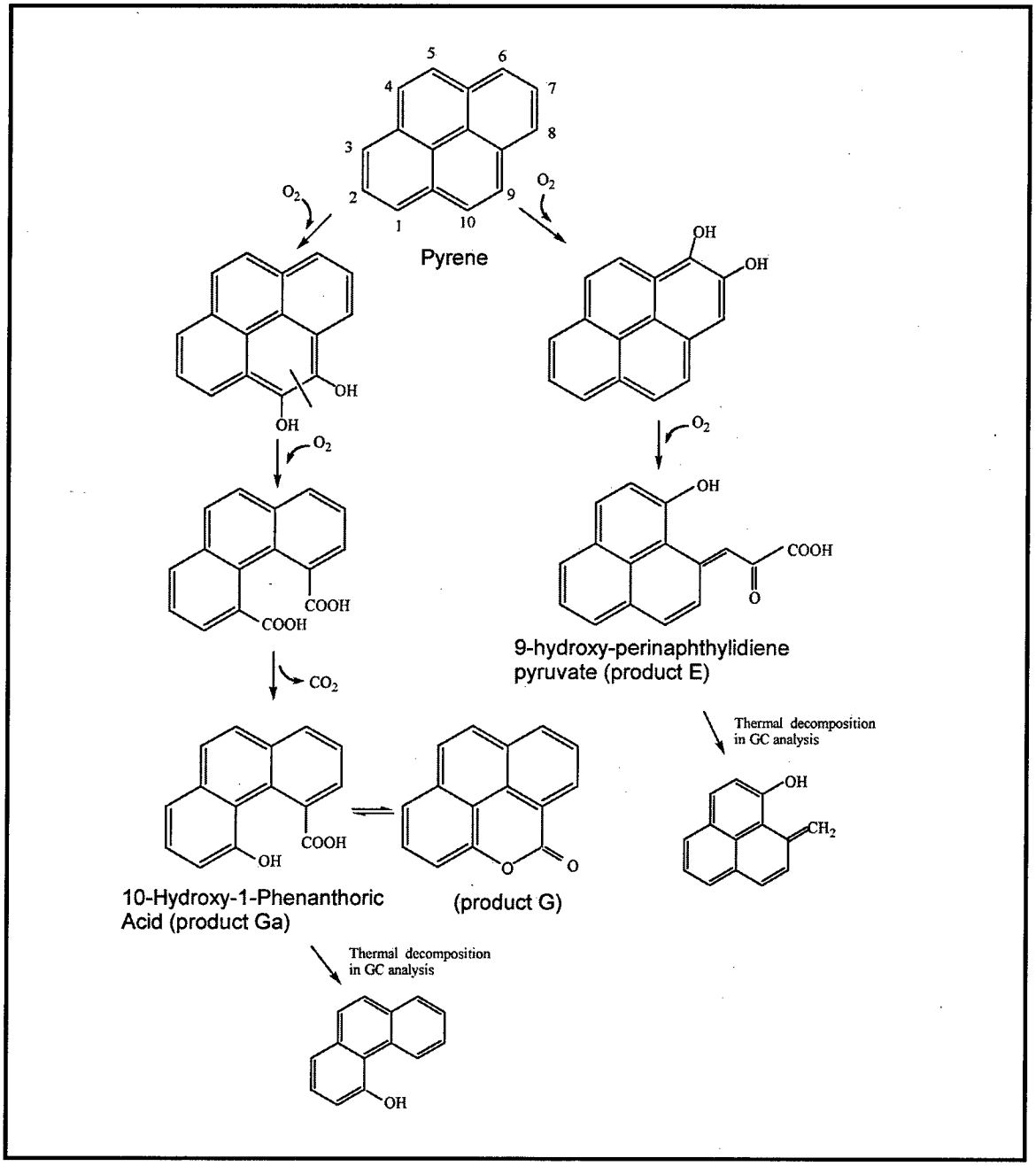


Figure 6. Proposed pathway for the co-metabolism of pyrene by *S. paucimobilis* strain EPA 505

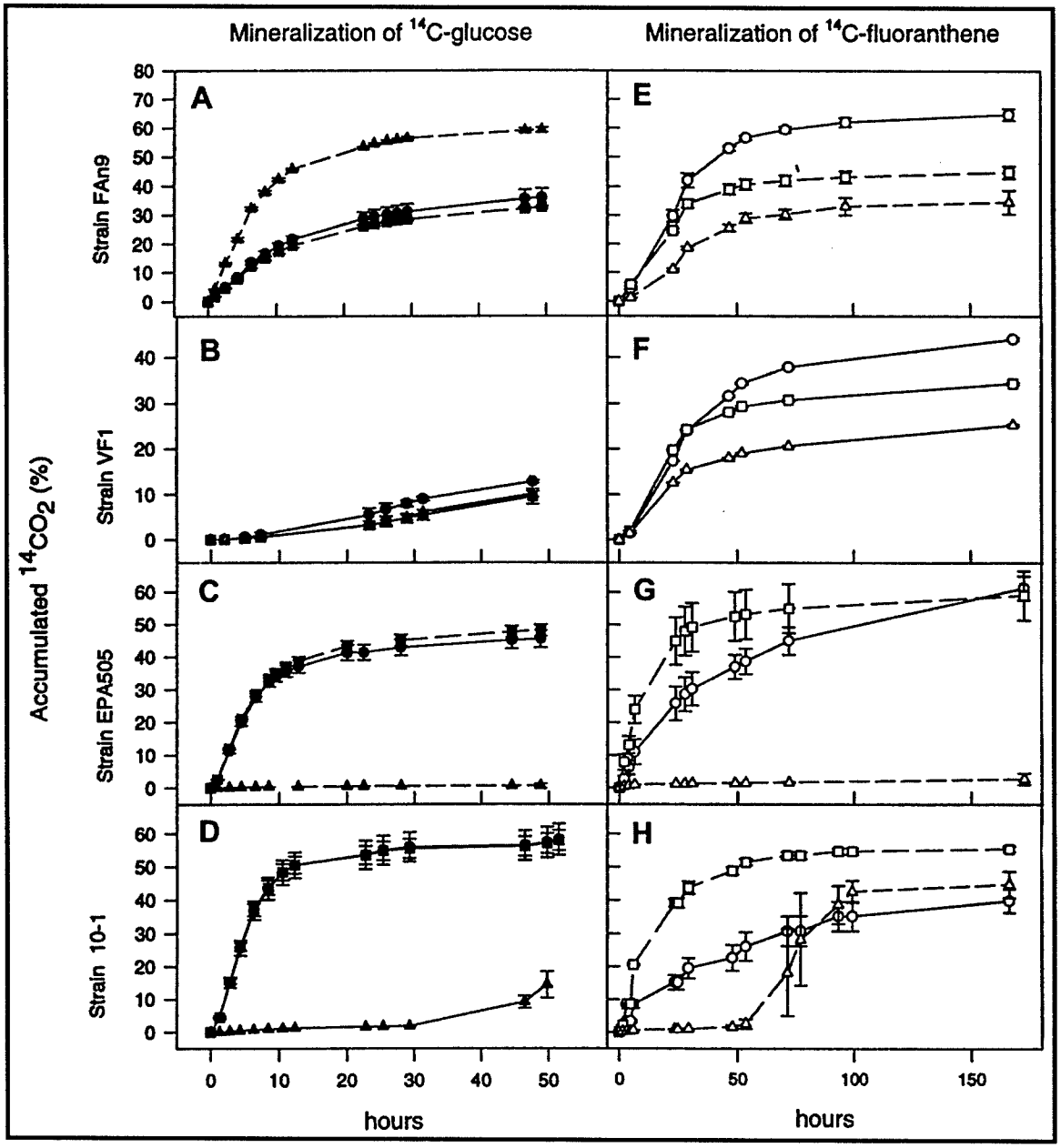


Figure 7. Mineralization of fluoranthene and glucose by four fluoranthene-degrading strains, in the presence (squares and triangles) and absence (circles) of surfactants. Surfactants were Tween 80 (squares) and Triton X-100 (triangles)

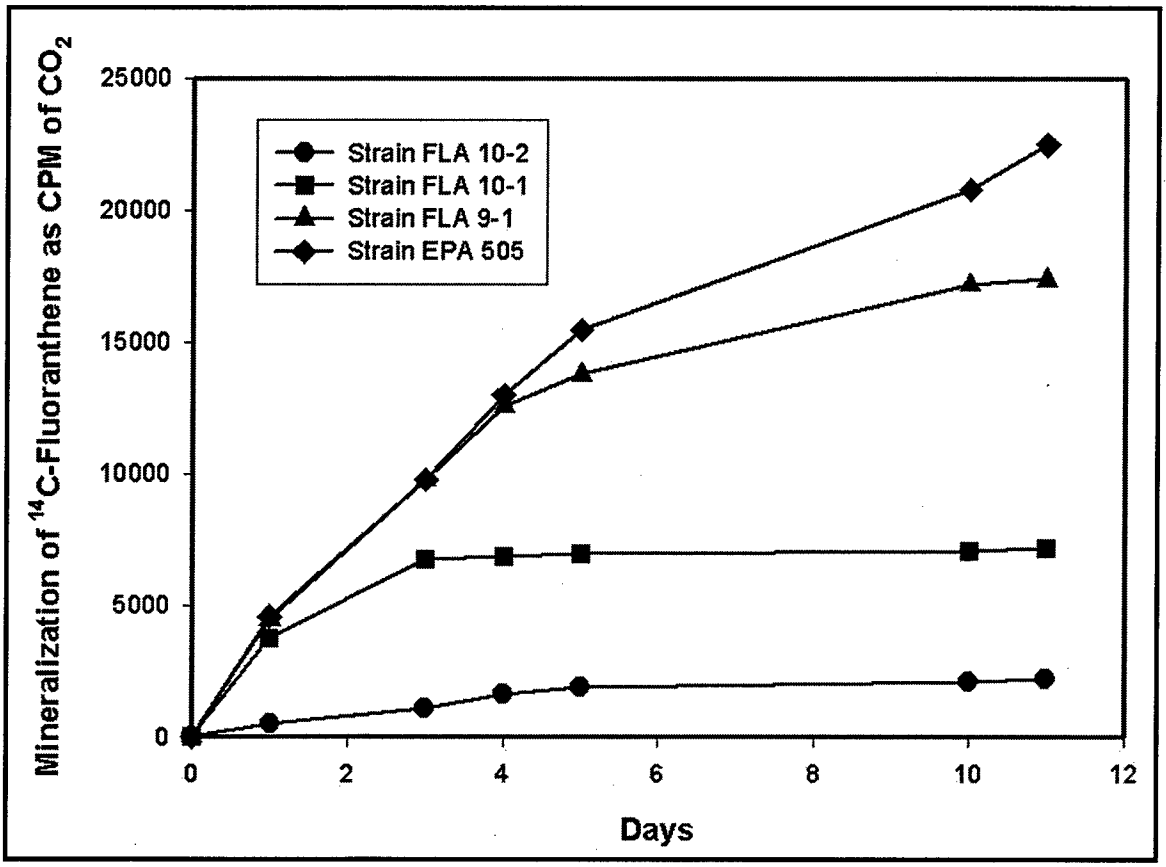


Figure 8. Effect of 0.48 mM Triton X-100 on the mineralization of radiolabelled FLA in minimal medium by 4 different fluoranthene degraders

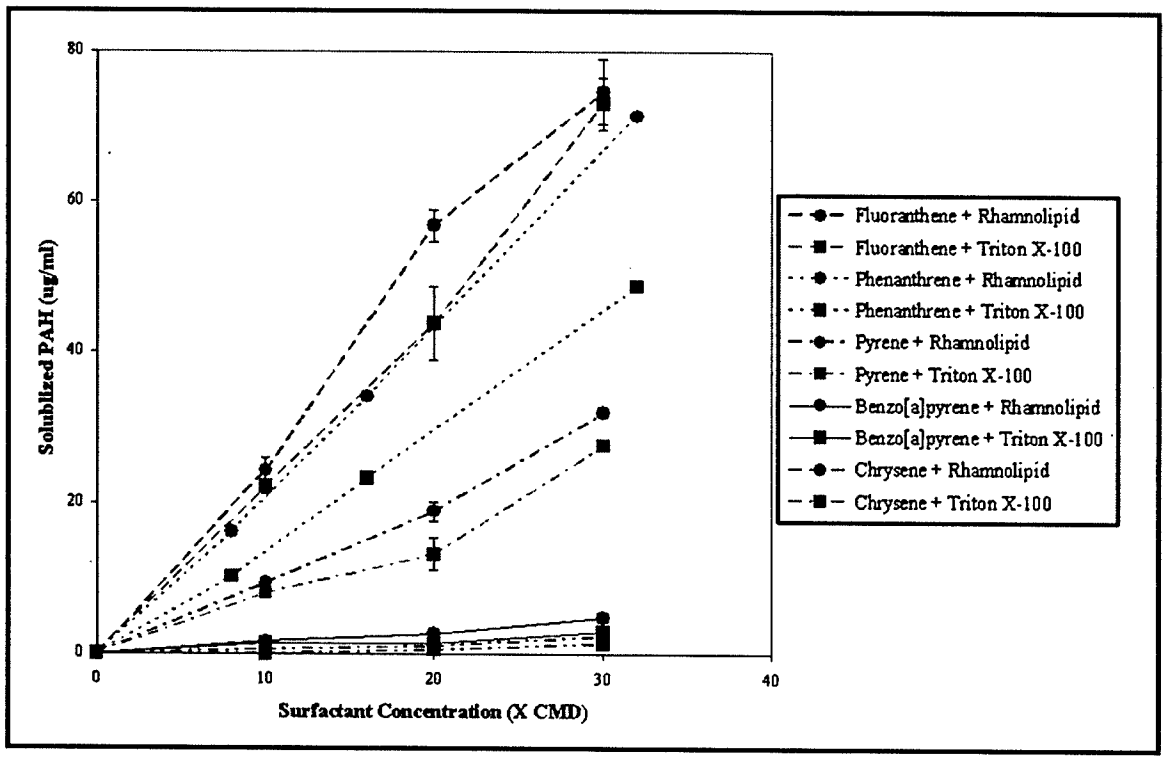


Figure 9. The solubilization of PAHs (n=3) by *P. aeruginosa* strain 64 rhamnolipid and by Triton X-100

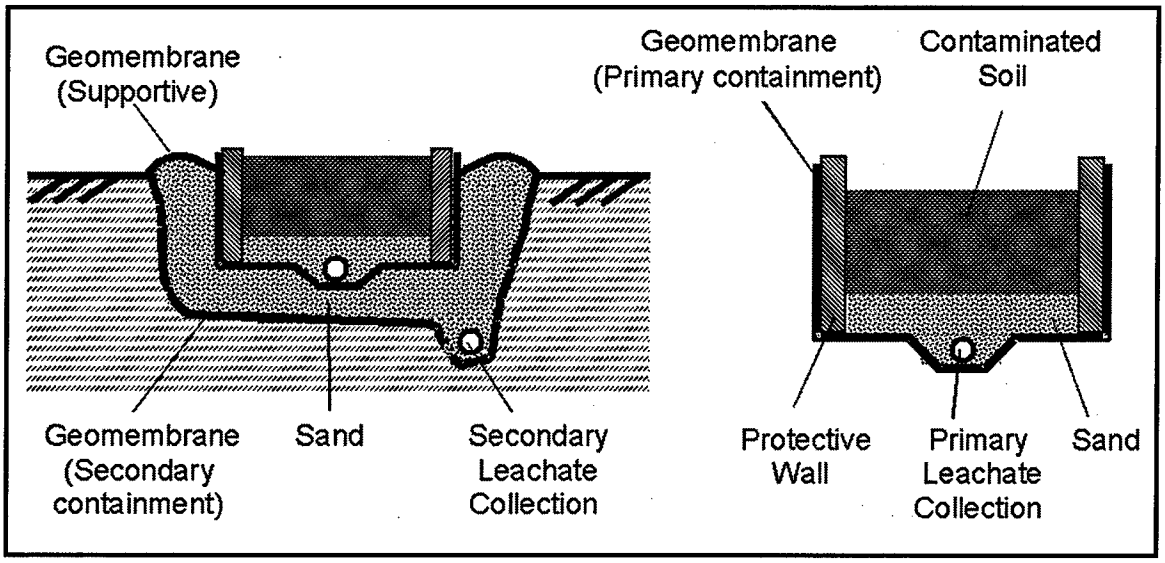


Figure 10. Design of the LTU primary and secondary containment systems

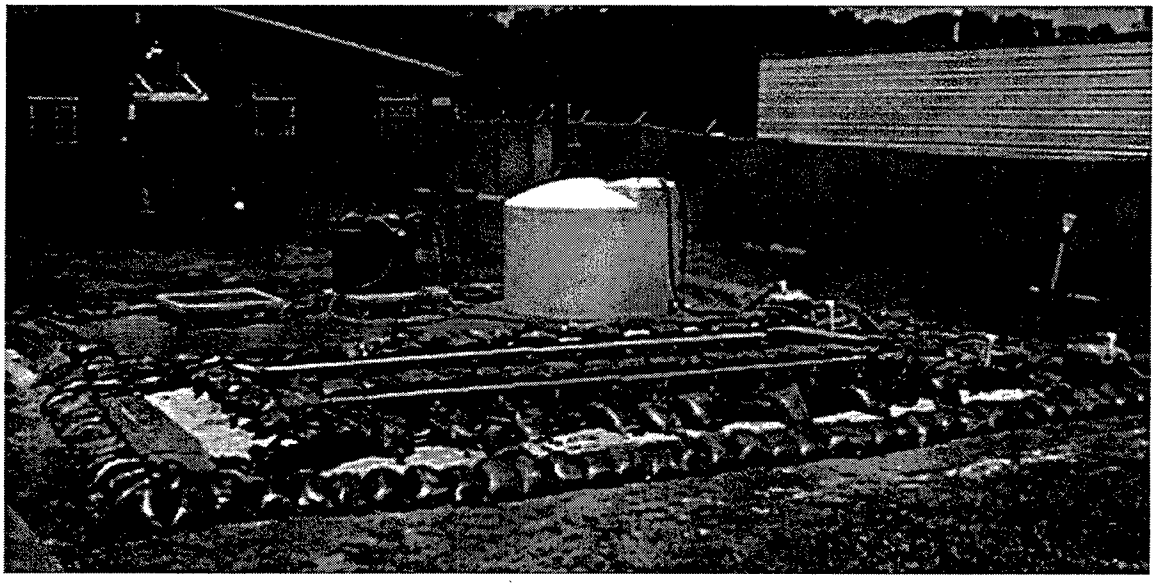


Figure 11. Photograph of the LTU pilot project illustrating the land treatment units, the primary and secondary containment systems, and the leachate collection system

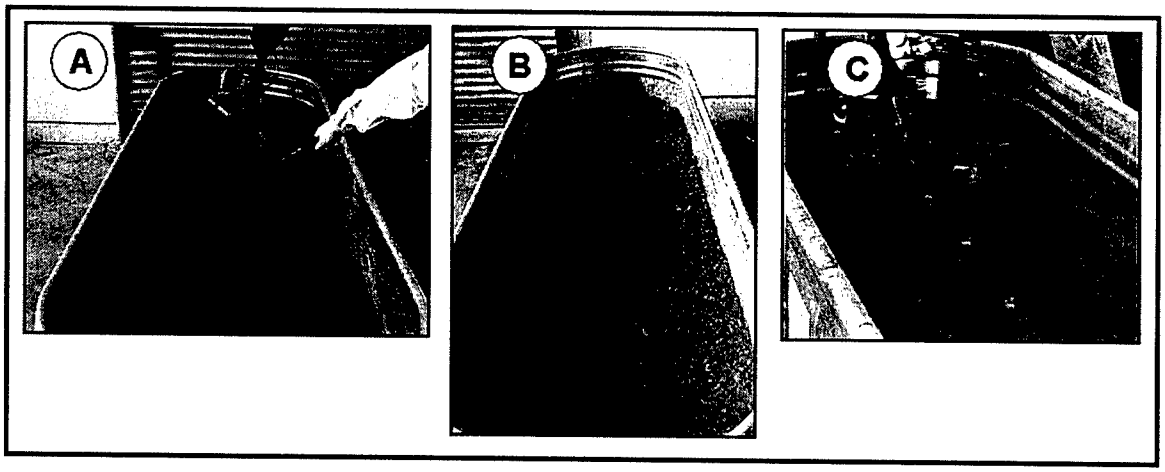


Figure 12. Trough assembly

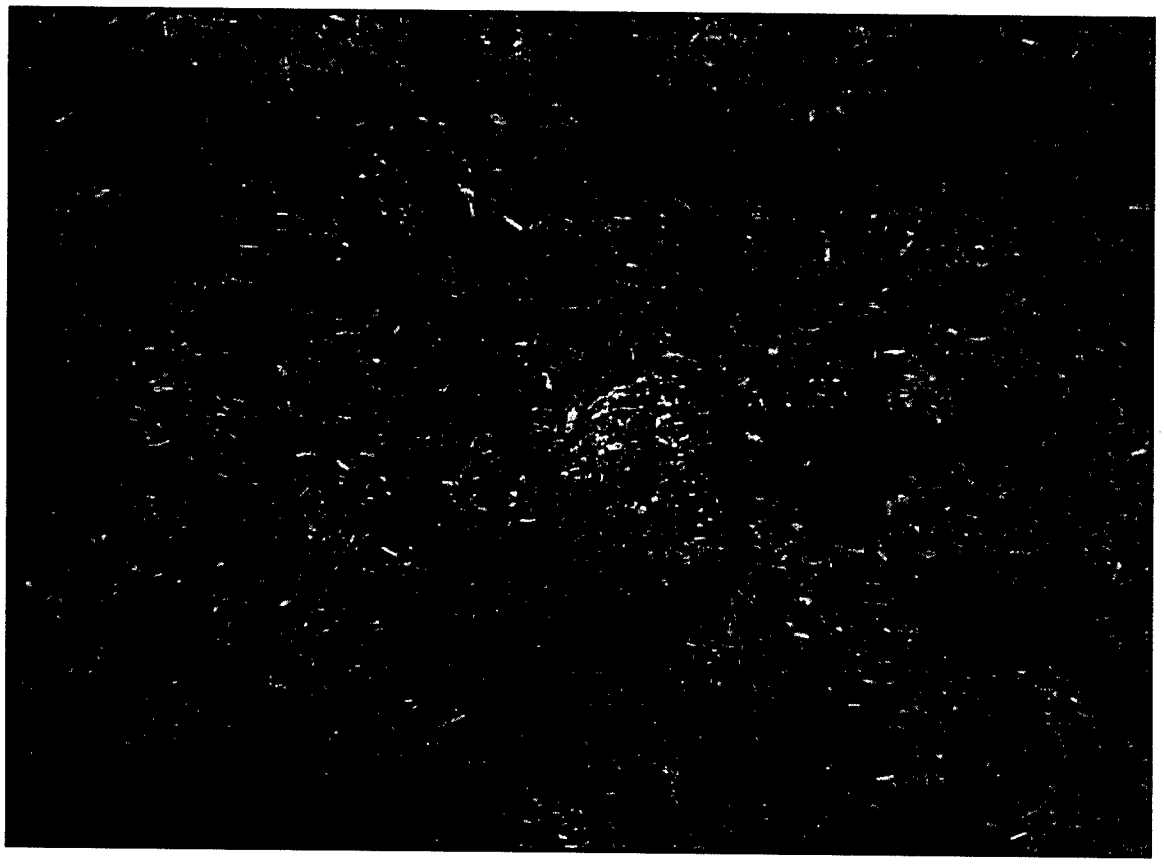


Figure 13. Change in soil texture following addition of the rice hulls as bulking agent

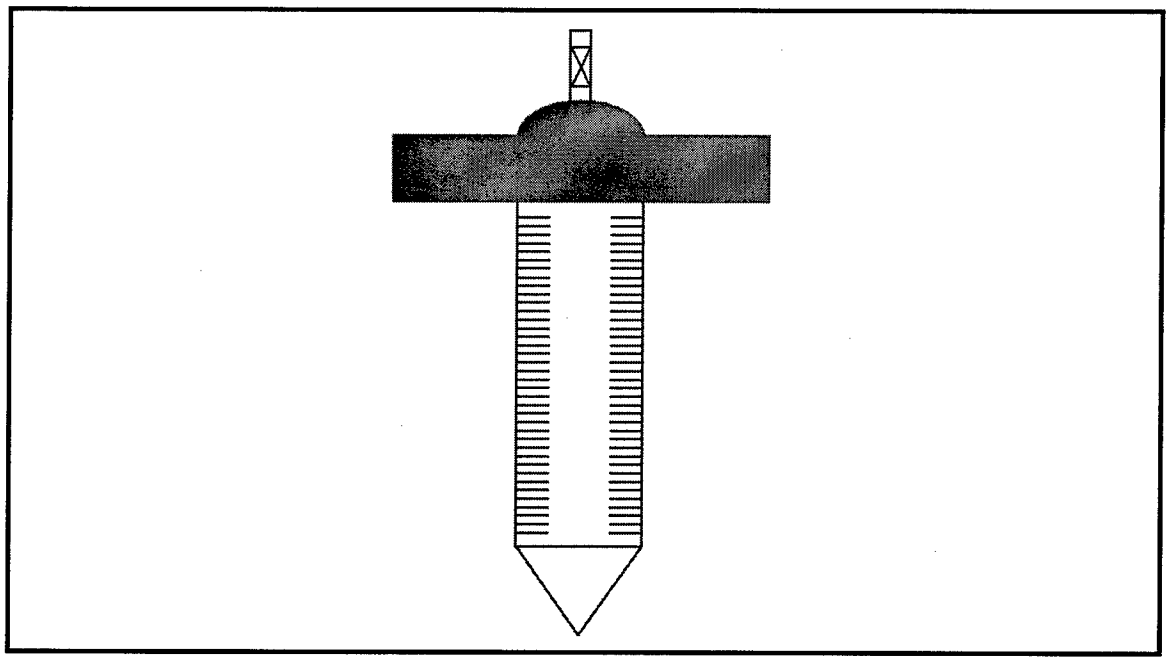


Figure 14. Diagram of soil gas sampling probe used to monitor microbial respiration in situ

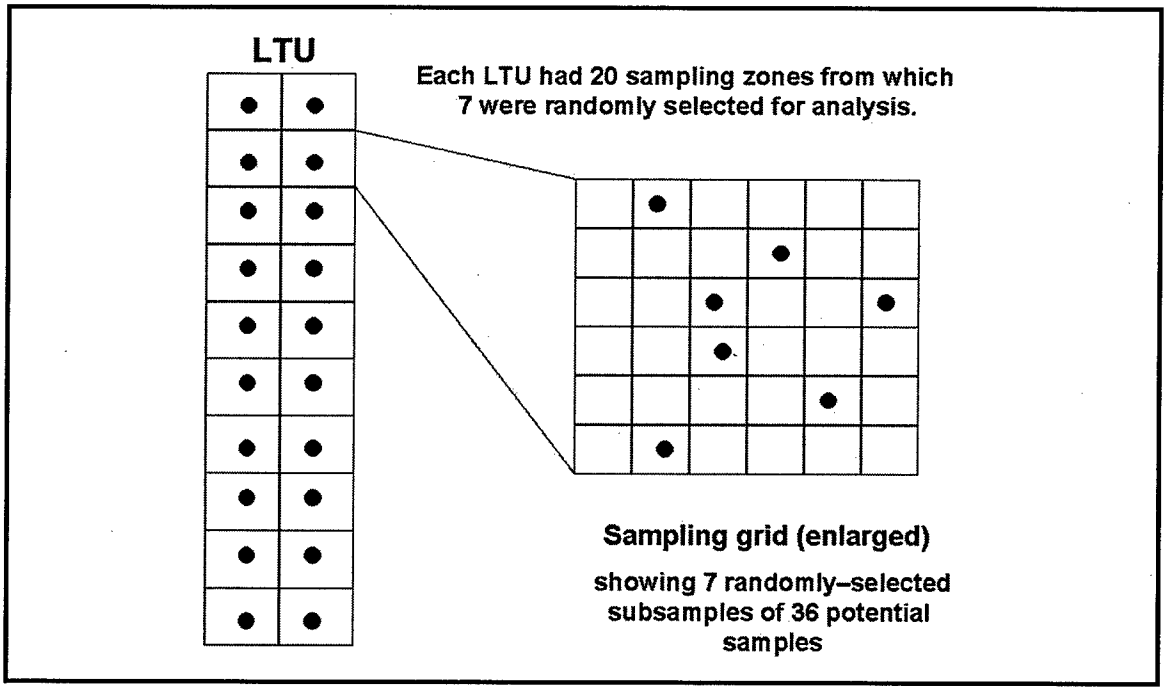


Figure 15. Design of random sampling process for the LTUs

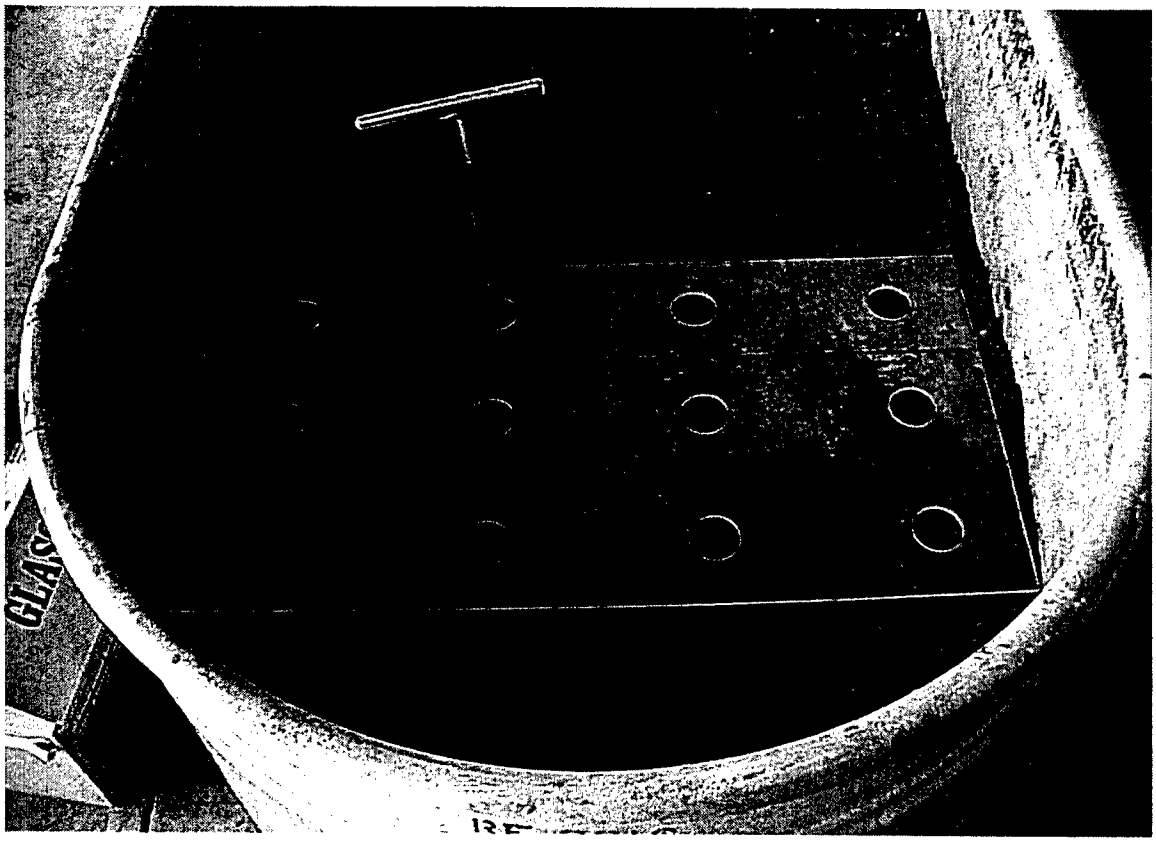


Figure 16. Photograph of sampling grid and soil corer used to collect samples from the troughs

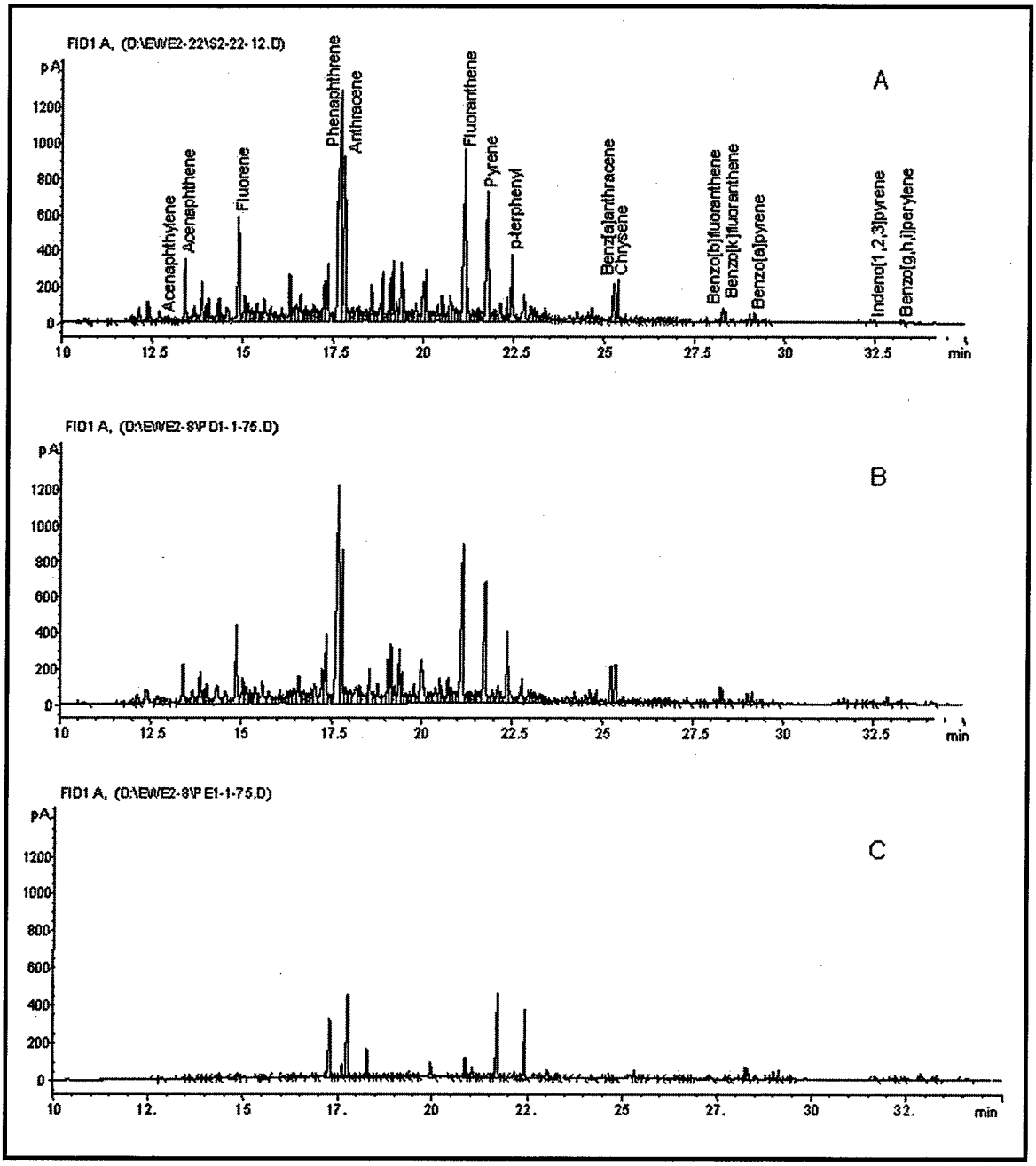


Figure 17. Chromatographs of solvent extracts from microcosms containing POPILE soil after 11 months

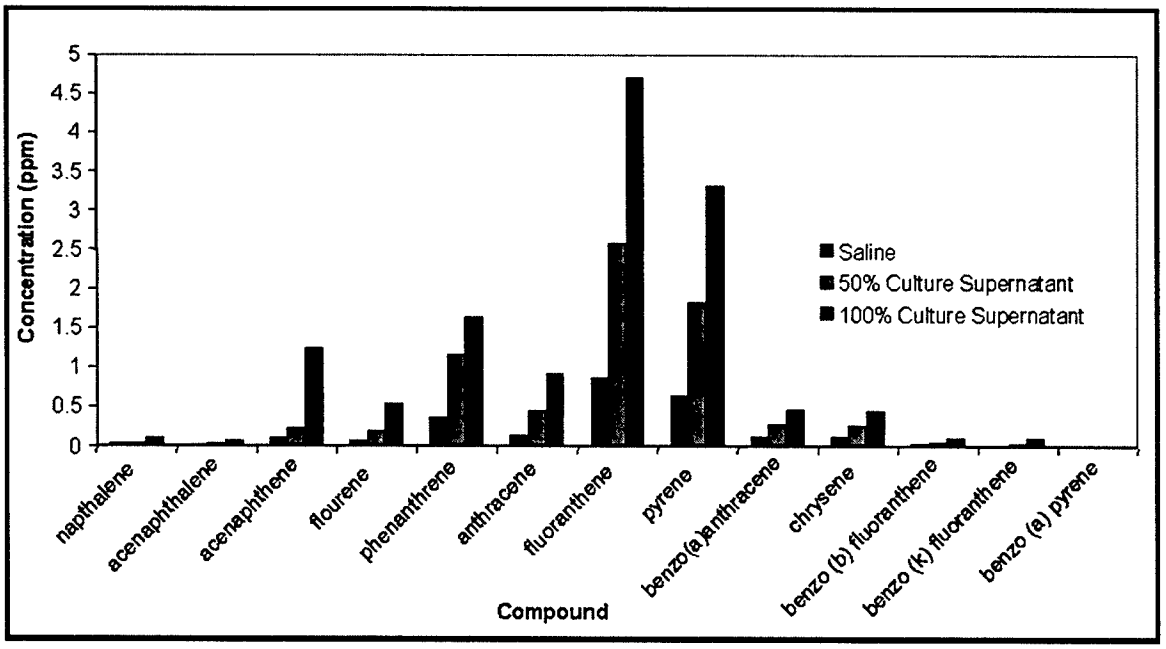


Figure 18. Concentration of PAHs in aqueous extracts of SMWT soil with saline (control) and with the addition of *P. aeruginosa* strain 64 culture supernatant

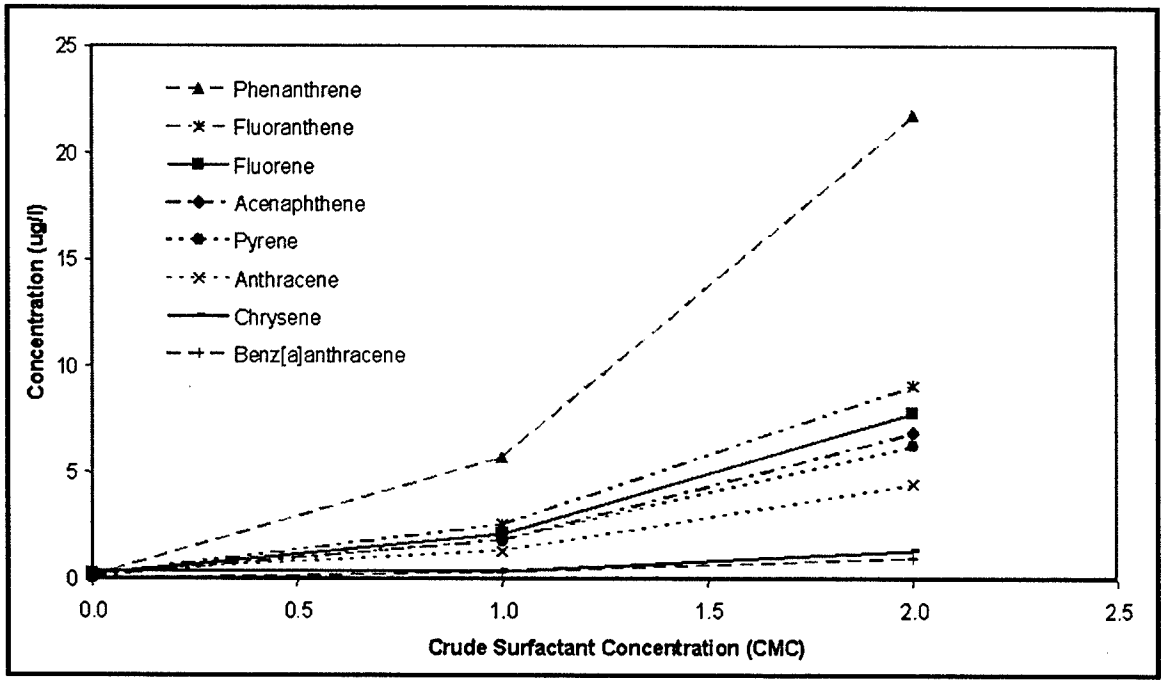


Figure 19. Concentrations of PAHs in aqueous extracts of POPILE wood treatment site soil at two concentrations of crude biosurfactant produced by *P. aeruginosa* strain 64

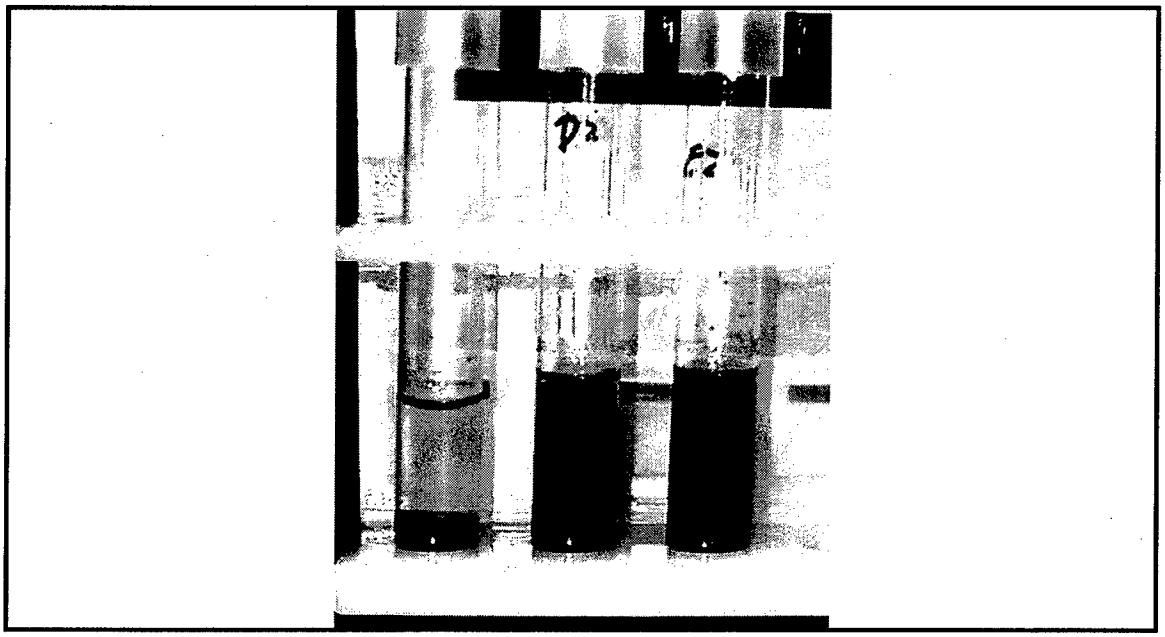


Figure 20. Emulsification of PAH-contaminated soil from microcosms

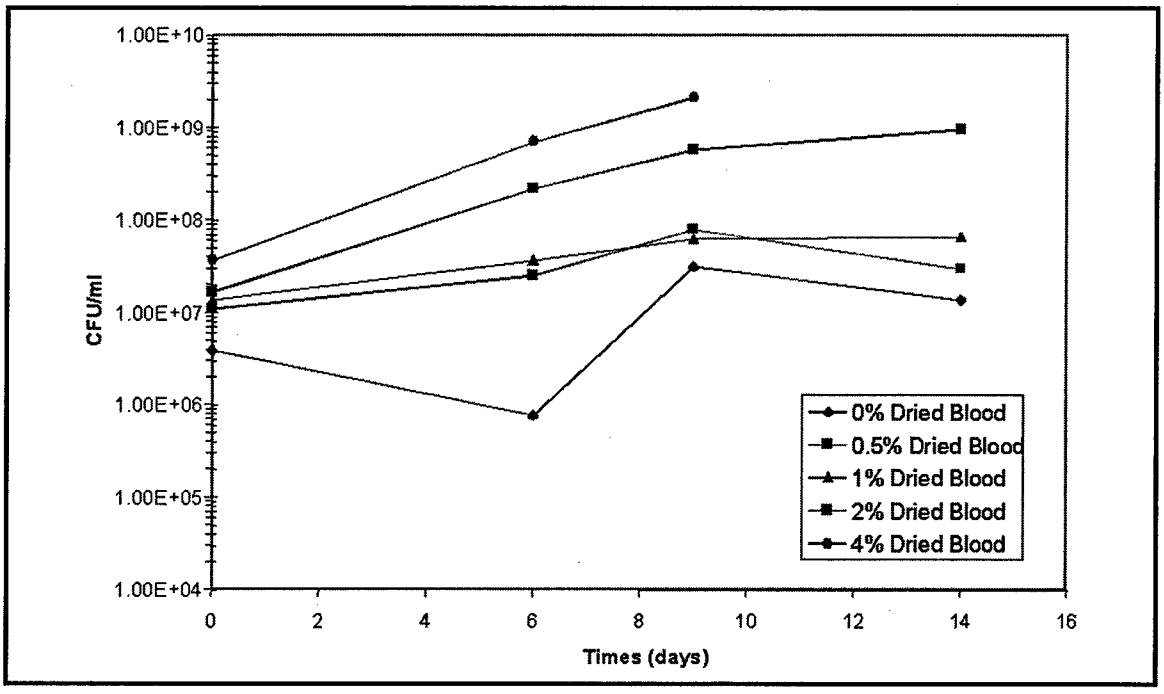


Figure 21. The effects of varying concentration of dried blood fertilizer on bacterial growth in SMWT site soil inoculated with *P. aeruginosa* strain 64

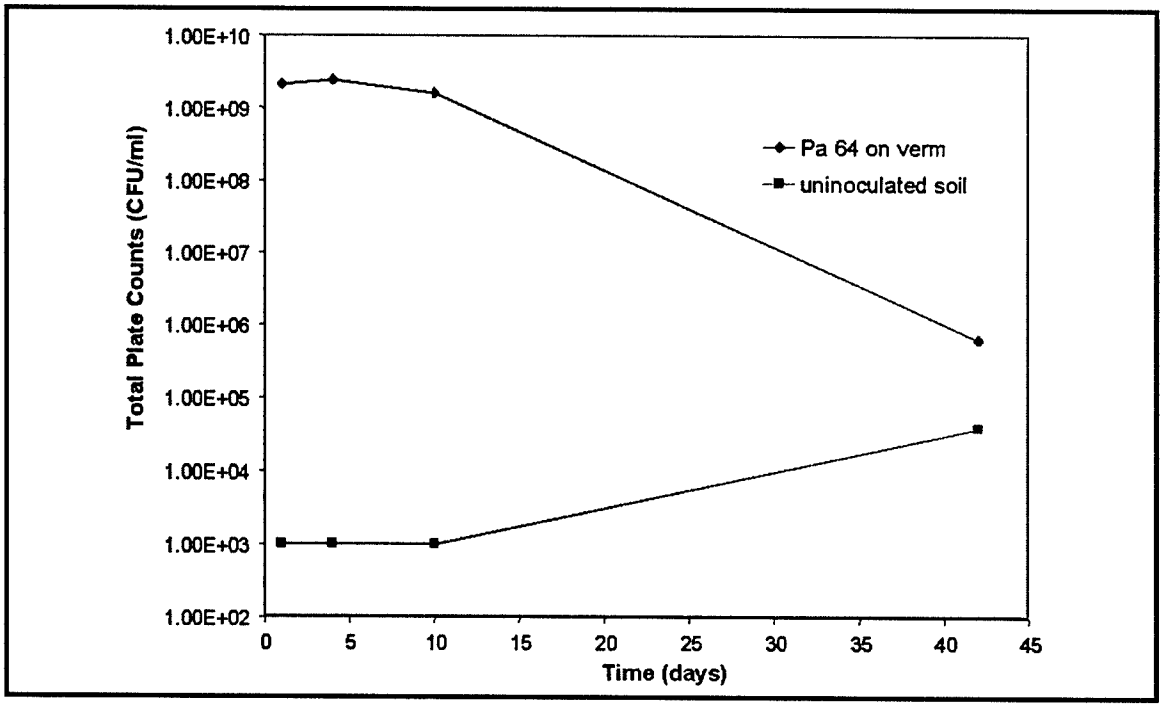


Figure 22. Survival of *P. aeruginosa* strain 64 on the vermiculite carrier in SMWT site soil

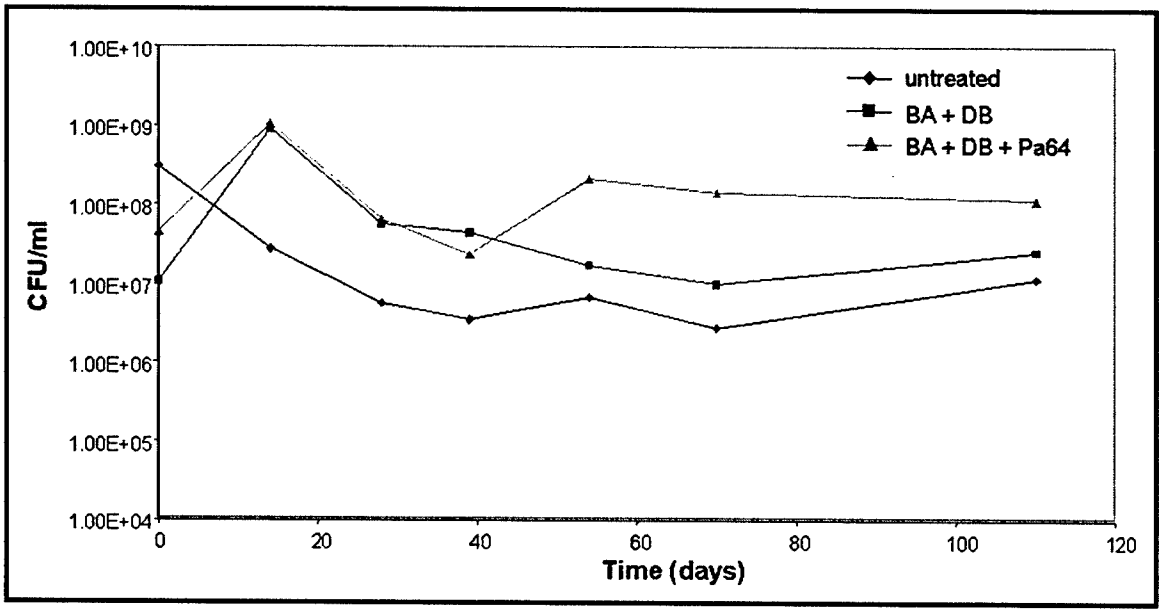


Figure 23. Plate counts from POPILE soil incubated in flask microcosms with and without soil amendments

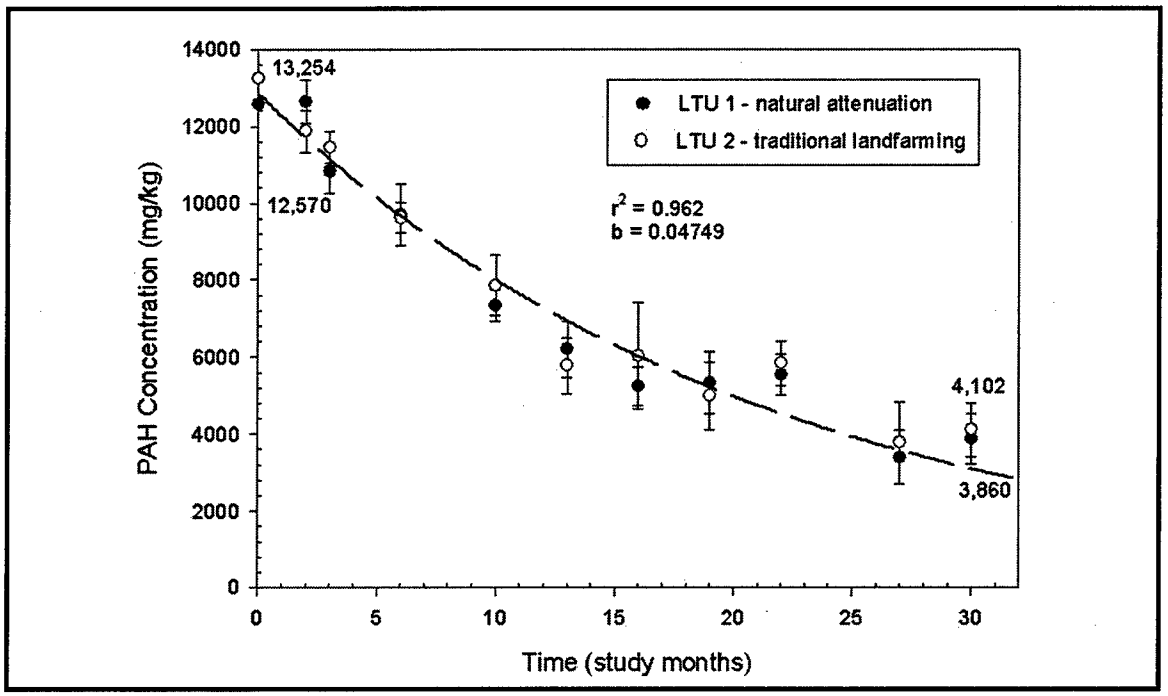


Figure 24. PAH concentration in LTU 1 and LTU 2

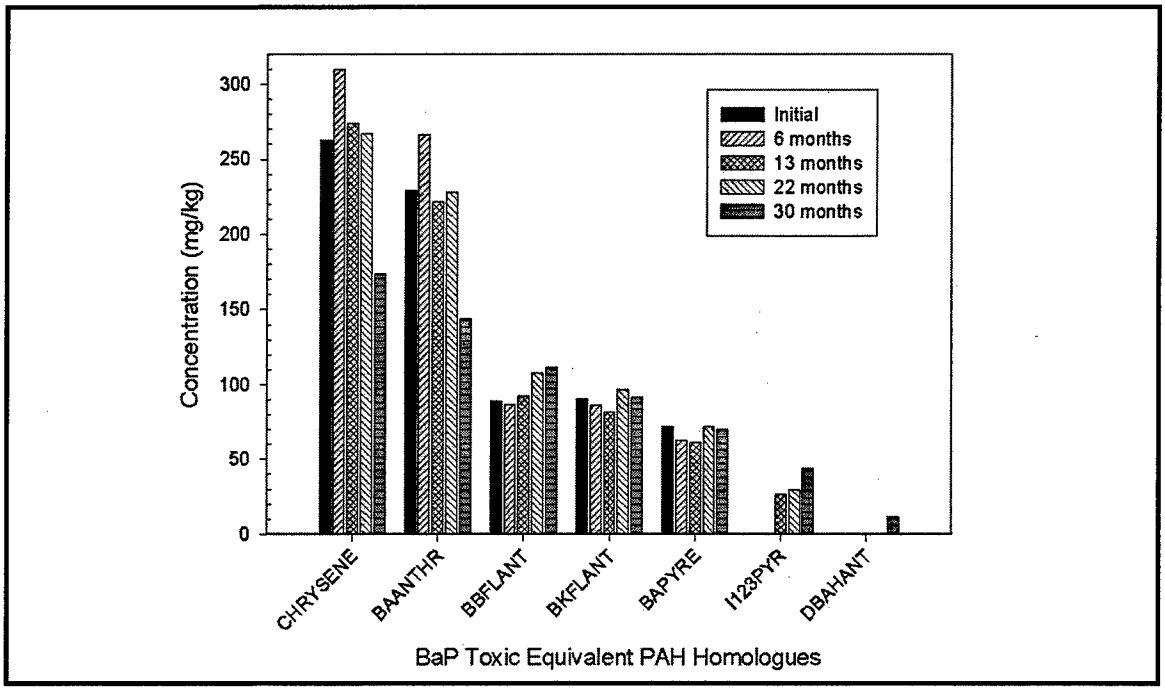


Figure 25. LTU 1 BaP Toxic Equivalent Homologues

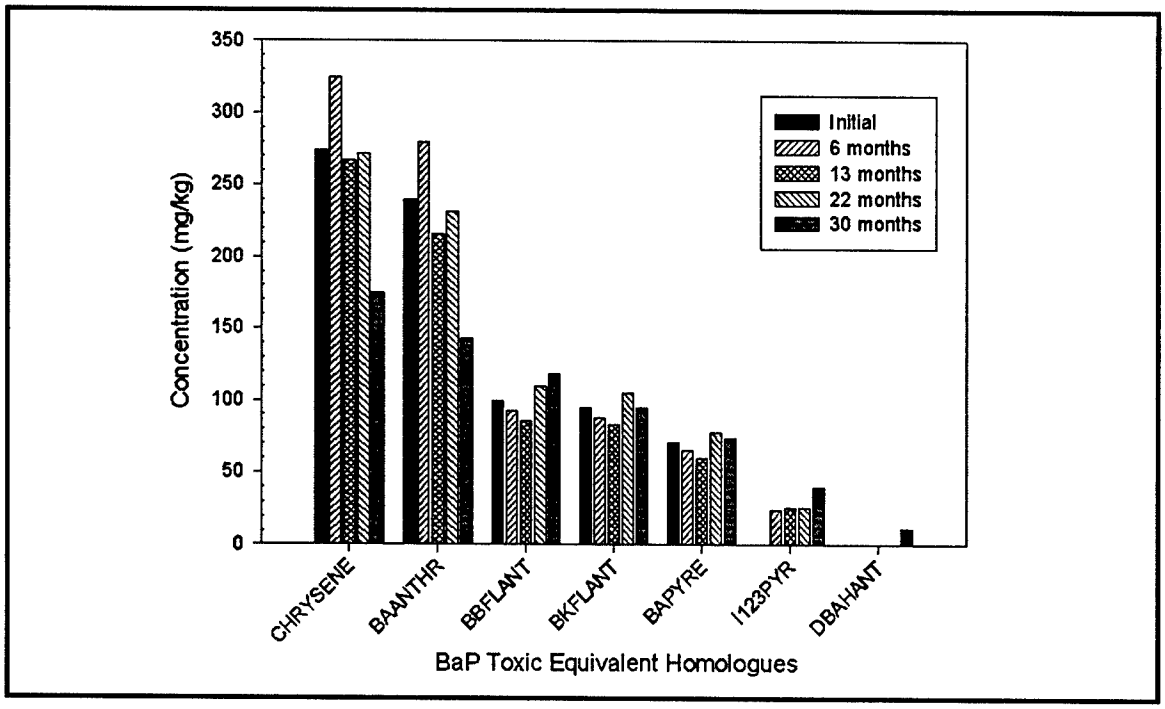


Figure 26. LTU 2 BaP Toxic Equivalent Homologues

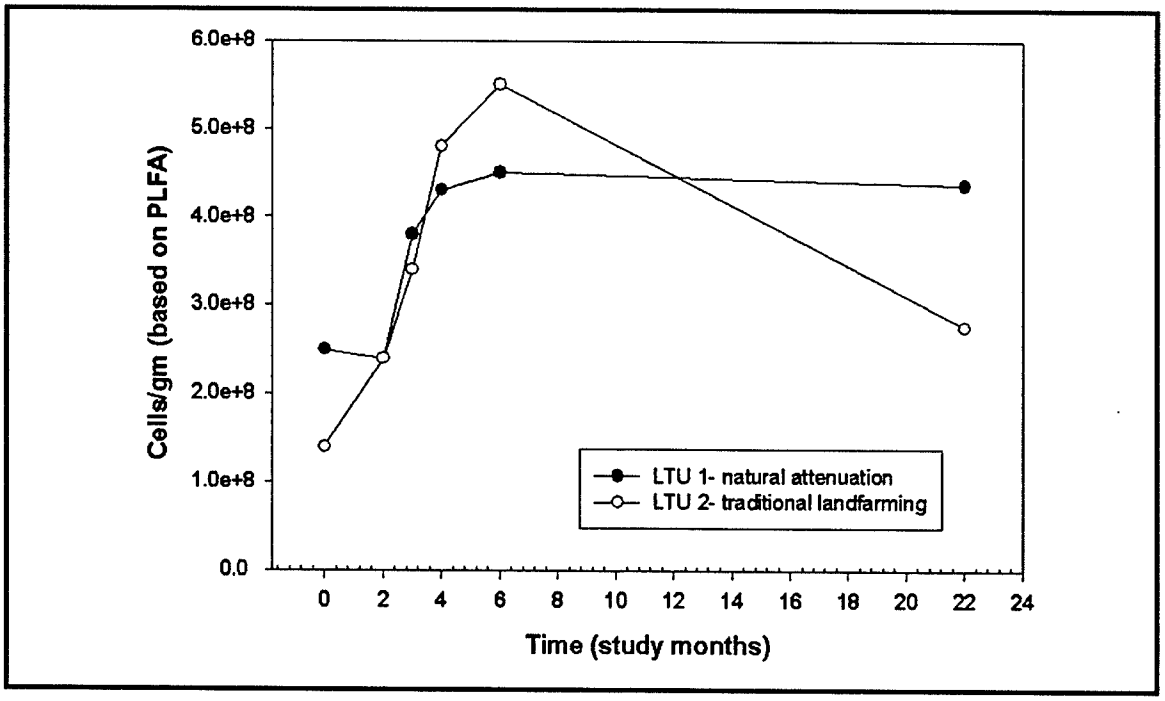


Figure 27. Changes in biomass in the LTUs

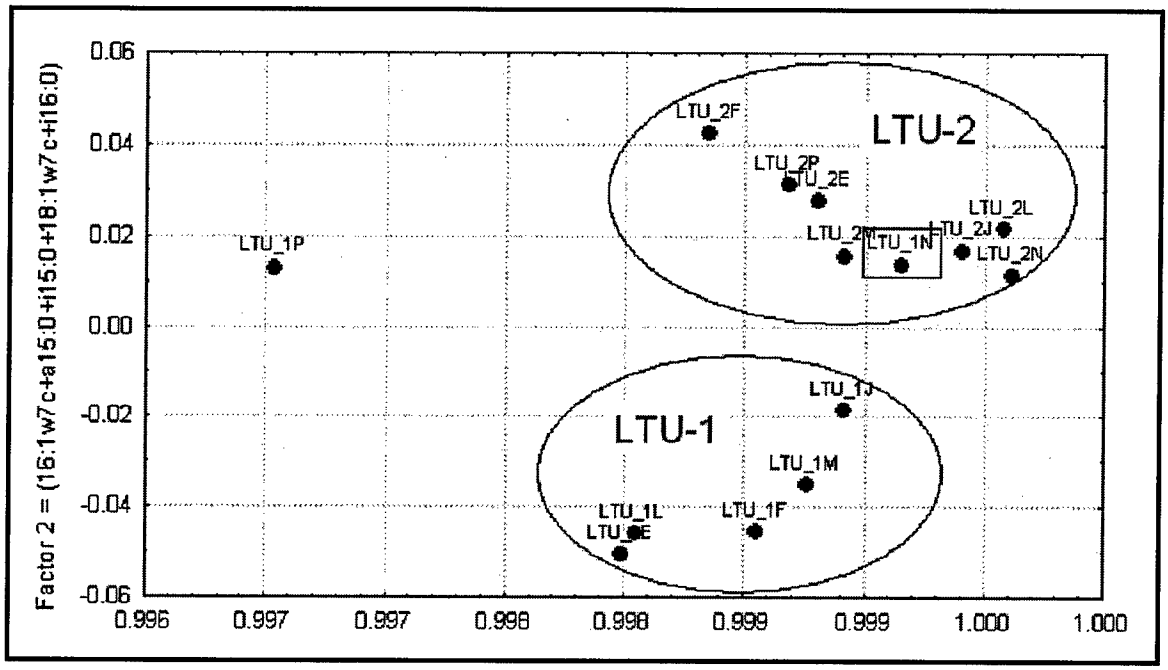


Figure 28. Final community composition in the LTUs by hierarchial cluster analysis

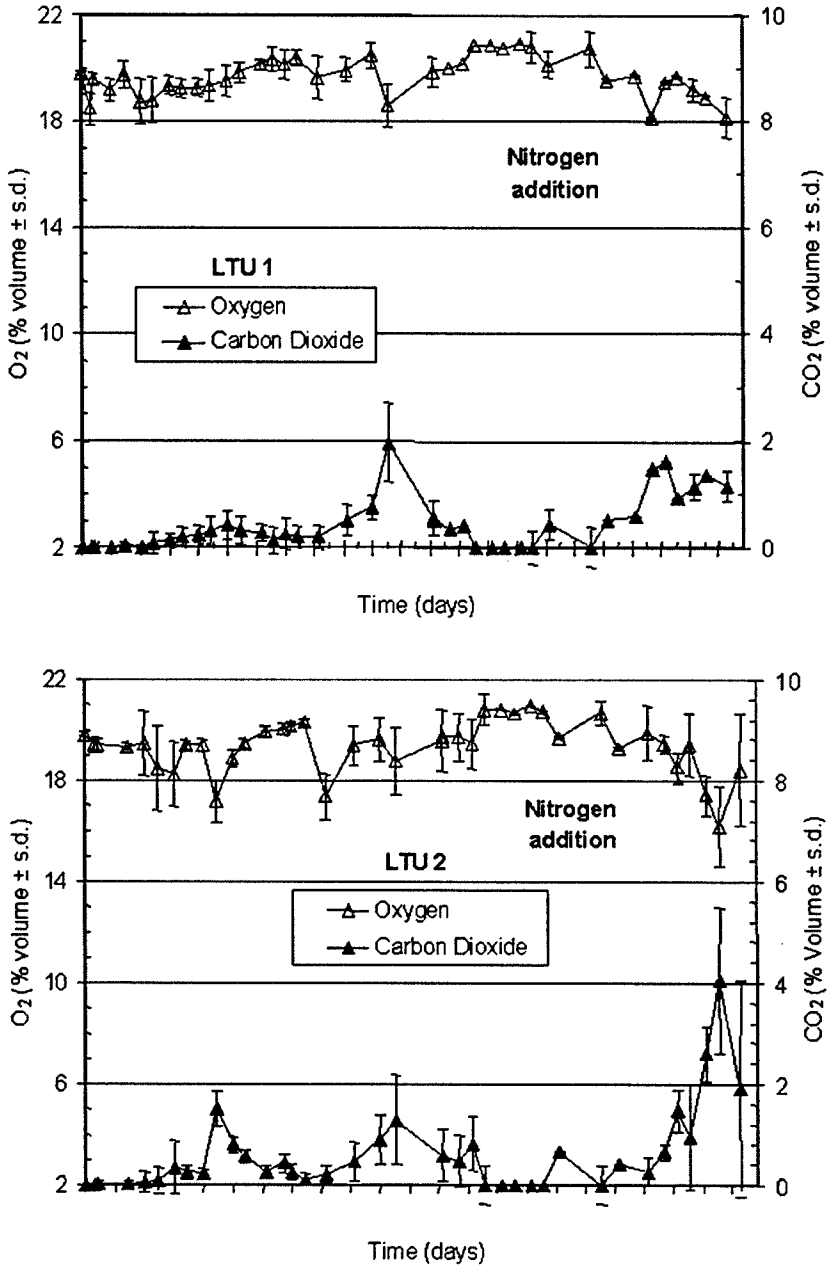


Figure 29. Respiration patterns recorded from the LTUs during the initial six month pilot study illustrating microbial response to tilling and nutrient addition

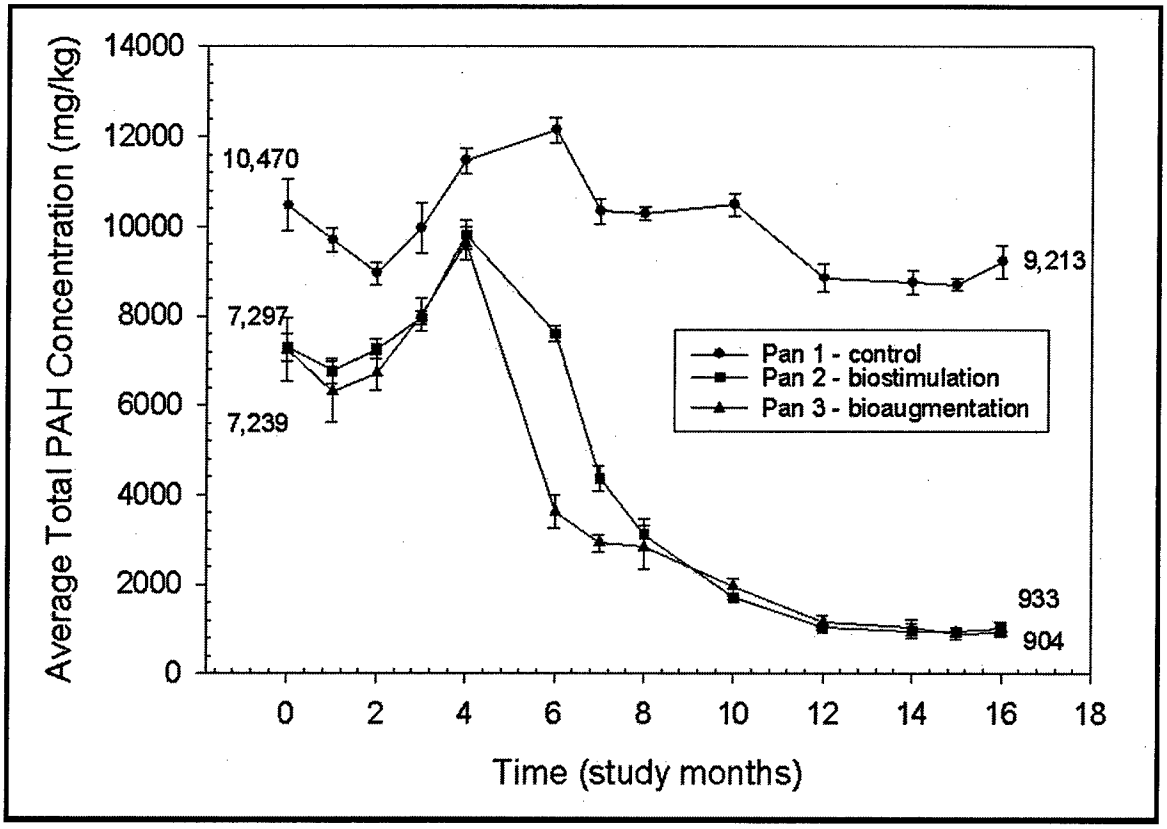


Figure 30. A comparison of treatment effect on PAH removal from the troughs

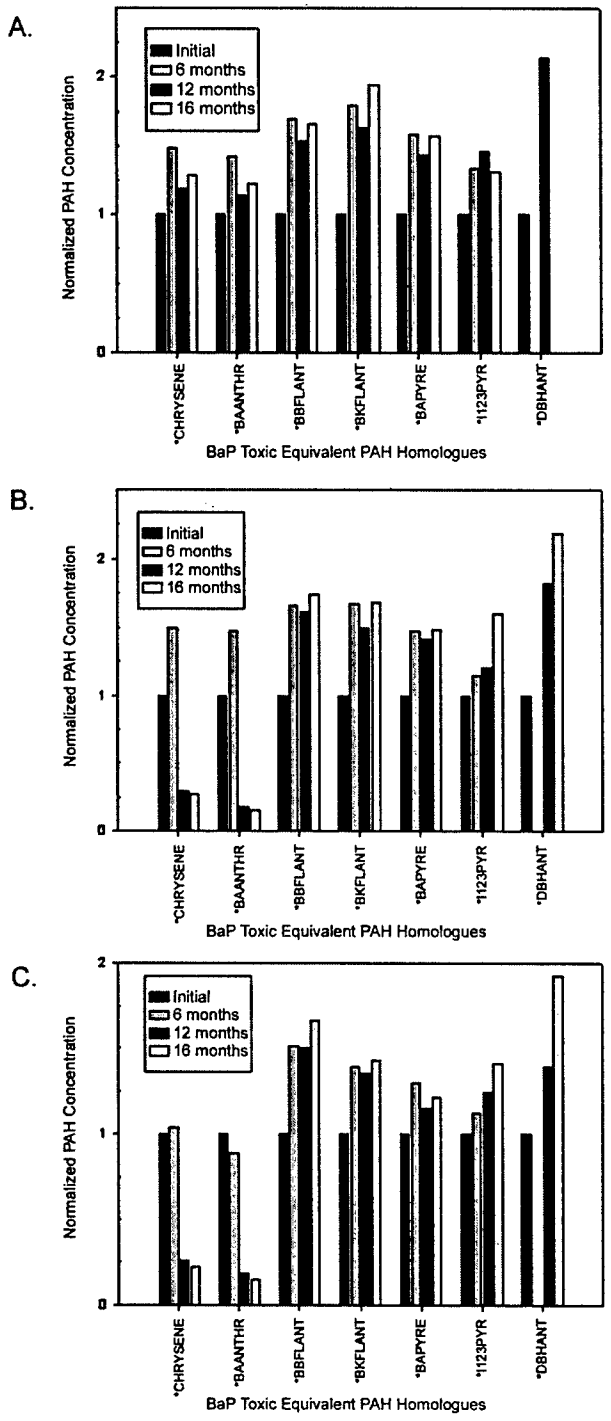


Figure 31. Changes in BaP TEQ compounds in the trough study

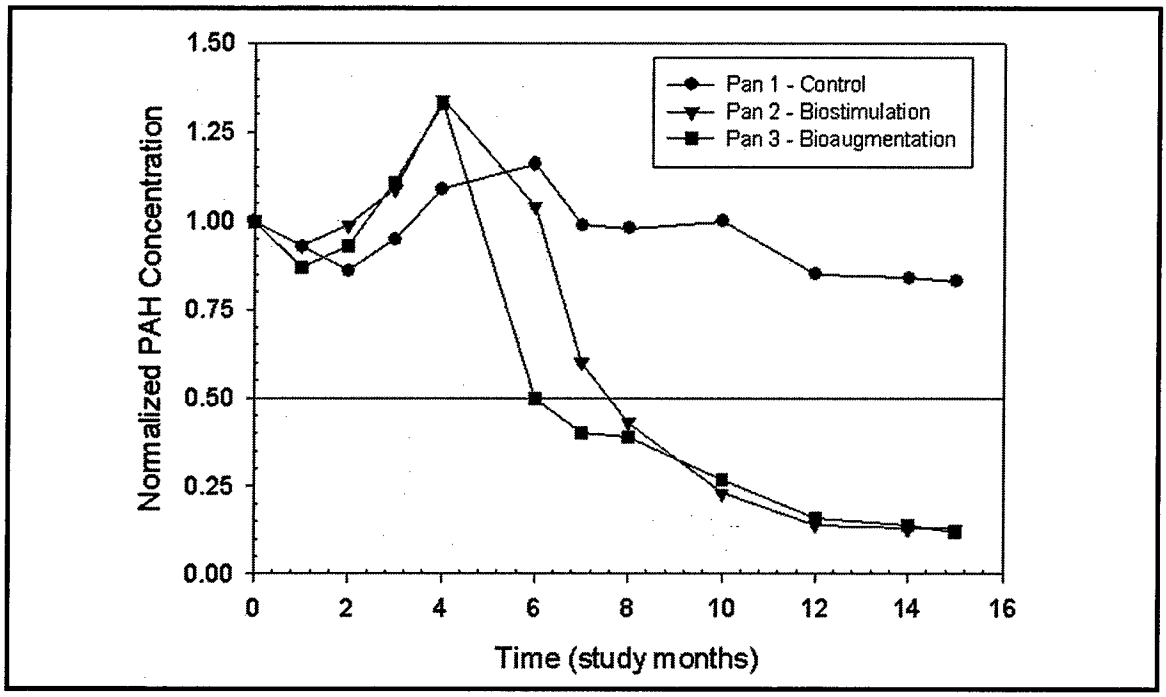


Figure 32. Normalized concentrations of PAH removal in troughs

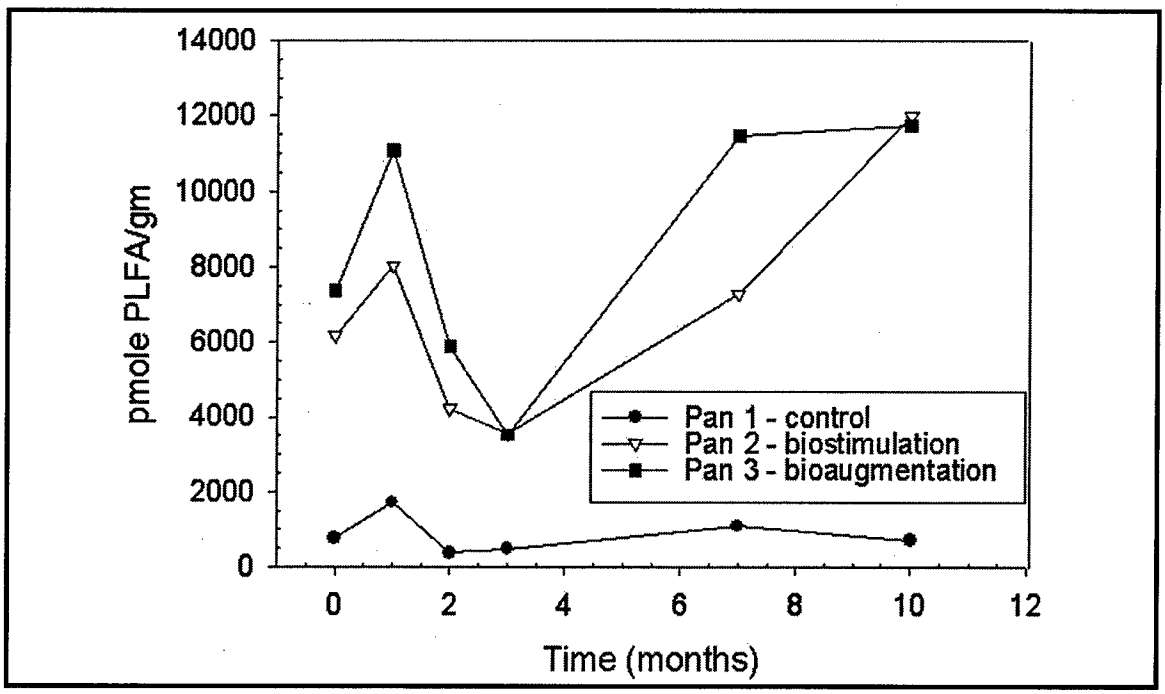


Figure 33. Effect of different amendments on biomass in trough pilot study

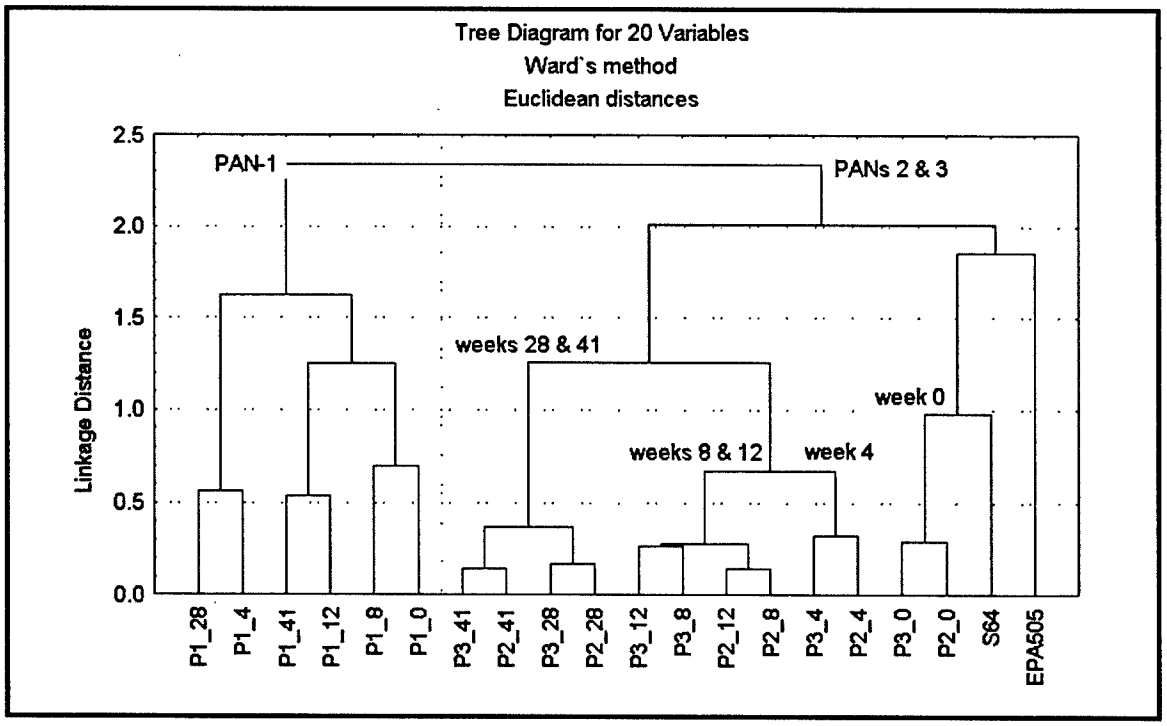


Figure 34. Effect of different amendments on community composition as demonstrated by PLFA hierarchial cluster

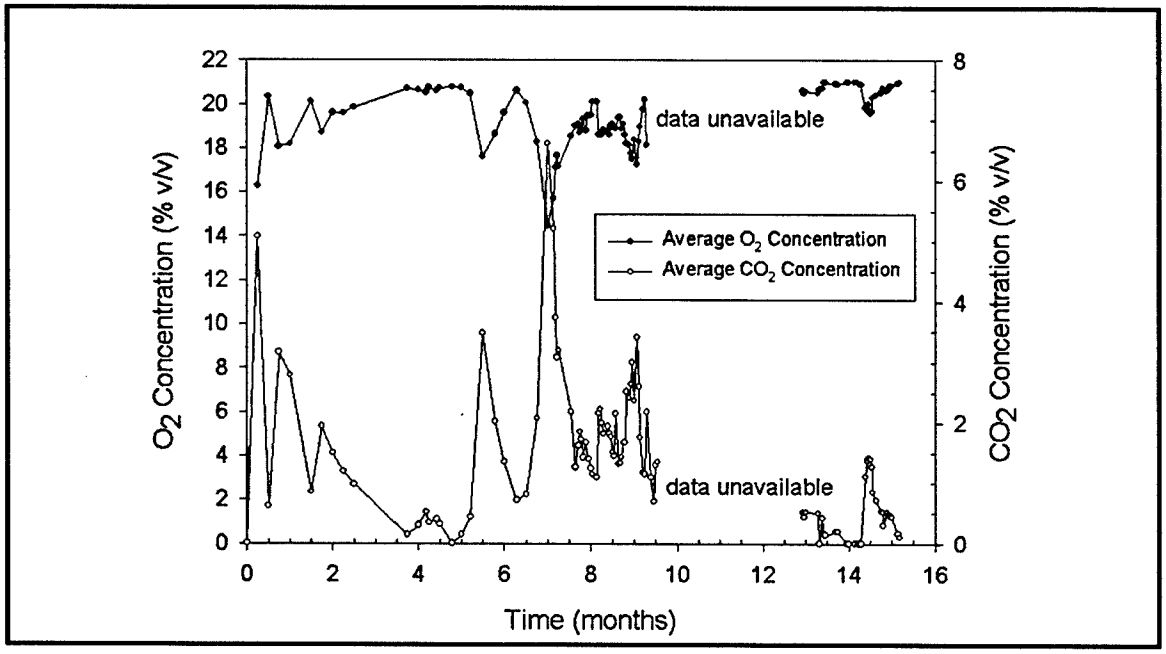


Figure 35. The microbial respiration observed over 16 months of treatment in Trough 2 (biostimulation).

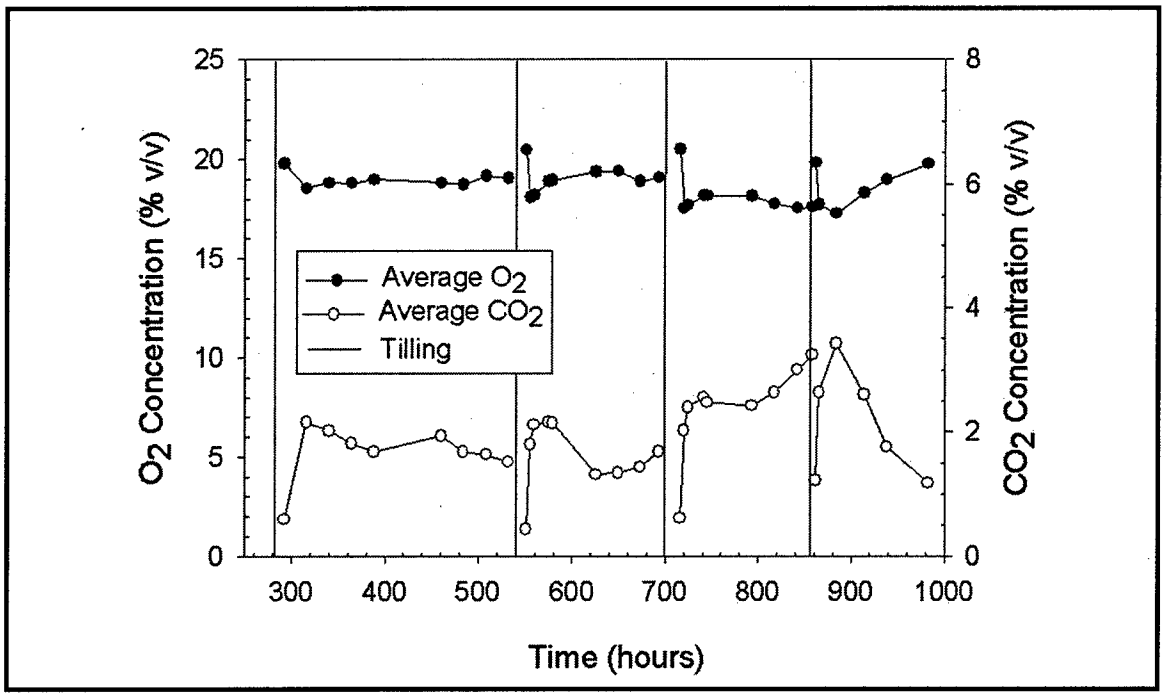


Figure 36. The effect of tilling on microbial respiration in Trough 2

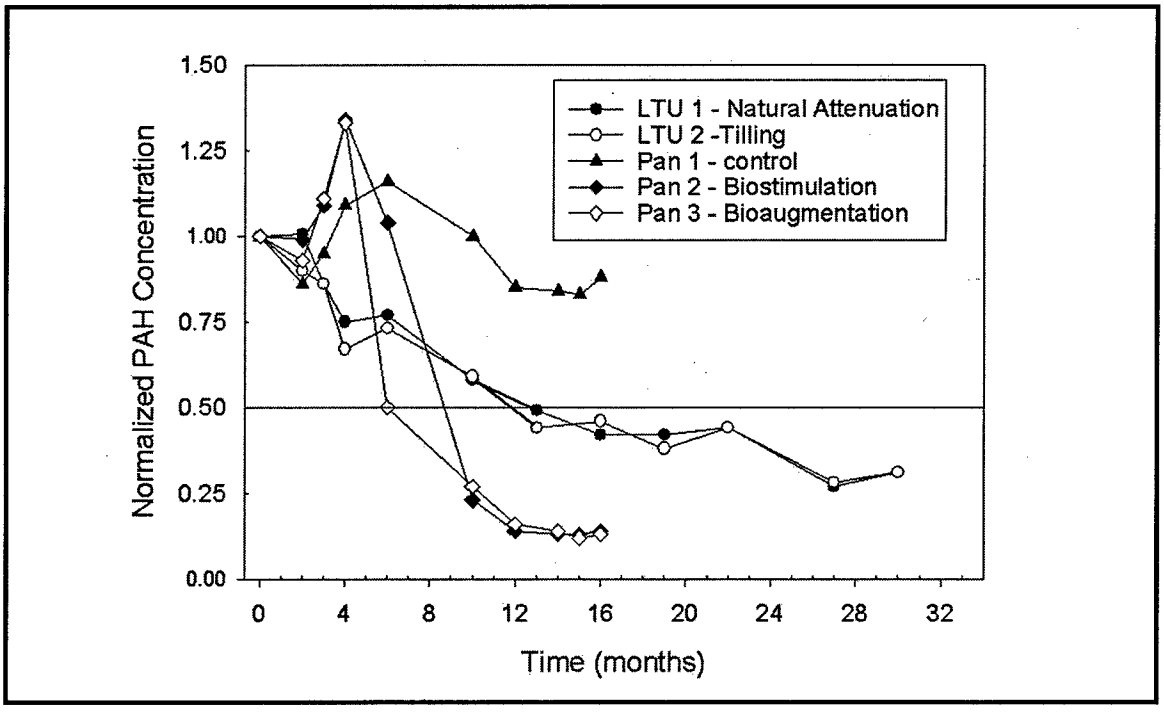
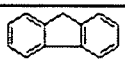
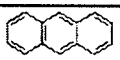
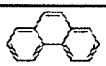
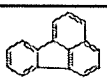
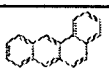
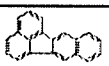
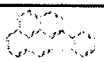


Figure 37. Comparison of PAH removal in LTUs and troughs

Table 1
Molecular Weights and Structures for the 16 PAH Homologues Analyzed During this Study

PAH homologue	Abbreviation	Molecular weight (amu)	Structure
Naphthalene	NAPHTH	128	
Acenaphthylene	ACENAY	152	
Acenaphthene	ACENAP	154	
Fluorene	FLUORE	166	
Anthracene	ANTHR	178	
Phenanthrene	PHENAN	178	
Fluoranthene	FLANT	202	
Pyrene	PYR	202	
*Benzo(a)anthracene	BAANTHR	228	
*Chrysene	CHRYS	228	
*Benzo(k)fluoranthene	BKFLANT	252	
*Benzo(a)pyrene	BAP	252	
*Benzo(g,h,i)perylene	BGHIPY	276	
*Indeno(1,2,3-c,d)pyrene	I123PY	276	
Dibenzo(a,h)anthracene	DBAHANT	278	

* Indicates a BaP Toxic Equivalent compound. See Table 2 for values.

Table 2
Toxic Equivalency Factors for the Seven PAHs of Greatest Environmental Significance

Compound (abbreviation)		Toxic Equivalent Factor (by Nisbet and LaGoy, 1992)
Benzo(a)anthracene	(BAANTHR)	0.1
Chrysene	(CHRYSE)	0.01
Benzo(b)fluoranthene	(BBFLANT)	0.1
Benzo(k)fluoranthene	(BKFLANT)	0.1
Benzo(a)pyrene	(BAP)	1.0
Indeno(1,2,3-c,d)pyrene	(I123PYR)	0.1
Dibenzo(a,h,)anthracene	(DBAHANT)	1.0

Table 3
Summary of Existing Treatment Options for PAH-Contaminated Soil

Technology	Examples	Benefits	Limitations	Factors to Consider
Solid Phase	Land farming Composting Engineered soil cell Soil treatment	Cost Efficient Modestly effective for HMW PAHs Low O&M costs Perform on site, in place	Space requirements Extended treatment time Control of abiotic loss Mass transfer Bioavailability	Catabolic capabilities of indigenous microflora Presence of metals and other organics pH, temperature, moisture control Biodegradability
Bioreactors	Aqueous reactors Soil slurry reactors	Most rapid degradation Controlled conditions Enhanced mass transfer Use of surfactants and inoculants	Material input requires physical removal Relatively high capitol costs	Same as solid-phase Toxicity of amendments Toxic concentrations of contaminants
In situ	Biosparging Bioventing GW circulation *In situ* bioreactors	Least cost Non-invasive Complements natural attenuation processes Soil/water treated simultaneously	Physicochemical control Extended treatment time Monitoring progress and effectiveness	Same as solid-phase Chemical solubility LNAPL/DNAPL present Geological factors Regulatory aspects for groundwater

(Modified from Mueller at al., 1997a)

Table 4
Amount of PAHs Remaining Following Biodegradation of PAH Fraction by Selected Strains of PAH Degraders

Isolate/Strain	2-Ring PAHs	3-Ring PAHs	4,5,6-Ring PAHs	Total PAHs
Initial	149.5 (0)	168.1 (0)	60.0 (0)	377.6 (NA)
Uninoculated	71.3 (NA)	107.0 (NA)	55.9 (NA)	234.1 (NA)
CRE 7[1]	1.7 (98)	14.8 (86)	47.2 (16)	63.7 (73)
N3P2[2]	0.5 (99)	4.9 (95)	46.1 (18)	51.5 (78)
UN1P1[2]	0.5 (99)	3.4 (97)	43.0 (23)	46.9 (80)
EPA 505[2]	0.3 (100)	2.2 (98)	18.6 (67)	21.1 (91)
PRY-1[3]	4.8 (93)	70.6 (34)	40.3 (28)	115.7 (51)

[1] Phenanthrene degrader
[2] Fluoranthene degraders
[3] Pyrene cometabolizer
NA indicates.

Table 5
Mass Balance of Radioactivity and Fluoranthene after 48 h Incubation of EPA 505 Cells with Different Concentrations of Triton X-100

Triton	Recovery of Radioactivity	Soluble Radioactivity	Cell Mass Radioactivity[a]	Carbon Dioxide Radioactivity	FLA Residual (mg)
0%	81	4	54	42	4.73
0.005%	100	4	48	48	0.25
0.03%	95	9	28	63	0.15
0.1%	95	12	26	62	< 0.005
2.0%	96	26	16	58	NA
Killed Cells	100	<1	99	<1	30.3

[a] Radioactivity retained on 0.22μ filter.
NA, not analyzed (surfactant concentration too high for GC analysis)

Table 6
Comparison of the Mass Solubility Ratios of Several PAHs in Commercial and Biosurfactants

Compound	Chemical Surfactant Triton X-100	Biosurfactant *P. aeruginosa* st. 64 rhamnolipid[a]
Phenanthrene	7.0×10^{-2}	1.3×10^{-1}
Pyrene	7.5×10^{-3}	2.9×10^{-2}
Fluoranthene	6.5×10^{-4}	3.8×10^{-3}
Benzo(a)Pyrene	N.D.[b]	3.8×10^{-3}
Chrysene	N.D.[b]	1.1×10^{-3}

[a] Lichenysin, lipopeptide biosurfactant from *B. licheniformis* ATCC 39307, was tested only with phenanthrene and the MSR was 1.1×10^{-1}.
[b] too low for detection

Table 7
Initial Characterization of the Original POPILE Soil Used in the Pilot Studies.

Test/Compound	POPILE Soil
Atterburg Limits	Plastic limit 17 Liquid limit 23 Classification: clay/silt
Particle size distribution	Sand 33% Fines 67%
Metals (mg/kg)	
Iron-combined forms	10,500
Magnesium	3,800
Barium	700
Zinc	30
Lead	10
Mercury	<1

Table 8
PAH-Degrading Microorganisms Isolated from PAH-Contaminated Soil and Characterized by PLFA Analysis

Sample Location	Contaminant	FLA[1] Degraders	PYR[2] Degraders
1. Gainesville, FL	Creosote	FLA 1-1*	PYR 1-1
2. Nashua, NH	Creosote	None	PYR 2-1
3. Berlin, Germany	Diesel Fuels	FLA 3-1, FLA 3-2, FLA 3-3	PYR 3-1, PYR 3-2
4. Hocomonco Pond, MA	Creosote	FLA 4-1*, FLA 4-2	None
5. Pensacola, FL	Creosote	FLA 5-1, FLA 5-3*	PYR 5-1, PYR 5-3
6. Pensacola (ACW), FL	Creosote	FLA 6-1*, FLA 6-2	None
7. Fence Post, FL	Creosote	FLA 7-1	PYR 7-1
8. Tyndall AFB, FL	Diesel & jet fuel	FLA 8-1	None
9. Port Newark, NJ (sediment)	Fuel	FLA 9-1*	PYR 9-1
10. Port Newark, NJ	Fuel	FLA 10-1*, FLA 10-3	PYR 10-1
11. Rade, Norway(#1)	Creosote	FLA 11-1	PYR 11-1, PYR 11-2, PYR 11-3
12. Rade, Norway (#2)	Creosote	FLA 12-1, FLA 12-2	PYR 12-1, PYR 12-2
13. Lillestrom, Norway (#3)	Creosote	FLA 13-1*	PYR 13-1, PYR 13-2
14. Lillestrom, Norway (#4)	Creosote	FLA 14-1	PYR 14-1
15. Drammen, Norway	Creosote	FLA 15-1*, FLA 15-2, FLA 15-3	PYR 15-1, PYR 15-2
16. Hommelvik, Norway	Creosote	None	PYR 16-1, PYR 16-2
17. Boston, Ma (sediment)	Fuel	UMB*	
18. Boston, Ma (sediment)	Fuel	CO6*	
19. Pensacola, FL	Creosote	EPA 505*	

[1] FLA: fluoranthene degrader.
[2] PYR: pyrene degrader

Table 9
Concentrations of PAHs in Soils Used To Evaluate the Effectiveness of Biosurfactant for Desorption

PAH Homologue	Concentration (mg/g)		
	Southern Maryland Wood Treatment Site[a]	Popile Wood Treatment Site (Arkansas)	Land Treatment Units (LTU)
Naphthalene	0.5	2273	92
Acenaphthalene	7	19	11
Fluorene	14	1073	803
Anthracene	3	2080	1308
Phenanthrene	39	3810	2756
Fluoranthene	21	1880	1600
Pyrene	15	1237	991
Benzo(a)anthracene	3	291	225
Chrysene	3	324	261
Benzo(b)fluoranthene	1	101	97
Benzo(F)fluoranthene	1	96	85
Benzo(a)pyrene	1	76	65
Indenopyrene	<0.5	29	24
Total PAHs	236	1519	9150
PCP	280	2307	2340

[a] GC-MS analysis done by Dr. Sabine Apitz and Kathy Meyers-Schulte at SPAWARSYSCEN in San Diego, CA.

Table 10
Fertilizers Investigated as Sources of Carbon and Nitrogen Supplementation for PAH Degradation Through Biostimulation

Fertilizer	Formulation (% nutrient, N;P;K)
Miracle Gro Plant Food	15:30:15
Acid Plant Food	30:10:10
Bone meal	4:12:0
Dried blood meal	12:0:0

Table 11
Experimental Design of the Flask (microcosm) Study

Variable	Control	Test A	Test B	Test C
Contaminated Soil (100 g)	X	X	X	X
Bulking agent; rice hulls (1:1)		X	X	X
Fertilizer; dried blood (2%)		X	X	X
Biosurfactant producer; *P. aeruginosa* strain 64 (2%)			X	X
HMW PAH degrader; *S .paucimobilis* strain EPA 505 (2%)				X

Table 12
Comparison of Experimental Design of the Pilot Studies

	Pilot Studies				
	LTUs		Troughs		
Variable	1	2	1	2	3
Initial PAH concentration (mg/kg)	13,000	13,000	10,500	7,000	7,000
Mixing/Tilling	**Phase 1**: initial and day 71 **Phase 2**: none	**Phase 1**: every 2 weeks **Phase 2**: quarterly	Weekly	Weekly	Weekly
Soil Moisture	**Phase 1**: 30 – 80% FMC **Phase 2**: Natural precipitation	Same as LTU 1	30-80% FMC	30-80% FMC	30-80% FMC
Nutrient addition	**Phase 1**: liquid and solid fertilizer 1x each, **Phase 2**: no addition	Same as LTU 1	No addition	Slow release, solid nitrogen	Slow release, solid nitrogen
Bulking agent,	No	No	No	Yes	Yes
Bioaugmentation	**No**	No	No	No	Yes
Respiration monitoring	**Phase 1**:yes **Phase 2**: no	Same as LTU 1	Yes	Yes	Yes

Table 13
Decrease in Total PAH, Individual Homologues and Total BaP Toxic Equivalent Compounds in POPILE Soil Following 11 Months of Treatment in Microcosms

	No Treatment (1/2)		Bulking Agent + Dried Blood Fertilizer		Bulking Agent + Dried Blood Fertilizer + *P. aeruginosa* strain 64	
PAH	**mg/kg**	**% Decrease[a]**	**mg/kg**	**% Decrease[a]**	**mg/kg**	**% Decrease[a]**
Acenapthene	201.0[c]	26.3	157.5[c]	42.2	0[e]	100.0
Fluorene	352.1[c]	9.9	251.7[d]	35.6	18.2[e]	95.3
Phenanthrene	1339.8[c]	37.1	1052.3[d]	50.6	42.7[e]	98.0
Anthracene	705.5[c]	17.3	739.7[c,e]	13.2	178.4[e]	79.0
Fluoranthene	779.0[c]	20.4	652.8[c]	33.3	43.9[e]	95.5
Pyrene	612.3[c]	0.2	517.8[c]	19.8	286.7[e]	55.6
Benzo[a]anthracene	148.0[c]	16.8	120.2[d]	32.5	36.5[e]	79.5
Chrysene	168.5[c]	7.7	153.5[c]	15.9	31.0[e]	83.0
Benzo[b]fluoranthene	65.4[c]	0.0	61.0[c,e]	0.0	52.2[e]	0.0
Benzo[k]fluoranthene	57.2[c]	0.0	57.2[c]	0.0	51.8[c]	0.0
Benzo[a]pyrene	48.4[c]	0.0	39.8[c,e]	1.0	35.6[e]	11.3
Total PAH	4477.3	22.6	3803.7	34.3	777.2	86.6
Total BaP Equivalents	70.5	48.4	58.7	57.1	45.1	67.1

[b] Average of 2 microcosms
[c] controls and the biostimulation treatment were the averages of 3 microcosms.

Table 14
Initial Nutrient Analysis of PAH-Contaminated Soil from the POPILE Site

Nutrient	Concentration (mg/kg)
Total Kejldahl nitrogen (TKN)	159±18
Nitrite nitrogen (NO_2-N)	1.88 *
Nitrate nitrogen (NO_3-N)	17±4
Ammonia nitrogen (NH_3-N)	4±1
Total phosphate (TP)	456±89
Ortho phosphate (OPO_4)	32±10
Total organic carbon (TOC)	28,672±3245

*estimated value, below machine detection limits

Table 15
PAH Reduction from Initial Concentration in LTU 1 and 2 Following 30 Months of Treatment

	LTU 1		LTU 2	
PAH	[Initial]	% Reduction	[Initial]	% Reduction
Naphthalene (2-ring)	2101±168	100	2186±252	98
Acenaphthene (3-ring)	878±59	79	940±103	75
Fluorene (3-ring)	980±81	84	1022±107	79
Phenanthrene (3-ring)	3169±221	83	3360±344	80
Anthracene (3-ring)	1453±317	68	1520±274	63
Fluoranthene (4-ring)	1460±76	49	1593±186	52
Pyrene (4-ring)	1030±123	43	1064±96	44
*Chrysene (4-ring)	263±19	29	274±30	33
*Benzo(a)anthracene (4-ring)	229±13	39	240±24	41
*Benzo(b)fluoranthene (5-ring)	89±10	NR	99±8	NR
*Benzo(k)fluoranthene (5-ring)	91±8	NR	95±7	NR
*Benzo(a)pyrene (5-ring)	72±7	4	71±4	NR
Total PAH	12,570±1,043	73	13,254±690	72

Note: All data is significant at the 95% confidence level, n=7
* denotes a BaP Toxic Equivalent compound
NR=no reduction

Table 16
Initial Physical Characterization and Nutrient Composition of the POPILE Soil Used in Control and Amended Troughs of the Bioaugmentation Pilot Study

Test/Compound	Trough 1	Trough 2	Trough 3
Cation exchange capacity (meq/100g)	13	23	25
Soil buffer capacity	4.57E-04	8.48E-04	2.91E-03
Field moisture capacity (FMC)	23%	45%	45%
pH	8	8	8
Total phosphate (TP)	500	600	600
Total organic carbon (TOC)	21,500	46,700	31,500

Table 17
Initial Soil Concentrations of PAHs in the Troughs

Contaminant	Trough 1	Trough 2	Trough 3
Naphthalene (2-ring)	938±59	40±13	14±5
2-methylnaphthalene (2-ring)	726±34	94±23	33±11
Acenaphthylene (3-ring)	19±1	14±1	14±1
Acenaphthene (3-ring)	837±43	682±21	685±65
Fluorene (3-ring)	969±55	780±28	782±87
Phenanthrene (3-ring)	2696±215	2156±87	2172±190
Anthracene (3-ring)	1129±50	1002±67	941±156
Fluoranthene (4-ring)	1498±118	1274±95	1288±121
Pyrene (4-ring)	947±48	707±31	744±78
*Chrysene (4-ring)	235±15	184±8	190±20
*Benzo(a)anthracene (4-ring)	226±10	173±9	175±17
*Benzo(b)fluoranthene (5-ring)	70±2	57±4	61±5
*Benzo(k)fluoranthene (5-ring)	64±4	53±3	56±4
*Benzo(a)pyrene (5-ring)	53±2	44±2	46±3
*Indeno(1,2,3)pyrene (6-ring)	24±0	21±1	22±1
*Dibenzo(a,h)anthracene (6-ring)	5±0	4±0	4±0
Benzo(g,h,i)pyrene (6-ring)	15±1	12±1	12±1
Total PAH	10,470±577	7297±314	7239±716
Total BaP Equivalents	91	74	77

Note: a * indicates a BaP Toxic Equivalent compound
error is calculated at the 95% confidence level, n=5

Table 18
PAH Reduction in Trough 3 by Individual Homologue as Well as Total PAH and Total BaP TEQ

Contaminant	[Initial]	6 months	12 months	16 months
Naphthalene (2-ring)	14	64%	100%	100%
2-methylnaphthalene (2-ring)	33	78%	82%	100%
Acenaphthylene	14	68%	100%	100%
Acenaphthene (3-ring)	685	90%	99%	100%
Fluorene (3-ring)	782	92%	95%	97%
Phenanthrene (3-ring)	2172	88%	95%	97%
Anthracene (3-ring)	941	25%	60%	72%
Fluoranthene (4-ring)	1288	33%	89%	92%
Pyrene (4-ring)	744	NR	82%	84%
*Chrysene (4-ring)	190	NR	**74%**	**78%**
*Benzo(a)anthracene (4-ring)	175	**11%**	**82%**	**85%**
*Benzo(b)fluoranthene (5-ring)	61	NR	NR	NR
*Benzo(k)fluoranthene (5-ring)	56	NR	NR	NR
*Benzo(a)pyrene (5-ring)	46	NR	NR	NR
*Indeno(1,2,3-c,d)pyrene	22	NR	NR	NR
*Dibenzo(a,h) anthracene	4	**100%**	NR	NR
Benzo(g,h,I)perylene	12	NR	NR	NR
Total PAH	7239	50%	84%	87%

NR=no reduction

Table 19
Summary of Conditions and Results in Each Pilot Study

System	Conditions	Removal Summary
LTU 1	Control No tilling	Good total removal Slow rate
Trough 1	Control Weekly tilling	Poor removal, Different microbial community than LTU 1
LTU 2	Tilling biweekly for 6 months, followed by tilling quarterly	Good total removal, slow rate Different microbial community than LTU 1
Trough 2	Nutrients, bulking agent Tilling weekly	Good total removal, faster than LTUs Different microbial community than LTUs
Trough 3	Nutrients, bulking agent Bioaugmentation Tilling weekly	Good total removal, faster than LTUs Similar microbial community to Pan 2.

Attachment: Technology Transfer Documents

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Appendix C Chlorinated Solvent Contaminated Soils and Groundwaters: Field Application of the Solvent Extraction Residual Biotreatment (SERB) Technology

Strategic Environmental Research and Development Program

Chlorinated Solvent Contaminated Soils and Groundwaters: Field Application of the Solvent Extraction Residual Biotreatment (SERB) Technology

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Draft Report

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Preface

Project Title

Chlorinated Solvent-Contaminated Soils and Groundwater. Field Application of the Solvent Extraction Residual Biotreatment (SERB) Technology.

Performing Organizations

This project was conducted under the guidance of Dr. Guy W. Sewell (USEPA/NRMRL/SPRD-Ada, OK) with assistance from Ms. Susan C. Mravik and Dr. A. Lynn Wood. Dr. Michael Annable and associates from the University of Florida-Gainesville and Dr. Randy Sillan and Mr. Kevin Warner of LFR Levine Fricke conducted the cosolvent extraction test. LFR Levine Fricke was the site contractor and responsible for coordinating activities at the site and ground water sampling. Dr. James M. Tiedje from Michigan State University conducted the microbial ecology studies.

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1 Project Background

Chlorinated solvents were used and released to the environment in massive amounts during the 1950's, 60's, and 70's. These contaminants have migrated through the subsurface and impacted ground water at over 1000 DoD sites. Their widespread use and the physical/chemical properties of these compounds have resulted in the chloroethenes being the most commonly detected class of organic contaminants in ground water. Parent chloroethenes can become human health hazards after being processed in the human liver or via reductive dehalogenation in the environment. This has generated a high degree of interest in efficient and cost effective technologies to remediate soils and ground waters contaminated with tetrachloroethylene (PCE) and trichloroethylene (TCE).

Remedial technologies for chlorinated solvents have progressed in three major phases since the environmental challenge they present was recognized during the 1970s. Up until the late 1980s, physical-based technologies such as pump-and-treat for dissolved phase contaminants and excavation (where possible) for residual phase materials were utilized to address this problem. The limitations of these approaches both in terms of efficacy and performance, as well as a more developed understanding of the nature of DNAPL source areas and plume dynamics, precipitated the research and testing of second phase technologies, such as source zone treatment by mobility agents (co-solvents, surfactants, steam), reactants for source zones (permanganate) and dissolved phase (permeable reactive barriers), and biological-based technologies focused on the dissolved phase. We are entering a third phase of technology development driven by the twin forces of risk management and cost-to-restoration. This third phase of technology development is characterized by multiple technology application and focused on comprehensive site restoration approaches.

In-Situ Flushing Technology

Nonaqueous phase liquids (NAPLs), such as fuels, oils, and industrial solvents, may act as long-term sources of groundwater pollution when released into aquifers because of their low aqueous solubilities. Dense nonaqueous phase liquids (DNAPLs) are denser than water and are more difficult to remediate because of their tendency to sink and pool in the aquifer. Conventional remediation such as pump-and-treat can take many decades to remove DNAPLs (Mackay and Cherry, 1989). Enhanced source-zone remediation can expedite the removal of contaminants. One enhanced source zone remediation technique is *in situ* cosolvent flushing, which involves the addition of miscible organic solvents to water to increase the solubility or mobility of the NAPL (Imhoff et al., 1995; Falta et al., 1997; Lunn and Kueper, 1997; Rao et al., 1997;

Augustijn et al., 1997; Lowe et al., 1999). In the case of DNAPLs, increased mobility can result in greater contaminant risk due to the potential for downward migration, and density modification of the NAPL has been proposed to prevent this risk (Roeder et al., 1996; Lunn and Kueper, 1997; Lunn and Kueper, 1999). Alcohols have principally been used as cosolvents for enhanced source zone remediation (Lowe et al., 1999).

In situ cosolvent flushing provides four primary advantages over the traditional pump-and-treat remediation approach (Augustijn et al., 1994a,b; 1996): 1) enhanced solubilization as a result of reduced polarity of the displacing fluid, 2) enhanced mobilization of the NAPL as a result of reduced interfacial tension between the NAPL and the cosolvent and because of swelling of the NAPL ganglia by uptake of cosolvent, 3) enhanced desorption and a concomitant reduction in retardation, again due to an increase in solubility, and 4) increased dissolution/desorption kinetics due to an increase in the mass transfer rate constant, and an increase in the driving force (i.e., the concentration gradient).

A number of laboratory studies have examined the dissolution and mobilization of NAPL by cosolvents (Luthy et al., 1992; Brandes and Farley, 1993; Peters and Luthy, 1993, 1994; Milazzo, 1993; Imhoff et al., 1995; Roy et al., 1995; Lunn and Kueper, 1996, 1997). These studies have shown that NAPL may be effectively removed using cosolvents such as alcohols. The laboratory studies have also shown that cosolvent floods can be designed to favor either enhanced dissolution or mobilization by judicious selection of the organic cosolvent. For systems designed to promote NAPL dissolution (as with the study reported herein), the solubility enhancement as a result of the addition of the organic cosolvent can be estimated using the log-linear cosolvency model (Yalkowsky and Roseman, 1981; Fu and Luthy, 1986a; Morris et al.,1988). The solubility of a nonpolar organic solute in a binary solvent mixture (S_m) increases in a log-linear manner with increasing volume fraction of cosolvent (f_c):

$$\text{Log } S_m = \log S_w + \beta \sigma f_c$$

where

$$\sigma = \log (S_c/S_w)$$

β is an empirical coefficient that accounts for water-cosolvent interactions; S_c is solubility in a neat solvent; S_w is solubility in water; and σ is the cosolvency power.

Technology Status: Cosolvent Flushing

A limited number of field-scale, cosolvent flushing demonstrations have been conducted. Two cosolvent flushing demonstrations were conducted at Hill Air Force Base (AFB), Utah in isolated test cells installed in a sand and gravel aquifer contaminated with a multi-component NAPL (Rao et al., 1997; Sillan et al., 1998a; Falta et al., 1999). Rao et al. (1997) demonstrated NAPL remediation by enhanced dissolution. The test cell was approximately 4.3 m long by 3.6 m wide, and the clay confining unit was 6 m below grade. A total of 40,000 L of a ternary cosolvent mixture (70% ethanol, 12% pentanol, and 18% water) was injected into the cell over a ten-day period. Based on several remediation performance measures (target contaminant concentrations in soil cores, target contaminant mass removed at extraction wells, and pre- and post-flushing target contaminant groundwater concentrations), the cell-averaged reduction in contaminant mass

was reported as >85%. They also reported an approximate 81% reduction in NAPL saturation based on pre- and post-flushing partitioning interwell tracer tests (PITTs). Falta et al. (1999) presented results from a second cosolvent flushing study at Hill AFB wherein the remedial mechanisms were NAPL mobilization and enhanced dissolution. Their test cell was approximately 5 m long by 3 m wide, and the clay confining unit was 9 m below grade. They injected 28,000 L of a ternary cosolvent mixture (80% tert-butanol, 15% n-hexanol, and 5% water) over a 7-day period. Reductions in target contaminant concentrations measured from pre- and post-flushing soil cores were reported to range from 70% to >90%, and an 80% reduction in total NAPL content was reported based on pre-and post-flushing PITTs.

Brooks et al. (2001) described a third cosolvent flushing field demonstration at the Dover National Test Site (DNTS) at Dover Air Force Base, Delaware. This test was conducted in an isolated test cell into which a known volume of PCE had been injected. The dimensions of the cell were approximately 5 m by 3 m by 12 m deep, with a clay layer forming the lower boundary of the cell. DNAPL was injected into the formation at approximately 10.5 m below the surface, creating a source zone of approximately 1.5 m thickness. A total of 41,700 L of 95 % ethanol were used during this flushing demonstration. Upon extraction from the test cell, the cosolvent solution was treated on-site to remove PCE and re-injected into the test cell. Because of this recycling system, a total of 69,700 L of ethanol were displaced through the test cell. Based on mass balance measurements 71% of the resident PCE was removed by this flushing process.

These results and others have indicated that PCE and TCE constituted DNAPLs are amenable to extraction based mass reductions. However, while the potential for substantial mass removal have been demonstrated, the debate over the relative and absolute benefit of partial source removal, in terms of costs and risk reduction, is currently unresolved. It is clear that even under the best of extraction scenarios, residual amounts of contaminants will remain at levels which could preclude meeting regulatory requirements for site closure.

Biotransformation of Chlorinated Solvents and Subsurface Microbial Ecology

Heterotrophic organisms (humans and most bacteria) oxidize organic compounds to obtain energy. In this process, electrons or reducing equivalents from the oxidizable organic compound (substrate/electron donor) are transferred to, and ultimately reduce an electron acceptor. The electron acceptor may be an organic or inorganic compound. During this electron transfer process, usable energy is recovered through a complex series of oxidation-reduction (redox) reactions by the formation of energy storage compounds or electrochemical gradients. The oxidation of organic compounds coupled to the reduction of molecular oxygen is termed aerobic heterotrophic respiration. When the oxidation of the organic compounds is linked to other electron acceptors (i.e., sulfate, nitrate, ferric iron, chloroethenes) by bacteria it is referred to as anaerobic respiration. The oxidation of the organic mater, linked to oxygen reduction or other electron acceptors, to yield energy and carbon compounds for microbial growth is termed oxidative catabolism.

Pristine (oxygenated) ground water environments are electron donor limited. When readily degradable organic matter enters the subsurface in sufficient quantities, it can produce a variety of chronologically and spatially defined metabolic zones. These zones are dependent on the availability of electron acceptors. As organic matter enters an oxygenated aquifer, whether it is a human produced (anthropogenic) contaminant or an influx of "natural" material, indigenous aerobic heterotrophic microorganisms metabolize the organic material and consume the available oxygen in the process. When this occurs, aerobic respiration slows and eventually stops. This allows for the development of anaerobic metabolic communities utilizing other electron acceptors. Under these conditions the subsurface environment becomes electron acceptor limited and the rates of transformations of the introduced organic(s) is controlled by the availability of the various electron acceptors. The introduction of the parent chloroethenes (PCE, TCE) does not supply electron donor to subsurface environments and thus will not drive ground water anaerobic. Chloroethenes, though, are often co-disposed with other organics, or may mobilize native or anthropogenic organics, which can serve as electron donors and can be biologically utilized for the reduction of dissolved oxygen, other anaerobic electron acceptors and/or the chloroethenes. It is important to remember that the reductive dechlorination process is one of several competing electron sinks and the amount of reducing equivalents which are utilized for dechlorination of chloroethenes is determined by the energetics and relative concentration of all of the available electron acceptors. The parent chloroethenes (PCE,TCE) are resistant to oxidative catabolism. This means that in the presence of oxygen the biodegradation potential of these compounds varies from not detected (for PCE) to low (for vinyl chloride). TCE and less chlorinated daughters can be degraded under co-metabolic conditions. However, since this process is not directly beneficial or may even be detrimental to the microorganisms and requires the presence of an inducer, its relevance is limited.

In the subsurface and other anaerobic environments, chloroethenes can be transformed by microorganisms through a process known as reductive dechlorination. This is a step-wise removal of chloride ions (Equation 1).

$$R\text{-}Cl_x + H_2 \rightarrow R\text{-}Cl_{x-1} + H^+ + Cl^-$$

Equation 1. Reductive Chlorination of Chloroethenes

This process transforms the chlorinated ethenes to non-chlorinated products (ethene, ethane), which do not pose a threat to human health or the environment. Halorespiration is the term used to describe the reductive dechlorination process when specific microorganisms obtain energy for growth using halogenated compounds, such as PCE, as terminal electron acceptors. Unfortunately the more mobile and toxic daughter products, such as dichloroethene (DCE) and vinyl chloride (VC), are intermediates in the halorespiration pathway (Figure 1). If the process "stalls," as it often seems to in the subsurface, before reaching non-chlorinated end products, the reductive dechlorination process may increase the potential risks to humans and the environment. Thus the reductive dechlorination process can exacerbate or attenuate the problems created by the release of chloroethenes to the subsurface and ground water environments. Under some conditions, the less chlorinated chloroethenes (vinyl chloride and perhaps DCE) may undergo oxidative catabolism under anaerobic conditions. This process may be an important mechanism for mass removal of vinyl chloride in locations where redox potentials increase, such as leading edges of contaminant plumes or areas with high dispersion or oxygen diffusion.

Technology Status: *In Situ* Bioremediation

Bioremediation and natural attenuation are two *in situ* technologies often used for remediation of dissolved phase contaminants. Bioremediation is defined as using the metabolic processes of microorganisms to degrade, detoxify or mineralize contaminants. Bioremediation in the context of contaminated subsurface environments (soil, sediments, ground water) usually involves the injection of nutrients to stimulate the desired biological processes. This is also referred to as bio-stimulation. A less common, although growing approach is to introduce or inject specific microorganisms, usually in combination with nutrients, which is a process referred to as bio-augmentation.

Natural attenuation is a remedial option, which takes advantage of the widely observed phenomenon that concentrations of contaminants in the subsurface tend to decrease over time. This is due to a combination of physical, chemical and biological processes, which degrade, immobilize or transform dissolved contaminants. For most contaminants, the most important of these attenuation mechanisms is biodegradation by native microorganisms. The relative treatment costs of these technologies is low, and in most cases lead to destruction of the target contaminant; however, these processes are often very slow and thus maybe impractical due to risk of exposure to the public from the potential of contaminants in ground water reaching water wells or surface water. The selection and implementation of both bioremediation and natural attenuation also suffer from a lack of familiarization by remedial managers, typically civil and environmental engineers, and to a degree the uncertainties as to long-term performance, particularly in the case of natural attenuation.

Beginning in the 1990's a number of successful demonstrations of bioremediation technologies were reported for chlorinated solvent sites. These included both active reductive dechlorination systems (Beeman Sewell/Pinellas), co-metabolic systems (Savanah River, Moffet), and later in the 1990's a variety of natural attenuations demonstrations (St. Joe, Dover). While these demonstrations fulfilled the proof of concept and lead to the development of defined protocols for chlorinated solvent sites (NACS, RABITT), a number of limitations on the use of biological treatment approaches became clear. Some of these limitations were applicable to all *in situ* bioremediation systems, including the challenges associated with delivery and mixing of nutrients, and rates of clean-up were often limited by subsurface transport rates rather than biological reaction rates. However some of the limitations were unique to DNAPL and chlorinated solvent sites. Active and passive bioremdiation technologies for oxidizable contaminants such as fuels and petroleum proved to be metabolically robust and redundant. This means that the genetic capability for the degradation of a variety of compounds was found to be present at almost all sites where toxicity was not a major issue. This catabolic capacity was found to exist for multiple populations and redox conditions, such that a variety of electron acceptor could be used in sequence to support degradation (Suflita and Sewell, 1991). However, for chlorinated solvents, the conditions that supported transformation were more limited and the capacity, at least for complete dechlorination, does not appear to be universal. These limitations resulted in much slower rates of activity and potential instability in active systems.

Another potential limitation is that the technologies are focused on dissolved phase contaminants and have not yet been shown to remediate areas contaminated with separate phase contaminants. It has been believed that the highly toxic nature of the separate phase residual contaminants

found in source zone environments suppresses bioactivity. Extensive research demonstrating the biological transformations of dissolved phase chloroethenes has been conducted (references). Recent research has shown that complete dechlorination can occur even with initial PCE concentrations as high as 91 mg/L (DiStefano, et al., 1991; Yang and McCarty, 2000). The slow transformation rates coupled to the low dissolution rates for DNAPLs and their significant residual mass leads to both very large plumes for treatment and extensive treatment times. These designed treatment times for chlorinated solvent sites can range from decades for active systems to centuries for natural attenuation systems.

Combining bioremediation with enhanced source removal, though, could be a mechanism for obtaining complete site restoration. With the bulk of the residual phase removed by enhanced source removal, *in situ* biotreatment could transform the remaining contaminants to non-hazardous compounds at a rate in excess of dissolution.

Flask to Field

Although the solvent flushing and bioremediation technologies have been shown in laboratory and limited field evaluations to enhance the remediation of contaminated aquifers, there has been no previous attempt to couple these technologies in the field. There are a number of factors that have not been fully evaluated for both the individual and coupled technologies. The high degree of spatial variability in the subsurface environment, the difficulty in obtaining *in situ* mixing of the remedial fluid, the changes in the hydraulic properties of the system as the DNAPL is removed, and the effects of high concentrations of remedial solvent mixtures on the microbial ecology are major factors that must be accounted for in the system design to ensure the success of implementation.

Beginning in the late 1990's EPA researchers began evaluating the effects of potential cosolvents and their breakdown products on the reductive dechlorination process (Gibson and Sewell, 1992; Gibson et al., 1994). Observations from the Hill AFB source control trials also indicated that native biological transformations were occurring during NAPL source removals. These early observations, laboratory experiments, and field tests supported the development of a process conceptual model and testable pilot system.

SERB Concept

The Solvent Extraction Residual Biotreatment (SERB) technology is a treatment train approach that combines both cosolvent extraction and *in situ* bioremediation to obtain complete site restoration. Advantages of this technology are:

- Rapid removal of large masses of DNAPL in a very short time
- In situ treatment train technique
- Cosolvent injection/extraction overcomes transport and mixing limitations of standard bioremediation infiltration and injection techniques

- DNAPL mass removal reduces toxicity to microbes for bioremediation
- Continued removal of dissolved contaminants following cessation of pumping
- Combination of an active process, Solvent Extraction, with an essentially passive process, Residual Biotreatment

The overall SERB process is presented in Figure 2, which shows the initial characterization of the site (Figure 2a), the application of the cosolvent flushing technology (Figure 2b), the potential for mixing of electron donor and electron acceptor (Figure 2c) and the creation of reactive zones where bioremediation can occur (Figure 2d).

Site characterization activities are used to identify the location and extent of the source area and whether separate phase DNAPL is present, but most often these procedures are not conducted to give the level of detail required for the successful application of many remediation technologies. Figure 2a shows the complex distribution of DNAPL that can exist in the subsurface and how the DNAPL moves through the aquifer due to gravity and pools on less permeable layers. Site characterization, ground water flow characterization, and optimization modeling must be conducted to determine the number and placement of injection and recovery wells for the cosolvent extraction process.

The selection of a cosolvent is based on the properties of contaminant to be removed, cosolvency properties, and regulatory constraints concerning injection of compounds. The cosolvent extraction process utilized in the SERB demonstration was based on the principle of enhanced dissolution of the contaminant into the cosolvent, which was then removed through the recovery/extraction wells. PCE was the contaminant at the SERB demonstration site and ethanol was selected as the cosolvent for enhanced dissolution. Some *in situ* extraction technologies are also designed to mobilize the residual contaminant, which is also removed through the recovery/extraction wells. Mobilization of the contaminant requires excellent hydraulic control, especially in the case of DNAPL contaminants, to ensure the DNAPL is not allowed to contaminate the aquifer further. Upon selection of the cosolvent and determination of the injection/extraction design, the cosolvent flushing test is conducted (Figure 2b). Maximum contact and mixing of the cosolvent and residual contaminant is the most important criterion in the design of the injection/extraction system for successful application of the technology. Because of the complex nature of the subsurface environment, it is known that some portions of DNAPL will not be contacted by the cosolvent and the majority of the flow will be through layers of higher hydraulic conductivity.

Following the application of the cosolvent extraction technology and cessation of active pumping, it is envisioned that there will be separate areas of DNAPL contamination and cosolvent remaining in the aquifer, but that there will be areas where the DNAPL and cosolvent are mixed (Figure 2c). The areas where the cosolvent (electron donor) and DNAPL (electron acceptor) are mixed contain the highest potential to stimulate bioremediation. The SERB technology application at the demonstration site was designed to enhance the reductive dechlorination of the residual DNAPL contaminant, PCE. Ethanol was selected as the cosolvent because it was both an acceptable cosolvent and an acceptable electron donor for the reductive dechlorination process.

Stimulation of the bioremediation processes should result in subsurface bioactive zones that will remove the contaminant from the ground water at a rate faster than it dissolves from the remaining residual DNAPL in the subsurface (Figure 2d). If this is achieved, complete site restoration is possible.

Mixing of the cosolvent and the contaminant for both dissolution and removal of the separate phase DNAPL and the subsequent biodegradation of the remaining contaminant is the most important criterion in the design of the injection/extraction system for the SERB technology. Delivery of the electron donor to the subsurface has been a problem for various *in situ* treatment technologies and is usually limited by transport considerations. By conducting the cosolvent flush, the concurrent exposure of the electron donor (cosolvent) to the electron acceptor (chlorinated solvent) is facilitated by the injection/extraction system and the cosolvency effect. Thus, subsequent biodegradation of the chlorinated solvent becomes a much more viable remediation strategy.

For application of the SERB technology at the demonstration site, the reductive dechlorination of PCE was the bioremediation process that was to be stimulated. The pathways for the biotransformation of chloroethenes shown in Figure 1 demonstrate why PCE and TCE are extremely recalcitrant contaminants. There is no evidence for oxidative mechanisms for degradation of these compounds, thus the reduction dechlorination pathway is the only known mechanism for break-down to non-toxic constituents. There is evidence for both oxidative and reductive pathways for dechlorination of the other daughter products.

The reductive dechlorination process is a step-wise removal of a chlorine atom. Production of cis-DCE rather than the other isomers is an indication that it is a product from the dechlorination of TCE. Monitoring of all daughter products of PCE gives an indication of the extent of dechlorination occurring in the system. The reductive dechlorination of chlorinated ethenes can be conducted by both halorespiration (catabolic) and cometabolic mechanisms. Different microbial populations are responsible for each of these processes. Because of the complexity and heterogeneity of the subsurface environment, it is hypothesized that there can be several competing mechanisms and organisms active at the same time, but in different portions of the subsurface. This is displayed in Figure 3, where a consortia of microorganisms is shown to be responsible for different oxidation-reduction reactions. Various electron donors and electron acceptors are present in the subsurface and are utilized and degraded by these reactions. Evaluation of the concentrations of electron donors and electron acceptors in the site ground water can an indication of the types of reactions that may be occurring. Because most monitoring wells are screened over a large interval and water will move more quickly through the layers with a higher hydraulic conductivity, this type of analysis gives a general view of the screened interval.

2 Objectives

The overall objective of this research, as for the entire Flask to Field (FTF) Program, was to design, develop and implement new biological based clean-up technologies to address environmental challenges faced by DOD, DOE, EPA and the Environmental Restoration Research community. These new technologies were to be evaluated both in terms of efficacy and performance. The specific objective in the chlorinated solvents area was to develop and test comprehensive site approaches, which addressed the unique nature of the environmental challenge presented by solvent/DNAPL sites. Where the FTF program differs from other government and industry driven programs is the recognition of the value of fundamental research in directly supporting the design and testing, and more importantly in the evaluation of the performance to these novel remediation approaches. Because of this the chlorinated solvents area project's specific goal was to demonstrate the feasibility of the SERB technology for the comprehensive remediation of a DNAPL contaminated site. This meant that the approach dealt not only with the dissolved/mobile phase contaminants, but also addressed the need to remediate the separate phase material (DNAPL/residual) and limit the potential of the source area to serve as a extremely long-lived input to ground water at the site. The project also focused on developing a coherent conceptual model of the interaction of the remedial approach, the subsurface microbial ecology and the observed performance. To this end, significant effort was made to develop and test new molecular ecology tools and, innovative site characterization tools and to support the overall test with concurrent laboratory research efforts. The regulatory goals of the SERB pilot test were to evaluate the potential and effectiveness of this technology for the Florida Department of Environmental Protection (DEP) Drycleaning Solvent Cleanup Program (DSCP) and the EPA's DNAPL initiative. The specific goals in this regard were to demonstrate that the system configuration could achieve and maintain hydraulic containment while satisfying long-term injection permit requirements and to collect sufficient data necessary for estimating the full-scale remedial technology design parameters for the selected site, and the post-flush assessment of ground water parameters to evaluate the potential for enhanced *in situ* microbial degradation of the chlorinated solvents with residual cosolvent from the extraction technology. Another objective was to evaluate the subsurface and environmental impact of large scale ethanol injection. Ethanol was selected as the cosolvent for the solvent extraction demonstration because it has acceptable cosolvency properties and is a suitable electron donor for stimulation of *in situ* reductive dechlorination by native microorganisms.

The specific objectives of the SERB demonstration were to:

- Develop a greater understanding of halorespiration and to evaluate its potential as a bioremediation technology for PCE and other halogenated compounds either by enhancement of native activity (biostimulation) or by introduction of active microorganisms (bioaugmentation)
- Develop a detailed understanding of site geomorphology, contaminant distribution and hydrology utilizing new and innovative tools and procedures

- Development of molecular probes for the detection of known PCE dechlorinating organisms
- Conduct molecular analysis of site material for PCE dechlorinating communities and isolates
- Perform laboratory microcosm tests to detect and evaluate microbial processes.
- Evaluate cosolvent injection/extraction design and performance
- Evaluate in situ bioprocess performance
- Evaluate cost and performance, predict long-term performance of SERB approach
- Design and produce an implementation manual for SERB Technologies

3 Technical Approach

Laboratory Process and Microbial Ecology

Attempts to achieve PCE dechlorination to ethene in the field have obtained variable success. This observation and previous work by researchers at the Center for Microbial Ecology at Michigan State University with materials from many different contaminated sites have suggested that this inconsistency may be due to site differences in microbial populations. Thus one objective of the chlorinated solvents thrust area was to develop our understanding of the relationship between the extent of PCE dechlorination and particular microbial populations. To support this objective the decision was made to develop a concerted effort focused on the analysis of microbial communities associated with successful PCE dechlorination, which was conducted in addition to and in support of the pilot test of the SERB Technology. The sub-objectives of the effort were as follows:

- Identification and development of molecular probes for the detection of known PCE dechlorinating organisms
- Molecular analysis of PCE dechlorinating communities and isolates.
- Enrichment of PCE-dechlorinating bacteria in materials from
- contaminated field sites.
- Testing of molecular probes for detection of chloroethene
- dehalogenating communities in site materials.
- Testing of molecular probes for detection of chloroethene
- dehalogenating communities in Sages/Jacksonville site material.
- Evaluating potential for ethanol for toxicity to native microorganisms.

Field Process and Site Evaluation

Site description

The study was conducted at a former dry cleaner site in Jacksonville, Florida that was operational from 1968-1973 and 1979-1989 and is currently abandoned. Prior to this, a gasoline service station was operated on the site from 1953 until the mid- to late- 1960's. Although the PCE release history is unknown, dry-cleaning fluids were not stored on site and were replenished by a delivery truck where occasional releases occurred. A former floor-drain sump was also used to collect drainage from the floor near the dry-cleaning machine and high subsurface PCE concentrations were found in this area. Suspected sources of PCE contamination include the sump area, the area outside the back door of the building where filter tubes were washed with PCE, and the area where a waste oil tank was removed that may have been used both for service

station activities (parts cleaning) and dry-cleaning waste. The sump area was targeted for the cosolvent flushing pilot test (Figure 4).

Site/source zone characterization

The subsurface site materials consist primarily of fine-grained sands with a discontinuous clay layer at approximately 10.7 meters below ground surface (bgs). The water table ranged from 2 to 2.6 m bgs during the 2 ½ years of ground water monitoring. The natural hydraulic gradient during this time ranged from 0.0025 to 0.006.

High PCE concentrations were measured at locations in the sump area over a vertical interval of 7.9 to 9.6 m bgs. A high-frequency sub-sampling technique determined the PCE in this area to be in thin layers (5-8 cm thick) that were not necessarily continuous over the extent of the source area (Jawitz et al., 2000).

Initial concentrations of PCE in ground water from wells in the targeted area ranged from 42 to 90 mg/L. More detailed characterization data can be found in the reports prepared for the Florida Department of Environmental Protection by LFR Levine Fricke (1997, 1998, 1999, 2000).

Evaluation of pre-injection natural attenuation potential

Initial evaluation of the Sage's site included an evaluation of natural attenuation. Ground water parameter data from the existing wells were evaluated for the potential for biodegradation based on scores for the natural attenuation parameters developed according to the weighting guidelines provided in Wiedemeier et al. (1996). The detailed results of this evaluation are reported by LFR Levine Fricke (1997) where the majority of the wells (5 out of 6) indicated "inadequate" to "limited" evidence for natural attenuation of chlorinated solvents (Table 1). Reductive dechlorination was found not to be favored because of aerobic conditions, lack of sulfate reduction or methane production, and low levels of dechlorination products, such as chloride, ethane, and ethane. Based on this assessment, the conclusion was that remediation by natural attenuation had limited potential as a significant component of the overall remedial strategy for effective clean-up of the site. It was also recognized that the DNAPLs present in the subsurface would serve as continuing sources of chemicals that would contribute as a long-term source to the dissolved ground water plumes. The recommendation was to conduct the cosolvent flushing pilot test to study the effectiveness of alcohol flooding for DNAPL remediation.

Well Description

Three injection wells (IW's) surrounded by six recovery wells (RW's) were used for implementation of the cosolvent flushing test and ground water monitoring. These wells were 10-cm diameter polyvinyl choride (PVC) and were installed with a 25-cm diameter hollow-stem auger. A sand pack was used in the screened interval and a bentonite plug was placed immediately above the screened zone. The three IW's were screened from 7.6 to 9.9 m bgs and the six RW's were screened from 7.9 to 9.6 m bgs.

Monitoring wells (MW's) that were 5-cm diameter PVC were also installed with a hollow-stem augor as describe above and screened from 7.9 to 9.6 m bgs. A series of 2.5-cm PVC wells (C's)

were installed with a cone penetrometer truck and screened from 8.3 to 9.1 m bgs. All wells were used for ground water monitoring and their locations are shown in Figure5.

A fully-screened well (MW-516) was installed outside of the contaminated area utilized for conducting flowmeter and aquifer pumping tests. The well was constructed of 5-cm schedule 40 PVC casing with machine 0.025-cm slotted screen from approximately 3 to 10.7 m bgs. This well was installed with a sonic drilling rig, which pushed the casing into the subsurface with no removal of material.

Seven multi-level samplers (MLS) were installed within the source zone and the ring of RW's. The five sampling depths at each MLS location were 8.1, 8.7, 9.1, 9.4, and 9.9 m bgs for a total of 35 sampling locations. These locations were sampled by the U.F. and data is reported elsewhere (Jawitz et al., 2000).

Ground water flow characterization

Characterization of the ground water flow was initially conducted by LFR Levine Fricke (1997). Ground water flow direction in the surficial aquifer was generally westward toward the St. Johns River. Ground water elevations monitored over the project period showed similar results (Figure 6). The flow in the adjacent drainage canal is to the north, which may impact ground water flow. The relationship of the drainage canal to ground water flow in the surficial aquifer was unclear from the initial investigation. During periods following major rainfall events, the depth to water table would be expected to decrease and the drainage canal could potentially impact local ground water flow direction. The hydraulic gradient during the project period ranged from 0.0025 to 0.006. A slug test conducted by LFR in MW-4, which was screened from 7-10.1 m bgs, gave an average hydraulic conductivity of 3.2 m/day. Slug tests conducted in MW-1, MW-2, and MW-3, which were screened from approximately 3 to 6.1 m bgs gave an estimated average hydraulic conductivity of 6.1 m/day. The results from this study were used in the design of the solvent extraction system.

In July 2000, an electromagnet borehole flowmeter was used by USEPA to define the relative hydraulic conductivity distribution of aquifer materials screened by well MW-516 (Acree, 2000). The electromagnetic borehole flowmeter is a commercially available system manufactured by Tisco, Inc. and consists of a 1.25-cm ID downhole probe, a 2.5-cm ID downhole probe, and an electronics module. The investigation was conducted using procedures based on the methods of Molz *et al.* (1994) and Young *et al.* (1998). Flowrate measurements using the electromagnetic borehole flowmeter were made under ambient and constant-rate pumping conditions at a measurement interval between 15 cm and 60 cm within the well screen. The 1.25-cm ID probe was used for flow measurements under ambient conditions and the 2.5-cm probe was used for measurements under pumping conditions. The test was performed at a constant pumping rate of 8.8 L/min and repeated at a rate of 4.0 L/min. The rates were chosen to induce sufficient flow from each test interval with negligible head loss across the 2.5-cm ID downhole probe. Measurements of vertical flowrates under ambient and constant-rate pumping (induced flow) conditions were analyzed using methods described by Young et al. (1998).

The estimates of hydraulic conductivity for this test represent averages over the measurement interval, which was between 15 and 60 cm within the well screen, and are dependent upon the interval thickness. The data collected indicated that relatively conductive materials were present

from the water table to a depth of approximately 7.9 m bgs at this location. Materials of lower hydraulic conductivity were present below this depth. Drawdown in adjacent monitoring wells was measured during the constant-flowrate extraction test performed for the flowmeter survey and this data was used for estimation of the hydraulic conductivity of the aquifer materials. The methods of Neuman (1975) were used to estimate the hydraulic conductivity using an aquifer thickness of 9.1 m, which corresponds approximately to the interval from the water table to the depth of the well screen. These estimates of aquifer hydraulic conductivity ranged from 4.3 m/day to 14.3 m/day using the drawdown data from five monitoring wells with an average of 9.1 m/day for the aquifer. This average value of 9.1 m/day was then used to estimate the hydraulic conductivity profile using the estimates of relative hydraulic conductivity for each interval measured during the flowmeter study (Figure 7).

The estimates of hydraulic conductivity for the individual intervals with the flowmeter test show variability with depth as would be expected based on the site geology (Figure 7). The average hydraulic conductivity for the materials in the overlying sands from 3 to 7.6 m bgs is approximately 14.9 m/day, which is much higher than the value of 6 m/day estimated by LFR. The value for the lower sands from 7.6 to 10.7 m bgs of 3 m/day is significantly lower than the upper sands and is similar to the value of 3.2 m/day estimated by LFR.

The site characterization showed the highest concentrations of PCE contaminant in the aquifer sediment (6000 to 90000 mg/kg) were from 8 to 9.2 m bgs (Jawitz, 2000). This corresponds approximately to the zone with the lowest measured hydraulic conductivities. Based on the properties of PCE, it has been shown that PCE will migrate downward through the aquifer to a zone of lower permeable materials where it will be trapped in the pore space. Depending upon the volume of DNAPL moving through the aquifer and the thickness of the zones with lower hydraulic conductivities, it is possible for the DNAPL to break through these zones and move deeper into the aquifer.

4 Materials and Methods

Laboratory Evaluations

Molecular ecology

A number of chloroethene sites were used to provide source material for evaluation. Microcosm experiments were conducted to evaluate sediment, soil and aquifer materials for their potential to reductively dechlorinate PCE and other chlorinated ethenes under anaerobic conditions, and to assess the ability of various enhancing agents to stimulate PCE dechlorination. Eleven freshwater sediments, four marine sediments, five soils and seven aquifer samples were used to construct microcosms containing 10-15 % (V/V) sample under anaerobic conditions with either anaerobic mineral medium or phosphate buffered saline (PBS) and amended with PCE. Dechlorination of PCE was monitored by gas chromatography (GC) under conditions that can resolve ethene and each of the chlorinated ethenes. The enrichments that completely dechlorinated PCE were further enriched on the lesser chlorinated ethenes, cis-DCE and VC separately. The resulting subcultures completely dechlorinated their respective chlorinated ethene and were maintained for more than 10 transfers in basal salts mineral medium.

Subcultures were subjected to an analysis of the 16S rRNA genes by denaturating gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). The changes in community structure were evaluated. This technique is performed by PCR amplification of 16S rRNA genes from DNA extracted from a microbial community in which one of the primers is labeled with a fluorescent molecule. The resulting fluorescently labeled PCR product is then subject to restriction enzyme digests. The fragments are resolved using an automated DNA sequencer, which allows for the detection of only the fluorescently labeled terminal fragments. The resolved terminal fragments provide a fingerprint of the community, and an estimate of the number of ribotypes in a community. The Sage's site material was subjected to direct, and post amplification, ribosomal gene probing with probes developed from known PCE-dechlorinating bacteria.

Ethanol toxicity

Soil samples were collected from the former dry cleaner site in accordance with methods described in the published literature, ensuring sample integrity. Microcosms were prepared in triplicate in glass serum bottles fitted with Teflon™-coated rubber septa with approximately 50 grams of soil. The amount of soil used for the microcosms was dictated by the quantity required for analyzing the phospholipid fatty acid (PLFA) profiles of the samples. The liquid phase in

each microcosm consisted of Byrd's Mill medium (BMM), ethanol, and other additives, such as KNO_3, Na_2SO_4, or PCE, depending upon the electron acceptor condition selected for the particular experiment. Byrd's Mill medium was made with Byrd's Mill water (Byrd's Mill Spring, OK) at 500 mL/L, distilled water at 500 mL/L, and $(NH_4)_2HPO_4$ at 1.32 g/L, and this supplied the basic nutrients to the sample's microbial community. Different concentrations of ethanol, ranging from 0.05% to 70% v/v, were used for the study. Anaerobic microcosms were prepared in a glove box with a nitrogen-hydrogen atmosphere, and the aerobic microcosms were prepared in a laminar flow hood. Samples from each microcosm were withdrawn at regular intervals to determine the degradation of added ethanol. Degradation of ethanol was expressed in terms of attenuation rates of ethanol and was measured with different electron acceptors (O_2, NO_3^-, SO_4^{2-}, and PCE) and different stressor levels. Exposure time to the stressor for the different microcosms was also varied, and can be divided into two groups: (a) long term exposure, and (b) transient exposure. Spent microcosms were frozen and sent for PLFA analysis.

Field Studies

Ground water sampling and analysis

Ground water samples were collected prior to the implementation of the cosolvent extraction technology to provide background conditions. Following the cosolvent extraction test, ground water samples were collected and analyzed quarterly over a 2 ½ year period for nutrients, chloroethenes, ethanol, dissolved gases, and volatile fatty acids.

Samples for ethanol and alcohol tracer analysis and for PCE and daughter product analysis were collected in 40-mL VOA vials without headspace. The alcohol compounds were separated by gas chromatography on a DB624 capillary column and detected with a flame ionization detector. Chlorinated compounds were analyzed by purge and trap gas chromatography which utilized a 0.32mm X 60 m RESTEK Rtx-Volatiles column for separation and a flame ionization detector for detection.

Samples for chloride and sulfate analysis were collected in 100-mL polyethylene bottles and analyzed using Waters capillary electrophoresis method N-601. Samples for nitrate, phosphate, and dissolved organic carbon analyses were collected in 100-mL polyethylene bottles and preserved with concentrated sulfuric acid. Nitrate and phosphate was analyzed using a Lachat System. Dissolved organic carbon samples were analyzed with a Dohrman Carbon Analyzer.

Sub-samples for volatile fatty acid analysis were collected from the 100-mL non-acidified polyethylene bottle described above. These samples were analyzed by high performance liquid chromatography (HPLC) with a Dionex system utilizing a Dionex ICE-AS1 IonPac column and AMMS-ICE MicroMembrane Suppressor. A Waters 431 conductivity detector was used for analyte detection.

Dissolved gas samples were collected in 100-mL glass serum bottles without headspace and sealed with teflon-lined septa and crimp caps. Samples were analyzed for methane, ethane, and ethene using an HP P200 Series micro gas chromatograph and a thermal conductivity detector.

Core material collection

Subsurface sediment samples were collected for direct molecular analysis (16S and FAME) prior to the cosolvent flushing test and at approximately one and two years after the cosolvent flushing test. Samples were collected from approximately 7.9 to 9.4 m bgs to coincide with the zone targeted by the cosolvent flushing test. The results of the molecular analysis are not available at this time. Subsurface materials collected from these locations and locations where additional MLS, injection/extraction wells, and monitoring wells were installed have also been used as a source of material and microorganisms for laboratory biotransformation studies.

Core materials collected for the molecular analysis prior to the cosolvent flushing test were put into sterile conical tubes and frozen by immersing into a liquid nitrogen canister for 2-5 min and stored frozen until analysis. Sterile spatulas were utilized to obtain samples of the undisturbed centers of the core. Between core sections all materials used were sterilized with isopropyl alcohol. Core materials were collected from IW-3 as this well was installed, from the area between RW-6 and RW-7 (C6), and near MW-2 (C5). Additional materials were collected during installation of the C-wells (C1-C4, C7). Locations of these samples are shown in Figure 8.

In July, 1999 and July, 2000 core materials were collected near the original locations using the direct-push Simco rig. These materials were collected into polyethylene sleeves that were sectioned by sawing and then capped with plastic caps. Materials used to handle these cores were sterilized with isopropyl alcohol between core sections. Cores were immersed into a liquid nitrogen canister for 5 min and stored frozen until analysis. A compilation of the core locations and time of collection is given in Table 2.

Cosolvent flush and partioning tracer tests

The pilot-scale field test of *in situ* alcohol flushing was conducted in August, 1998 as described by Jawitz et al., 2000. The alcohol flushing began on August 9, 1998, and ended on August 15, 1998. The injection/extraction system was designed so the IW's were screened deeper than the RW's to promote an upward flow of the injected fluids. The configuration of injection/ extraction wells was selected based on numerical model simulations conducted by Sillan (1999). A total flow of 15.1 L/min was distributed equally to the three IW's. The interior recovery wells (RW's 3,4,6, and 7) had extraction rates of 5.9 L/min and the outer two wells (RW's 2 and 5) had extraction rates of 3.4 L/min, which resulted in a 2:1 extraction-to-injection ratio. Steady-state flow was maintained so that the water table position remained constant during the tests. The test zone was flushed with 34 kL (equivalent to 2 pore volumes) of a 95% ethanol/5% water mixture over the 3 day period. In the initial 10 hours the ethanol concentration was ramped from 0 to 95% to minimize density problems. Post-test hydraulic containment began on August 15, 1998 until August 25, 1998, after the ethanol concentration in the treatment system influent dropped below the 10,000 mg/L termination criterion (LFR, 1998a).

A partitioning tracer test conducted prior to the alcohol flushing was used to estimate approximately 68 L of PCE in the zone swept by the injection/recovery well system (Jawitz et al., 2000). A post-flushing partitioning tracer test indicated that approximately 26 L of PCE remained in the swept zone so approximately 42 L of PCE was removed during the cosolvent flush. A more detailed description of the tracers used and data evaluation is given in Jawitz et al. (2000).

Hydrogen gas analysis

In July, 1999 hydrogen gas analysis was conducted on ground water from selected monitoring wells. Monitoring wells were pumped at a rate of 250-300 mL/min through a 250-mL gas sampling bulb containing a gas pocket of approximately 20 mL volume. A 2-mL gas sample was collected from the gas pocket with a gas tight syringe and injected into a RGA3 Reduction Gas Analyzer. Samples were collected and analyzed over time until equilibrium was reached at individual wells.

5 Accomplishments

Solvent Extraction (SE) Pilot Test

The cosolvent flushing pilot test activities were completed on September 10, 1998 with the collection of post-test groundwater samples. More detailed information concerning the cosolvent flushing test and an evaluation of the partitioning tracer data can be found in Jawitz et al. (2000) and LFR (1998).

Enhanced dissolution and solubilization of PCE was demonstrated as a result of cosolvent flushing. Analytical data from the recovery wells, except for RW-5, show the peak PCE concentrations were 3 to 80 times larger than the initial PCE concentrations (Fiure 9). According to the partitioning tracer test, RW-5 showed very low amounts of DNAPL present in the swept zone and the pre-cosolvent flush ground water PCE concentration was 1 mg/L. During the cosolvent flush, the maximum concentration of PCE in this well was 2 mg/L with a maximum ethanol concentration of 4% (Figure 9d).

The partitioning tracer test indicated that the other recovery wells contained a significant volume of DNAPL in the individual swept zones. These were: 3.2 L for RW-2, 11.6 L for RW-3, 4.6 L for RW-4, 7.2 L for RW-6 and 14 L for RW-7. RW-3 (Figure 9b), RW-4 (Figure 9c) and RW-6 (Figure 9e) showed peak PCE concentrations that were approximately 25 times the initial concentration. RW-7 showed the greatest increase in PCE concentration, which was over 80 times the initial concentration (Figure 9f). The PCE concentrations in RW-2 were variable at the beginning of the cosolvent flush and reached a concentration of approximately 2-3 times the initial concentration (Figure 9a). The maximum ethanol concentration in these wells ranged from 18 to 45%.

Ground water concentrations of PCE in all of the recovery wells decreased to initial concentrations following the cosolvent flushing test. Comparison of average ground water concentrations in the recovery wells prior to and post cosolvent flushing showed a high degree of variance and only 4% decrease in concentrations (Table 3). This same comparison for the three injection wells gave a 99% decrease in concentrations and a similarly large variance. Averaging the concentrations for both the recovery and injection wells showed a decrease in average concentration of 62% (Table 3). This calculation compares well with estimates of the amount of PCE removed during the cosolvent flushing test by two techniques explained in more detail in Jawitz et al. (2000). Comparison of core material collected prior to and following the cosolvent flushing test indicated a removal effectiveness of 0.65. The overall removal effectiveness for the recovery wells calculated from the partitioning tracer data was 0.62 to 0.63, but varied significantly at individual recovery wells depending upon the technique used for analysis.

The hydraulic containment system was adequate to maintain capture within the testing zone. Groundwater elevations measured during the course of the pilot test showed an inward gradient

from the outer monitoring wells (MW505, MW506, MW507, and MW509) toward the recovery wells. DNAPL PCE migration was not observed during the test and ethanol was only detected in two of the outer monitoring wells following the test. In the first sampling event following the cosolvent flushing test the ethanol concentration in MW505 was 4620 mg/L and in MW506 was 0.74 mg/L. MW506 is located up-gradient from the targeted area and MW505 is located to the north. Neither well is located downgradient of the direction of ground water flow.

Analysis of the partitioning tracer data by Jawitz et al. (2000) estimated 42 L of PCE was removed by the cosolvent flushing test. Remaining PCE DNAPL was estimated to be 26 L. Approximately 92% of the 34 kL of injected alcohol was recovered at the end of the hydraulic containment so that 2.72 kL of ethanol remained in the subsurface as an electron donor for enhancement of the microbial processes.

The cosolvent extraction demonstration at the Sage's site was a successful demonstration of the removal of a significant mass of DNAPL contaminant from a selected zone. Improvement in removal effectiveness could be obtained by selection of a remedial cosolvent with a higher cosolvency property and by increased volume of cosolvent pumped through the system. This test demonstrated the capability of this technology to be utilized at other Florida DEP DSCP sites. Information obtained from this test will aid in the design of a full-scale technology implementation for the Sage's site.

Residual Biotreatment (RB) Monitoring and Assessment

Currently the post-flush assessment of the ethanol enhanced *in situ* microbial degradation of the chlorinated solvents is on-going. This assessment of *in situ* reductive dechlorination, the residual biotreatment (RB) phase of SERB, is based on site monitoring of geochemistry and contaminant chemistry as well as laboratory and field evaluations of the impact of ethanol on site microbial communities.

Ground water samples from the monitoring wells at the site have been collected and analyzed quarterly for 2 1/2 years along with additional site characterization activities (Table 4). A database has been established that contains the measured results including residual ethanol concentrations, its possible metabolic products, PCE and potential biotransformation intermediates, and other geochemical parameters. A compilation of the parameters most indicative of microbial activity is given in Table 5.

The compiled data was graphed by individual well to show changes in concentration of the parameters over time. Relatively complete data sets were obtained for all MW and C wells. IW's and RW's were not sampled consistently throughout the field evaluation, thus the bar charts of this data are somewhat limited. IW1-3, RW-2, and RW-5 were not sampled after September 1999.

Contour plots of the data were made to better visualize the changes in the parameters over time. Surfer™ was used to create these contour plots with the data from the MW, C, RW, and IW wells. MW1-3, which were screened over a shallower interval and were outside the contaminated plume were not utilized in this exercise, nor was MW-508, which was screened over a deeper interval but showed high PCE concentrations. Changes in concentrations of the

measured parameters gives an indication of the change in subsurface geochemistry and the stimulation of microbial activity as a result of the SERB process.

Ethanol concentrations stayed at approximately 10,000 mg/L in the area of the co-solvent extraction during the first year of ground water monitoring and then decreased to less than 2,000 mg/L after the second year (Figure 10). MW-505 and MW-509 were the only monitoring wells outside the injection/extraction area to show significant concentrations of ethanol (>4,000 mg/L) following the cosolvent flush. These wells bound the injection/extraction system on the north and west. Down-gradient wells (MW-510, MW-512, MW-513, and MW-514) showed low concentrations of ethanol (<500 mg/L) after one year of monitoring. After 19 months the down-gradient C wells (C2, C3) had concentrations of ethanol similar to the MW's. C1, which is located closer to the injection/extraction area, had concentrations of ethanol over 2000 mg/L after 19 months. C4 had no measurable ethanol concentrations during the monitoring period. Differences in the concentrations in these wells may be because the C-wells were screened from 8.3 to 9.1 m bgs whereas the MW's were screened over a larger interval from 7.9 to 9.6 m bgs. This difference in screened interval can impact measured concentrations, especially in layered sediments of differing hydraulic conductivities.

The ethanol contour plots show the decreasing concentrations over time and the movement ethanol downgradient (Figure 11, Figure 12, and Figure 13). This data also shows that although the overall ethanol concentration decreased over time, a significant amount of electron donor is still available for subsurface microbial activity after two and one-half years.

PCE concentrations were somewhat variable in the IW's and RW's during the first year of monitoring (Figure 14). In general, concentrations appeared to decrease for the sampling event immediately following the cosolvent flushing test and then rebounded to near initial concentrations. After approximately one year of monitoring, concentrations began decreasing in all IW's and RW's. This trend is shown best in the more complete data sets from RW-3, RW-4, RW-6 and RW-7 (Figure 14). Interestingly, MW-509, which showed high ethanol concentrations, also showed a decrease in PCE concentration in the first sampling event and a general trend of lower concentrations over time. MW-505, which also contained ethanol immediately following the cosolvent flushing test, had low initial concentrations of PCE and maintained these low concentrations throughout the monitoring. In general, the other downgradient MW's showed an increase in PCE (from 10 to 60 mg/L) for the first six months following the cosolvent flush and then a decrease in concentrations to less than 5 mg/L after two years of monitoring (Figure 14).

C1 and C2 had initially high PCE concentrations (>90 mg/L) and it is suspected that these wells were completed in a previously undetected source area. C1 and C2 maintained relatively high concentrations and C3 and C4 appeared to have increasing concentrations of PCE for the first six months of ground water monitoring. Concentrations of PCE in all of these wells decreased to less than 10 mg/L after two years of monitoring. It is interesting to note that all wells showed a decrease in PCE concentration even though C1, C2, and C3 showed an increase in ethanol concentrations over this same period. The contour plots of the PCE data show dramatically decreasing concentrations in the ground water over the monitoring period (Figure 15, Figure 16, and Figure 17).

TCE and *cis*-DCE concentrations, which are daughter products that form by dechlorination of PCE, have increased in the ground water following the cosolvent flushing test. Prior to the introduction of ethanol to the subsurface, concentrations of TCE and *cis*-DCE were extremely low. TCE concentrations have increased in all wells during the first 19 months of monitoring up to as high as 15 mg/L and then have decreased to relatively low concentrations (<4 mg/L) (Figure 18). Prior to the cosolvent flushing test, *cis*-DCE was detected in the C-wells at concentrations of up to 50 ug/L and in MW-511 at 250 ug/L, while the other IW, RW, and MW wells contained none to less than 20 ug/L. After six months significant *cis*-DCE (up to 4 mg/L) had formed in the area of the cosolvent extraction and immediately downgradient. After 19 months significant *cis*-DCE formation (>10 mg/L) was detected in the further downgradient MW and C wells (Figure 19). Contour plots of the TCE and *cis*-DCE concentrations over time give a good visualization of the daughter product formation at the site (Figure 20, Figure 21, Figure 22, and Figure 23, Figure 24, Figure 25).

Ground water samples were also analyzed for the isomers of *cis*-DCE, *trans*-1,2-DCE and 1,1-DCE. These compounds were detected at low levels and made up less than 10 percent of the DCE analyzed. This ratio is indicative of biological transformations which seem to favor cis-DCE as the predominant product under most conditions.

Vinyl chloride was not detected in any ground water samples collected from the site during the monitoring period. Vinyl chloride is the next dechlorination product after DCE and is a known carcinogen and regulated contaminant. Interestingly, ethylene, which is the final dechlorination product in the biotransformation of PCE was detected at noticeable levels within 6 months following the cosolvent flushing test (Figure 26). Ethylene concentrations were somewhat variable, which may be due more to the difficulty in sampling and analysis rather than variability because of biological activity. The bar charts of ethylene show a consistent trend of formation in the same wells that showed relatively high ethanol concentrations over time (MW-505, MW-509, C1-3, RW-3 and RW-7). The contour plots for this data indicate the area of main activity is from the injection/extraction area downgradient to well C2 (Figure 27, Figure 28, and Figure 29).

The formation of the daughter products of PCE is an indication that *in situ* reductive dechlorination is beginning to occur. The process appears to have begun initially in the area where the cosolvent extraction was conducted and later in locations downgradient of the targeted source area.

Production of chloride is concomitant with the reductive dechlorination of PCE and because it is a relatively non-reactive anion, one would expect to see an increase in chloride concentrations over time. Background chloride concentration was less than 10 mg/L except in the injection/extraction zone where concentrations were as high as 20 mg/L. Overall, there was a trend of increasing chloride concentrations over time (Figure 30). In particular, monitoring wells that showed the formation of *cis*-DCE also had an increase in chloride concentrations. Data from the RW's did not show this relationship and had more variable concentrations of both *cis*-DCE and chloride. Contour plots of chloride do show an increase in concentrations in the area immediately downgradient of the injection/extraction zone within one month of the cosolvent flushing test (Figure 31, Figure 32, and Figure 33). After approximately one year, this area of higher chloride concentrations appears to have moved further downgradient to the area of C1 and C2.

The maximum measured chloride concentration was approximately 2 mM, which is almost four times the maximum measured PCE concentration of 500 uM. This compares well to the complete dechlorination of PCE, which would produce four chloride ions. It is not expected to see this relationship in an open system and these were the highest concentrations measured over the entire sampling period. The targeted zone does consist of materials with a relatively low hydraulic conductivity and the range in hydraulic gradient at the site suggests that overall movement of ground water through the site could be from 15 to 33 m/yr.

Partial oxidation of ethanol will result in the formation of one mole of acetic acid and 2 moles of hydrogen gas (Equation 2), whereas complete oxidation of ethanol will result in the formation of 2 moles of carbon dioxide and 6 moles of hydrogen gas (Equation 3). Acetic acid (>200 uM) was observed in all wells, except for MW-506, MW-507, MW-511, and C4 (Figure 34). Acetic acid was not measured in MW-506 (upgradient), MW-507 (south of injection/extraction zone), and MW-511 (furthest well southwest of monitored area) and these same wells never had detectable ethanol concentrations during the monitoring period. C4 (downgradient and furthest well northwest of monitored area) had measured concentrations of acetic acid from 200-600 uM after two years, even though ethanol was never detected at this location.

$$CH_3CH_2OH + H_2O \quad CH_3COOH + 2\,H_2$$

Equation 2. The Partial Oxidation of Ethanol to Acetic Acid and Hydrogen Gas.

$$CH_3CH_2OH + 3H_2O \quad 2CO_2 + 6\,H_2$$

Equation 3. The Complete Oxidation of Ethanol to Carbon Dioxide and Hydrogen Gas.

Initially the contour plots of acetic acid show increasing concentrations in the area of the cosolvent flushing test (Figure 35, Figue 36, and Figure 37). This trend continues for approximately 19 months, after which the overall measured concentrations decrease and the area of highest concentration is further downgradient in the area of MW-510 and MW-513. This coincides with the decrease in ethanol concentrations after 19 months, as would be expected, since ethanol is the parent product (Equaton 2). After the significant decrease in ethanol, it is expected that the microbial population would shift to using acetic acid as the electron donor so that there would be a decrease in these concentrations as well. The acetic acid contour plots show this trend (Figure 35, Figure 36, and Figure 37). The highest measured concentration of acetic acid was approximately 2.5 mM, which is one hundred times less than the maximum ethanol concentration of 260 mM. This would indicate that a significant amount of the ethanol is being completely degraded to carbon dioxide and hydrogen gas.

The hydrogen gas survey conducted approximately one year after the cosolvent flushing test indicated high levels of hydrogen were being produced (Figure 38). Concentrations of hydrogen gas in wells near the injection/extraction area were over 2 uM and downgradient locations measured from 5 to 30 nM. Hydrogen gas is produced from the oxidation of ethanol and can be utilized by microorganisms responsible for the catabolic and cometabolic reductive dechlorination of PCE and its daughter products.

Background concentrations of sulfate at the site were approximately 40 mg/L. All locations showed a decrease in sulfate concentrations over the monitoring period (Figure 39). Sulfate was removed from the area immediately downgradient and including the injection/extraction zone

within 3 months following the cosolvent flushing test (Figure 40, Figure 41, Figure 42). After approximately 14 months, the area of sulfate removal expanded to include most of the downgradient wells. Even MW-506, which was located upgradient of the flushing area, also showed a decrease in sulfate concentrations. The contour plots of this data depict the sulfate removal and give a strong indication that sulfate-reducing bacteria are becoming active and creating conditions conducive to the reductive dechlorination process.

Along with the removal of sulfate there was a production of methane, which indicates that methanogenesis has been enhanced. Methane production began after approximately six months and was measured in all wells (Figure 43). As with the sulfate removal, methane production began in the area near the cosolvent flushing test and concentrations downgradient increased after 14 to 19 months (Figure 44, Figure 45, Figure 46).

Microbial Ecology Assessment

The only known pure culture that is capable of complete reductive dechlorination of PCE to ethene is the obligately hydrogenotrophic organism *Dehalococcoides ethenogenes*. Hydrogen is generally considered the ultimate electron donor to stimulate the reductive dechlorination of chloroethenes. *Dsulfuromonas* sp. Strain BB1 is a dechlorinator that utilizes acetate as an electron donor to support the reductive dechlorination of PCE and TCE. Löffler et al., 2000 developed 16S rDNA-based PCR methods for detection of PCE-dechlorinating *Dehalococcoides* species and *Dsulfuromonas* sp. Strain BB1 and evaluated their presence in environmental samples.

Aquifer materials collected during installation of MW-510 and IW-001 were utilized in their study along with environmental samples from other sites. The MW-510 sample resulted in visible amplification in the initial PCR with the universal primers. Nested PCR with the Dehalococcoides-targeted primers yielded a positive signal with the MW-510 sample.

Microcosms studies conducted with the aquifer materials confirmed the results obtained with the molecular approach. MW-510 microcosms were the only aquifer material-based microcosms that indicated the presence of a hydrogenothrophic PCE-dechlorinating population. Vinyl chloride and ethene accumulated in the hydrogen-fed microcosms, whereas acetate-amended cultures showed only negligible dechlorination (Löffler et al. 2000).

The results of the molecular analysis and microcosm studies support the data from the Sage's site that indicates complete reductive dechlorination of PCE to ethene. Additional samples collected prior to the cosolvent extraction test and one and two years following the test are being analyzed to determine the changes in microbial ecology that may have occurred. These samples are currently being analyzed and the data is not available at this time.

Ethanol Toxicity Assessment

The effects of continuous and transient exposure of ethanol on subsurface microbial populations was evaluated in laboratory microcosms and in terms of available field data. Ethanol oxidation,

as evidence by concentration decreases, was observed at up to 1% (V/V) concentrations for transient exposure and 0.1% in continuous exposure microcosms (Table 6). Analysis of field ethanol concentration data yielded a first order degradation rate of 0.3 yr^{-1}. Laboratory rates appeared to be much faster ranging from 13 to 90/year (Table 7). We feel this is a reflection of the toxic effect of higher ethanol concentrations in the source area, and that the observed field rates are impacted by limitations on the size and location of bioactive zones. The observed acetate and methane formation rates are insufficient to account for ethanol losses and indicate the presence of other electron sinks such as dechlorination and sulfate reduction. Examination of PFLA profiles in laboratory microcosms suggest that higher concentrations of ethanol (at or above 5%) reduced microbial populations and that the decrease in biomass was influenced by exposure time (data not shown).

Evaluation of System Performance

Electron donor evaluation

The production of acetate and hydrogen gas is an indication that the biodegradation of ethanol is occurring at the site. The hydrogen produced by the oxidation of ethanol can be used for the dechlorination of PCE (Equation 1). With incomplete oxidation of ethanol (Equation 2), two moles of ethanol would be required to produce enough hydrogen for complete dechlorination of PCE. With complete oxidation of ethanol (Equation 3), only one mole of ethanol would be required.

The Surfer™ contour plots of PCE and ethanol ground water concentrations were used to estimate the mass of each compound in the dissolved phase. Calculations were made for the ground water concentration data collected immediately following the solvent extraction test and after two and one-half years of ground water monitoring (Table 8). The estimated residual PCE DNAPL was obtained from the partitioning tracer test data. Ethanol mass was also estimated based on concentrations measured in the recovery wells during the solvent extraction test, which was subtracted from the total mass injected (Table 8).

These estimates of compound mass were used to calculate the ratio of ethanol to PCE to evaluate the relative amount of electron donor available for the reductive declorination of PCE (Table 8). The estimate of ethanol mass from the recovery well data (47,234 moles) is ten-fold higher than that from the measured dissolved phase concentrations (5,119 moles). The total mass of PCE in the dissolved phase (11 moles) is also much lower than the estimate of the DNAPL residual from the partitioning tracer test (254 moles), as would be expected. The data indicates that excess electron donor (ethanol) is present to drive the reductive dechlorination of PCE to completion. The ratio of ethanol to PCE is approximately 180:1 when the estimate of ethanol from the recovery wells is used compared to 19:1 when the estimate of ethanol from the ground water concentrations is used. If we assume it takes 2 moles of ethanol for complete dechlorination and there are no competing reactions, then there is from 9 to 90 times the amount of ethanol to remove the 265 moles of PCE remaining in the subsurface.

The dissolved phase concentrations were used to estimate the mass of ethanol and PCE immediately following the cosolvent flush and after two and one-half years of ground water

monitoring. While these estimates to not take into account the residual PCE DNAPL that is present in the system, they do show that excess ethanol remains in the dissolved phase. The ratios from these estimates range from 465:1 immediately following the cosolvent extraction to 482:1 after two and one-half years of ground water monitoring. If we assume there are no competing reactions and there is complete oxidation of ethanol, these ratios indicate that there is over 200 times the amount of ethanol present in solution available for the complete dechlorination of PCE. Even though the concentrations of both ethanol and PCE have decreased over time, their ratio has remained constant.

While all of these estimates assume no competing terminal oxidation processes, such as methanogenesis or sulfate reduction, they show that enough ethanol is present in the subsurface that at even a low efficiency the theoretical demand could be met. Methane production and sulfate reduction indicates that competing processes are active at the site and will consume some of the ethanol in the system.

Estimates of transformation rates

As with most multi-component, ecologically competitive subsurface microbial processes, mathematical descriptions of the fate of PCE and ethanol at the Sage's site are problematic. Typically zero and first order degradation rates are use as a predictive tool for evaluating system performance. We have chosen to derive both zero and first order rates, based on data types, to provide values in a usable and generally accepted format for comparisons to other projects and literature values. Conceptually a first order rate should be more appropriate for ethanol oxidation since it is a catabolic process most likely controlled by a single enzyme (per species). Reductive dechlorination, on the other hand, is thought to be limited by available reducing equivalents (hydrogen), which is controlled by a complex set interactions between competing populations and processes. Thus, the dechlorinating populations seem to have a define/fixed capacity for transformation and the behavior of the dechlorination process often seems to fit the zero order model better (Gibson et al 1994). However both approaches are bounded by assumptions, such as limited growth (probably a good assumption under anaerobic conditions), single reactive species or fixed ratio of species (a questionable assumption), and conditions that do not vary spatially over the site (a poor assumption).

Single well rate constants

The accumulation of *cis*-DCE in the ground water from selected wells is plotted in Figure 47. The total range of rates of formation of *cis*-DCE for the site is shown in Table 9, which includes the rate constant in ug/L/day and correlation coefficient for the equation fit through zero. The swept volume for the solvent extraction test was 17.3 kL and the average rate of *cis*-DCE production for the RW wells was 5.54 ug/L/day. If we assume that the total mass of dissolved phase PCE measured during the last sampling event (659 g) was contained in this volume, we calculate that it would take approximately 19 years to consume the PCE in solution. Using the lowest rate of 1.09 ug/L/day and the highest rate of 57.29 ug/L/day, we calculate that it would take 96 to 2 years, respectively, for complete consumption of the dissolved phase PCE. These calculations show the potential for removal of PCE, but do not take into account any residual DNAPL PCE contained in the aquifer that would serve as a long-term source of contamination.

If we include the mass of residual DNAPL PCE (26L) that was estimated to remain in the swept volume and use the average rate of *cis*-DCE production of 5.54 ug/L/day, it would take over 1000 years for complete removal. This calculation assumes there are no mass transfer limitations.

Similar analysis of PCE removal rates for the RW wells gives an average rate of –37.9 ug/L/day, which is over six times the rate of *cis*-DCE formation (Figure 48). Table 10 is a compilation of the rates of PCE removal and correlation coefficients for the individual wells. Using the average rate of removal for the RW wells, it would take approximately 3 years for complete removal of the dissolved phase PCE and approximately 200 years for complete removal of the residual DNAPL PCE. Again, it is assumed that there are no mass transfer limitations.

Total mass removal

Total mass of cis-DCE and PCE was calculated for each sampling event based on the Surfer™ contour plots. The masses were put on a relative basis by dividing by the initial mass measured before the cosolvent flushing test. Rate constants for each compound were obtained by fitting a linear regression to the data (Figure 49).

The rate constant for PCE was $-0.56\ yr^{-1}$ and for cis-DCE was $0.81\ yr^{-1}$ for the site. Converting these rate constants to a half-life gives values of 1.2 and 0.9 years, respectively. These values indicate that the activity occurring at the Sage's Site in the dissolved phase is quite rapid the dissolved PCE plume could meet regulatory limit of 5 ug/L within ten to fifteen years.

6 Conclusions on Utility in Remediation

The goal of the chlorinated solvents thrust area was to develop and demonstrate biological remediation technologies for chlorinated solvents. This project focused on one such technological approach for source area treatment, a sub-area for which current technologies are limited, but which must be addressed if viable, cost effective approaches are to be implemented at solvent contaminated sites.

The SERB technology has several advantages for remediation of free-phase DNAPL in the subsurface. These include:

- Rapid removal of large masses of DNAPL in a very short time
- *In situ* treatment train technique
- Cosolvent injection/extraction overcomes transport and mixing limitations of standard bioremediation infiltration and injection techniques
- DNAPL mass removal reduces toxicity to microbes for bioremediation
- Continued removal of dissolved contaminants following cessation of pumping
- Combination of an active process, Solvent Extraction, with an essentially passive process, Residual Biotreatment

The ability to remove large masses of contaminant in a relatively short time is attractive to both regulators and stakeholders, who might be reluctant to support an extremely long natural attenuation option for the duration of natural DNAPL dissolution (assuming that natural attenuation processes were functioning in a sufficiently protective manner). It is also attractive to responsible or potentially responsible parties who might otherwise have no options other than expensive long-term and energy-intensive pumping and treating operations for containment. The *in situ* treatment train approach improves on either natural attenuation or enhanced bioremediation in the DNAPL scenario by both removing large masses of DNAPL and reducing toxicity for long-term biological processes to operate on the dissolved contaminants.

There are also disadvantages to the SERB technology, as there are for any remediation scheme:

- The technique is innovative and remains to be proven at sites having a variety of characteristics
- Both cosolvent and surfactant *in situ* floods have the potential and are implemented to mobilize and/or dissolve the contaminants. If hydrodynamic control is inadequate, the potential exists for contaminating larger volumes of the aquifer and the ground water
- The cosolvent flood itself will probably not reduce contaminant concentrations to the needed regulatory requirements, hence a successful residual biotreatment phase is very important
- The choice of cosolvent is critical for both the solvent extraction and residual biotreatment phases of the technique and success or failure can depend upon this choice. Laboratory studies must be considered before going to field scale
- There is considerable regulatory resistance to the injection of foreign materials (e.g.,

cosolvents) into the ground water, which may require a significant effort and large amounts of documentation and persuasion to overcome

- The Residual Biotreatment phase of SERB might still take a very long time to come to completion following the cosolvent flooding phase, depending upon the conditions created and the mass of contaminant remaining

7 Recommendations for Transitional Research

- Additional pilot-scale and full-scale demonstrations
- Further characterization of shifts in microbial ecology in response to SERB
- Evaluation of mixtures of optimal cosolvent/electron donor solutions
- Modeling of impact of source removal on long-term economics for site remediation costs

8 Technology Transfer

The environmental challenge posed by separate phase chlorinated solvents in the subsurface is a problem found at a large number of both military and industrial sites. The application of the SERB technology could have a significant effect on clean-up efforts for both DoD and private industry. Subsurface restoration practitioners should be able to make direct use of the results of the laboratory and field investigation. The results are also directly applicable to the needs of the regulatory community and should promote the acceptance of innovative technologies for subsurface remediation.

The SERB project was focused on a controlled field test, which demonstrated the capabilities of a treatment train technology that incorporated both active and passive remediation. The reports and presentations from this work will provide technical guidance as to the feasibility for implementation at full scale. The field demonstration was conducted in conjunction with an on-going collaborative field research project, which involved the State of Florida DEP, University of Florida, LFR Levine Fricke (site operations contractor), USEPA Technical Innovation Office, and the USEPA National Risk Management Research Laboratory (NRMRL). The involvement of this diverse group of collaborators should facilitate technology transfer efforts.

The USEPA-NRMRL's Subsurface Protection and Restoration Division located in Ada, Oklahoma houses the Superfund Technology Support Center (TSC), which provides a mechanism for technical assistance and technology transfer to move research results to the private sector. The TSC has provided technical assistance on over 300 Superfund sites since 1987 and has conducted numerous technology transfer seminars for EPA regional personnel, state personnel, and private contractors who are responsible for subsurface remediation at hazardous waste sites. The TSC provides a very effective means for transferring the results of this research to the user community.

An implementation manual for the SERB technology is currently in preparation. This manual will discuss the considerations that should be made before application of the technology, as well as the results from the SERB demonstration project. This manual will be produced as an EPA Report that will be widely distributed and available to government personnel and private industry. The Table of Contents for the implementation manual is provided in Attachment C-2.

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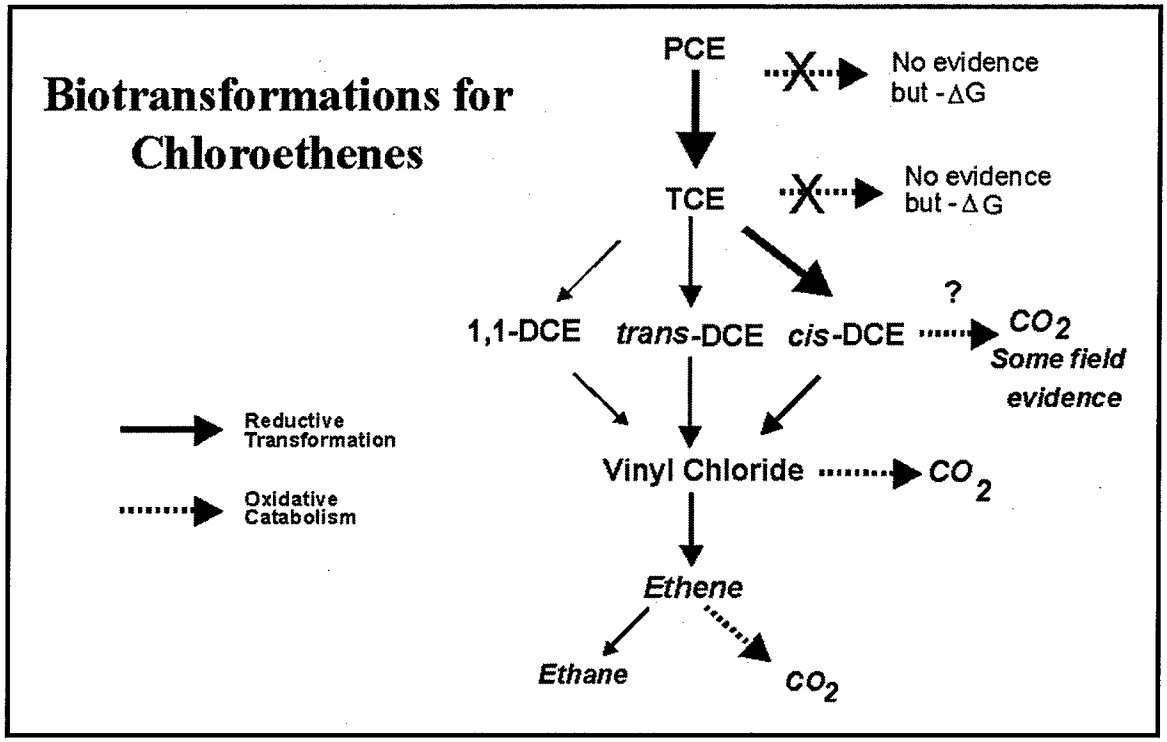
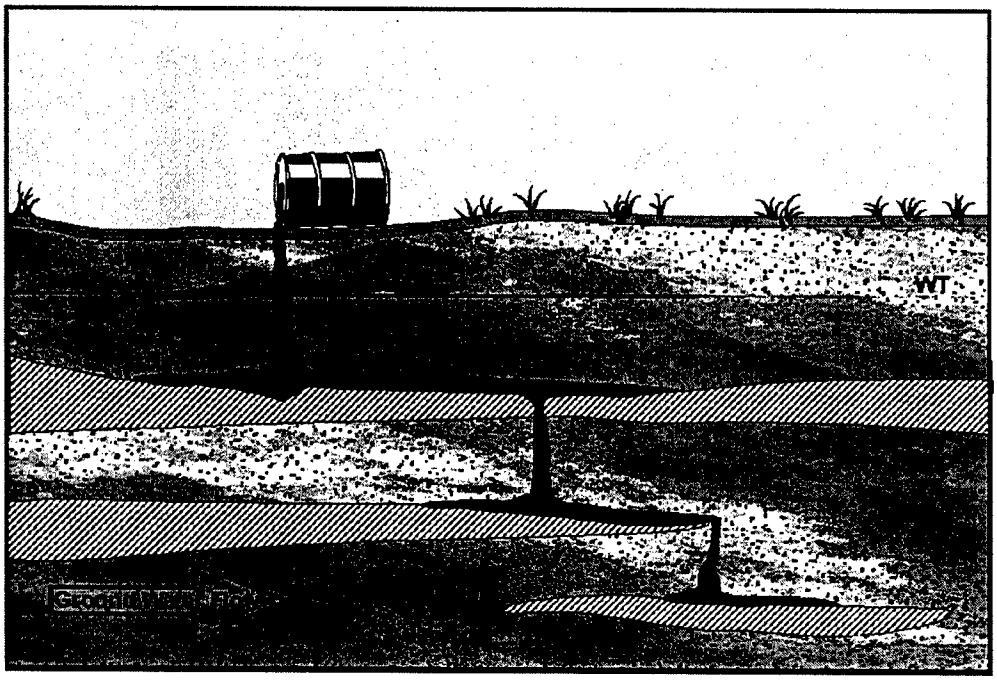
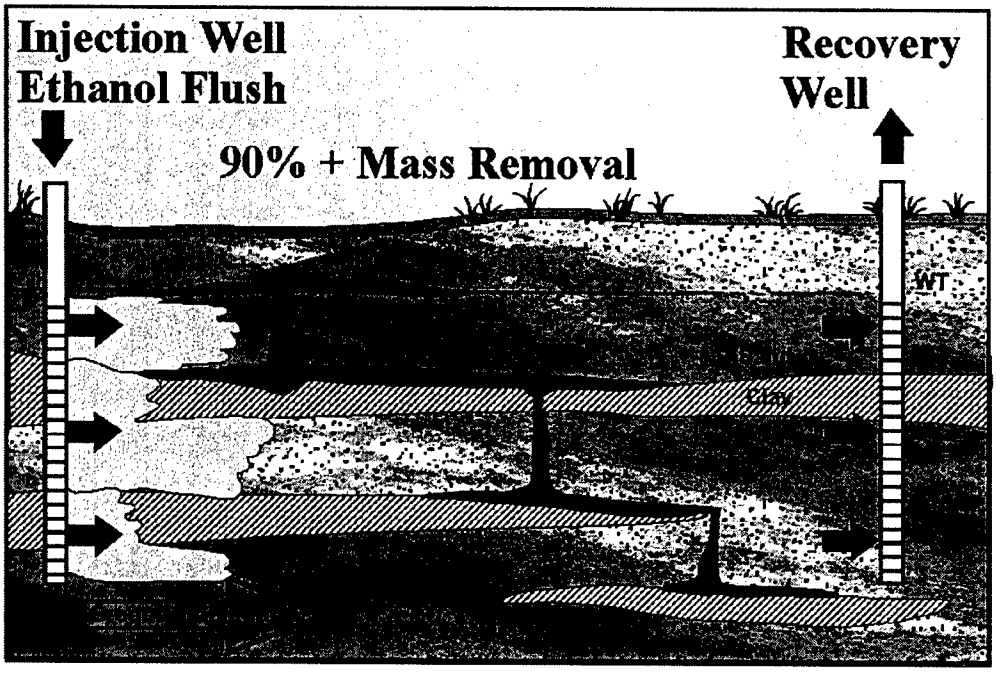


Figure 1. Biotransformation pathways for chlorinated ethenes

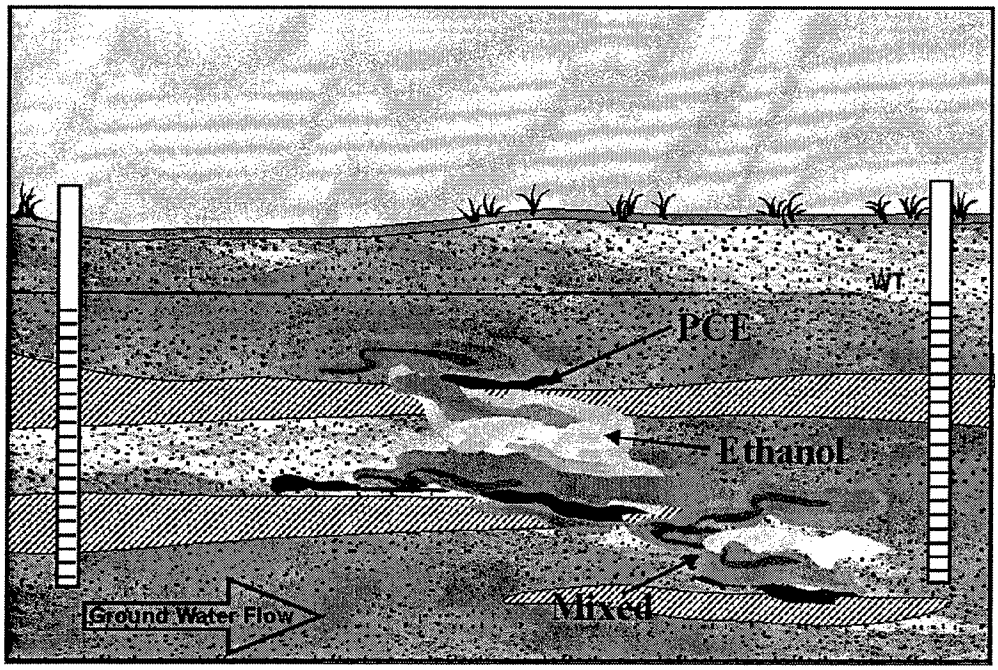


a. Complex distribution of DNAPL in subsurface

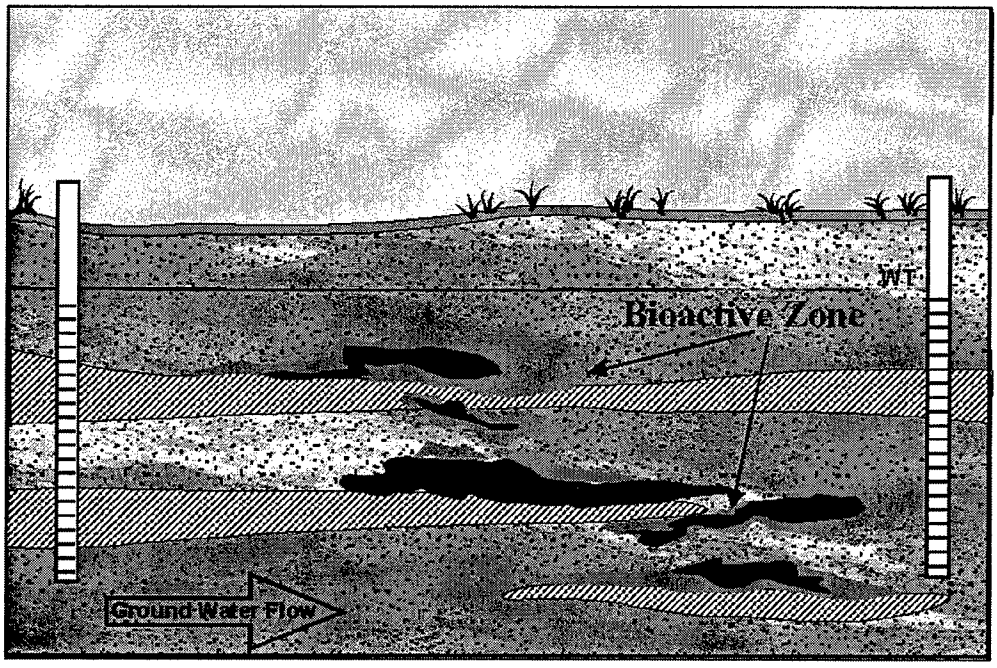


b. Dissolution of DNAPL by cosolvent flushing technology

Figure 2. Schematic representing application of the Solvent Extraction Residual Biotreatment (SERB) technology (Continued)



c. Separate and mixed areas of DNAPL and cosolvent exist in subsurface following cosolvent extraction



d. Development of bioactive zones conducive to the reductive dechlorination of DNAPL

Figure 2. (Concluded)

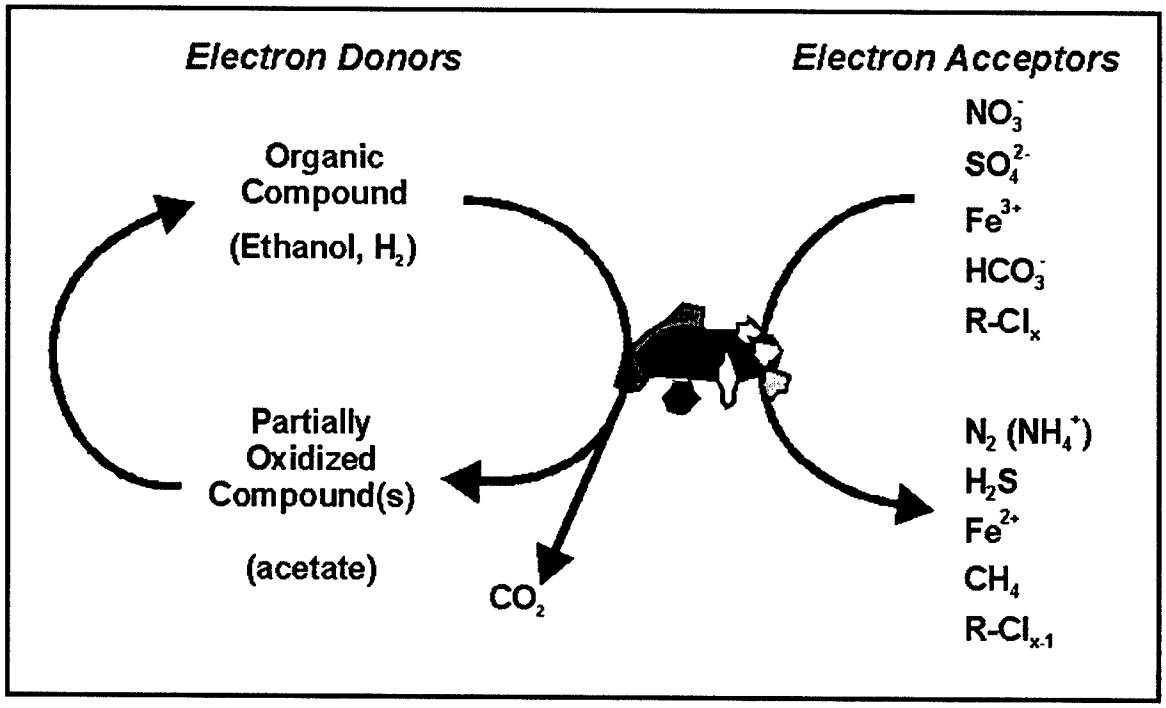


Figure 3. Complex oxidation-reduction reactions conducted by microorganisms in the subsurface environment

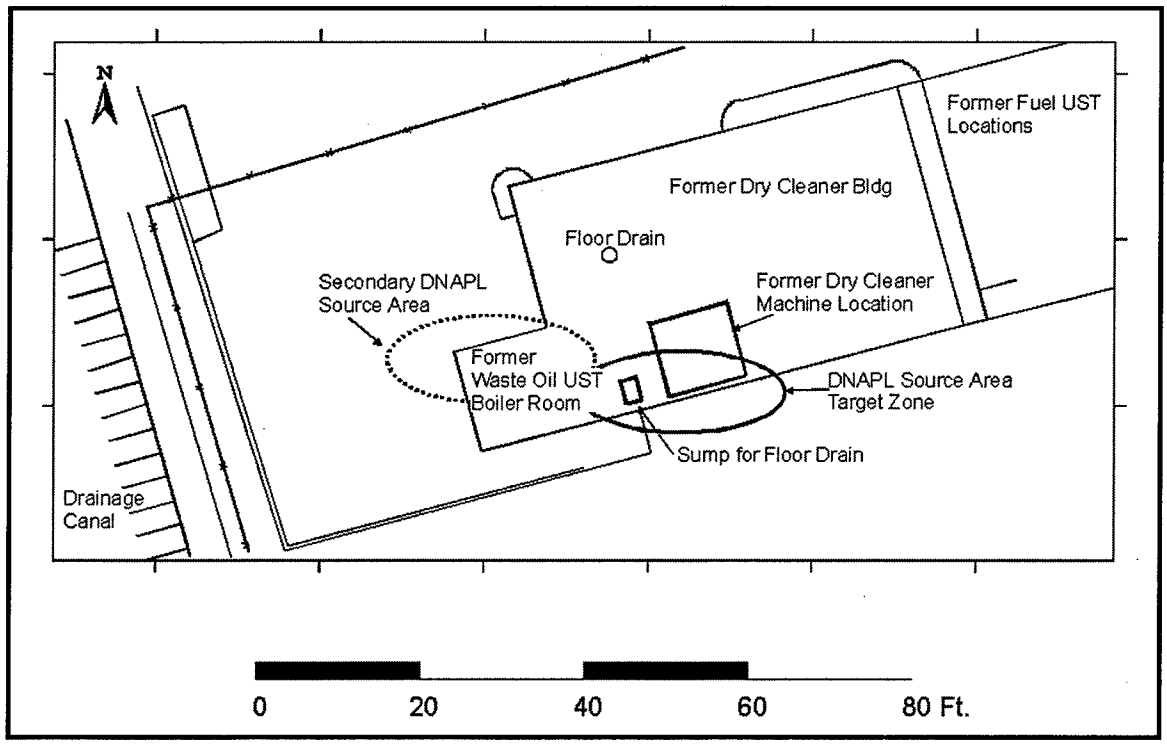


Figure 4. Sage's site map indicating former building and suspected contamination areas

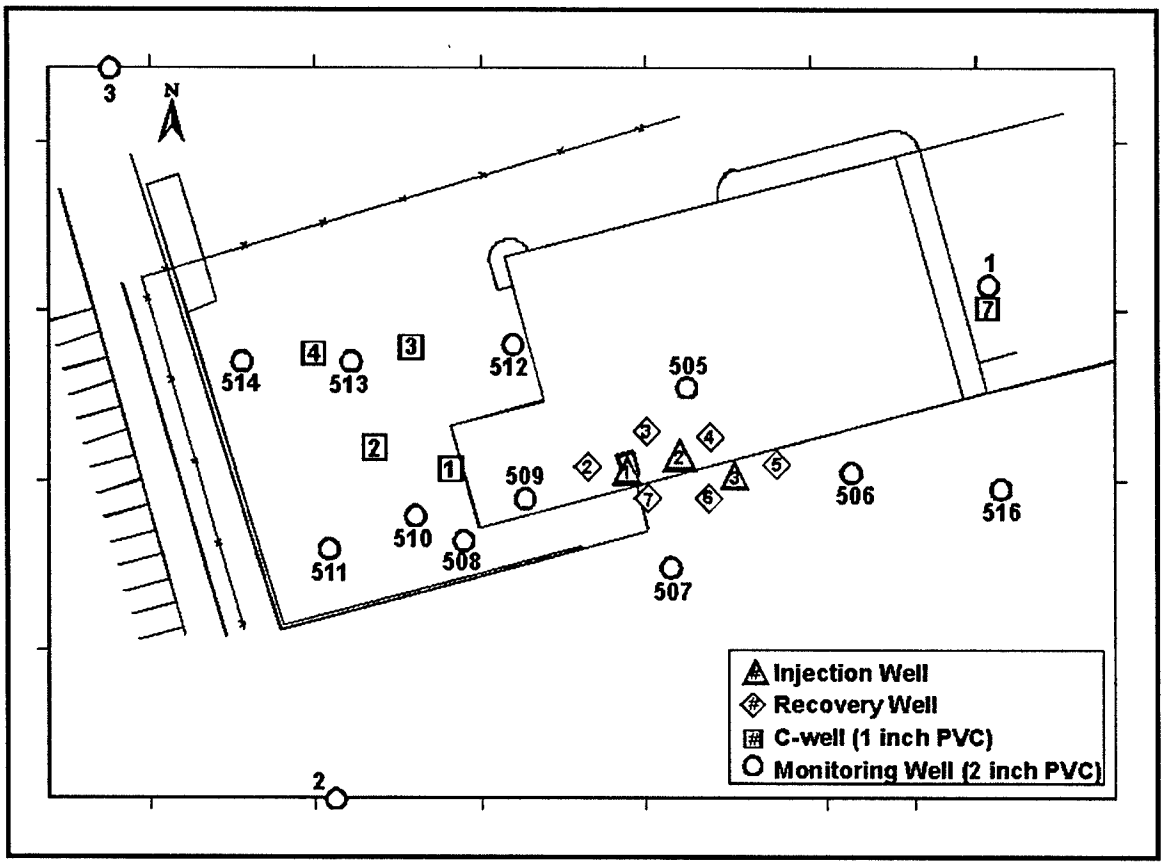


Figure 5. Well locations at the former Sage's Dry Cleaner site in Jacksonville, FL

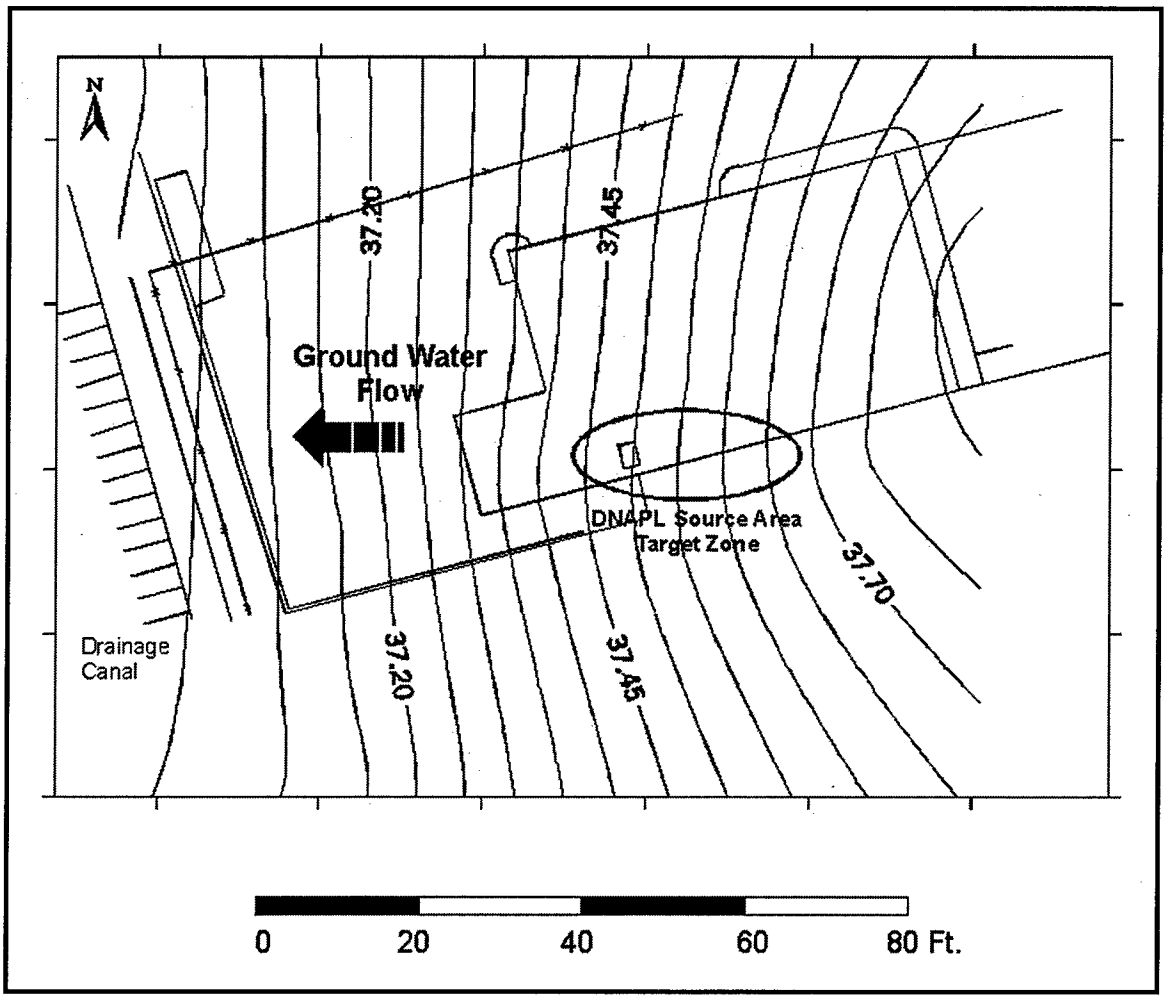


Figure 6. Ground water elevations at the Sage's site on 9/22/99 showing ground water flow to the west towards the drainage canal

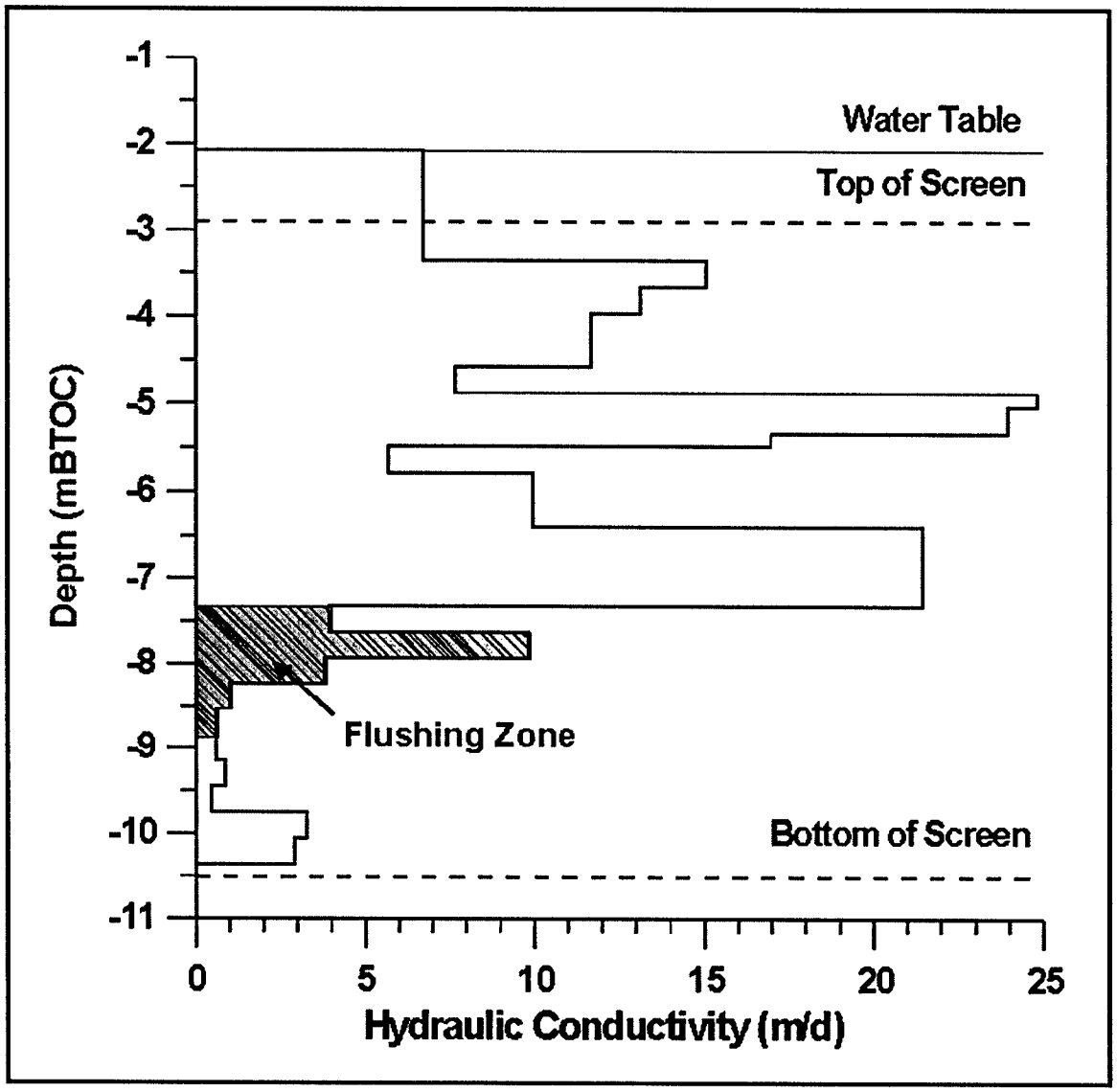


Figure 7. Estimated hydraulic conductivity profile for materials screened by well MW-516

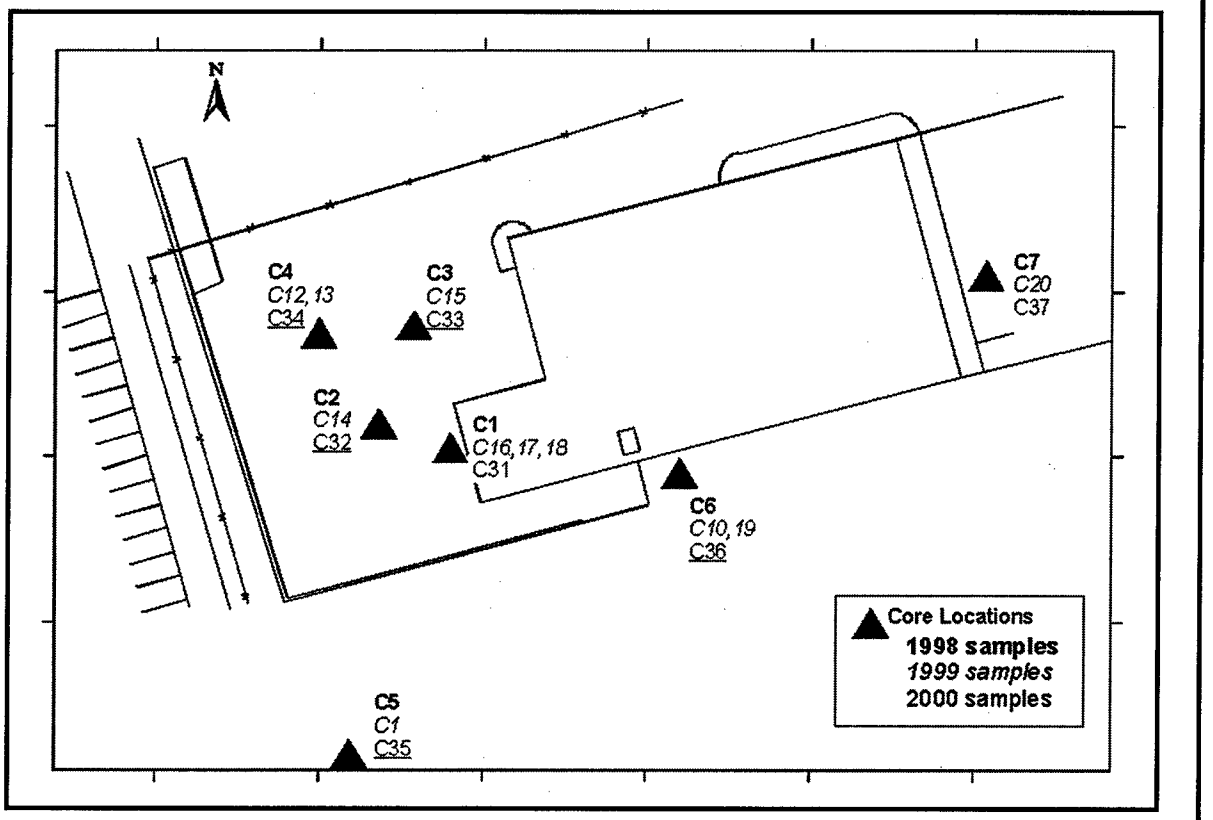


Figure 8. Locations of core materials collected for molecular analysis

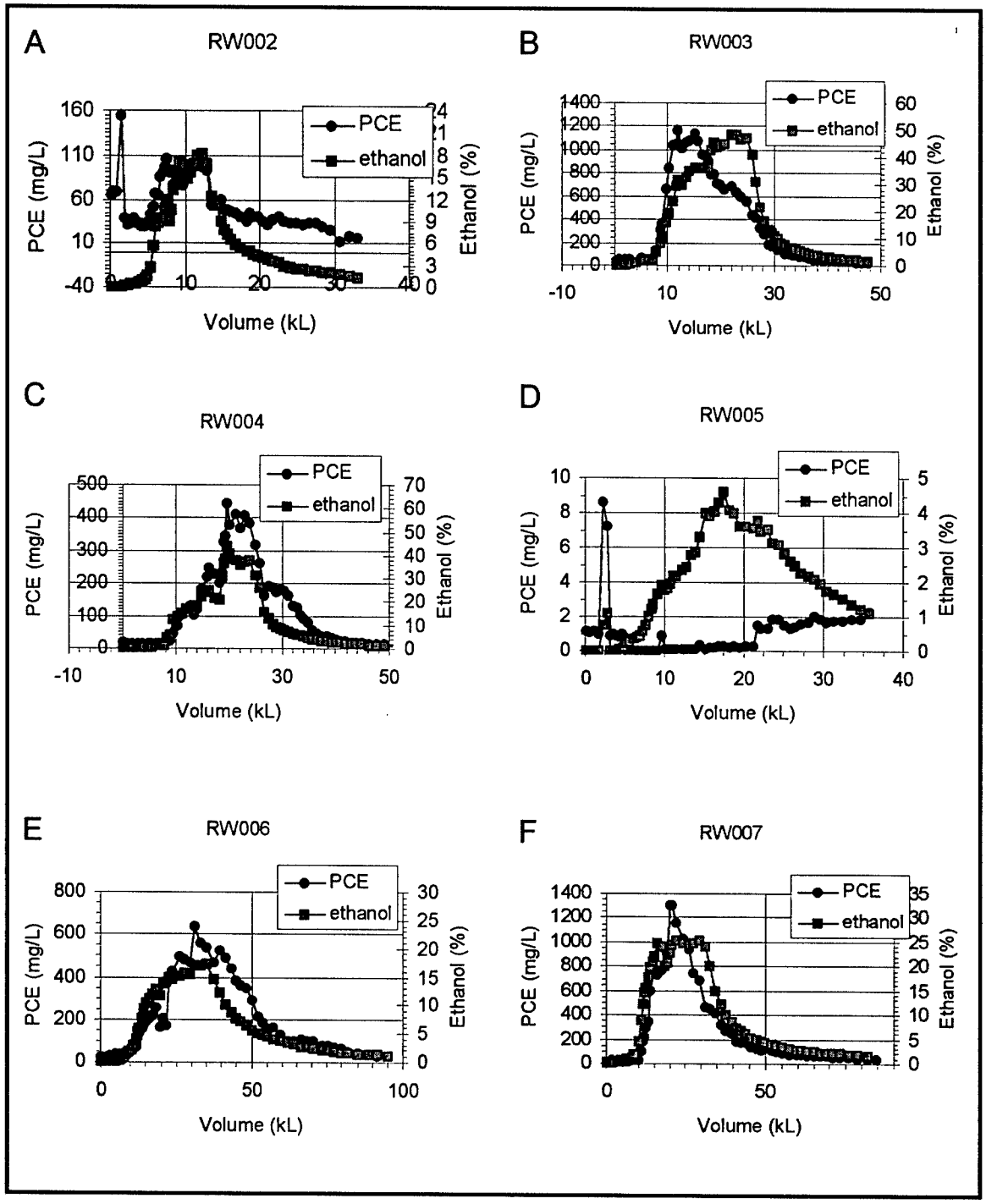


Figure 9. Ethanol and PCE concentrations in recovery wells during the cosolvent flushing test

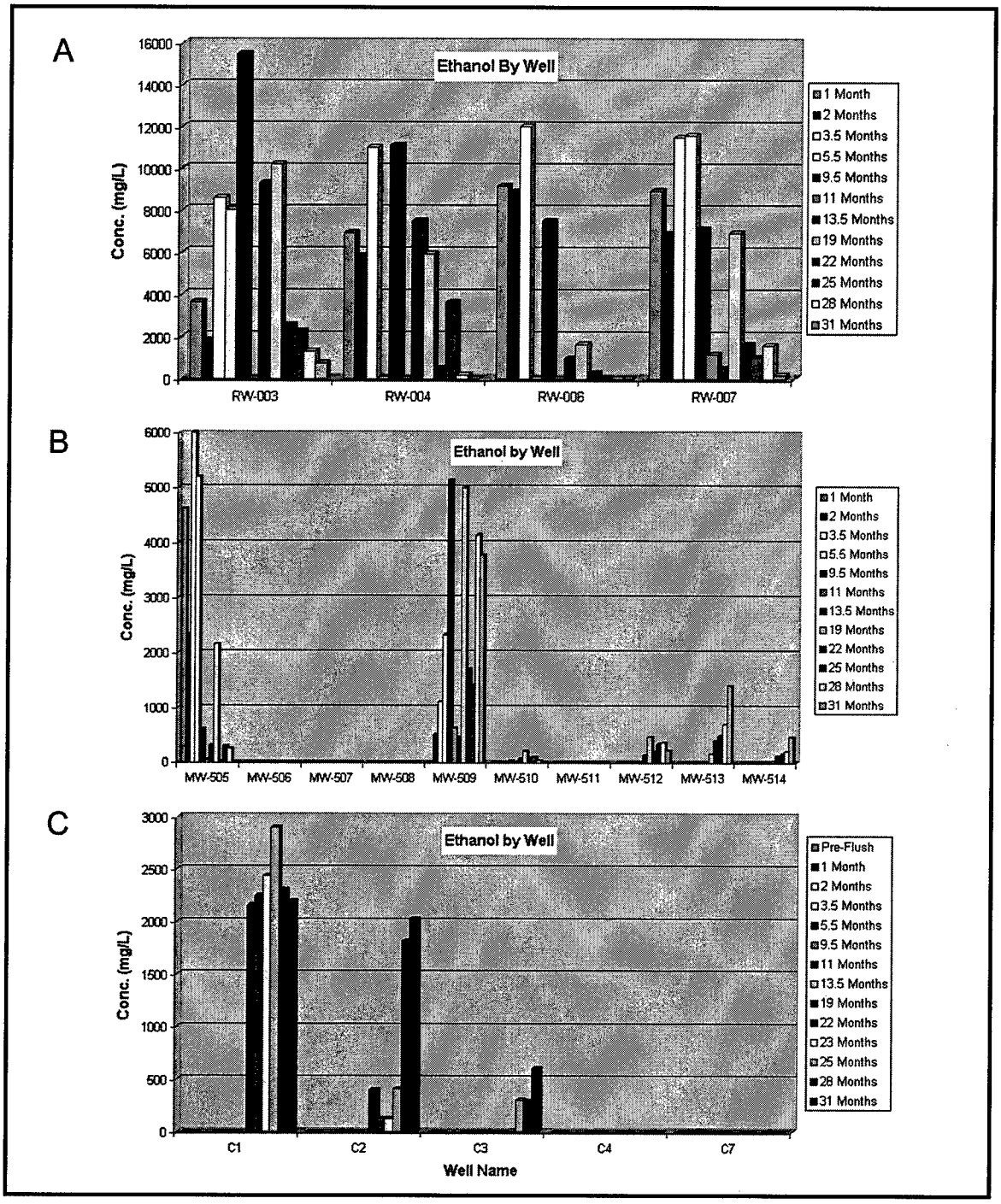


Figure 10. Ethanol concentrations by well during ground water monitoring

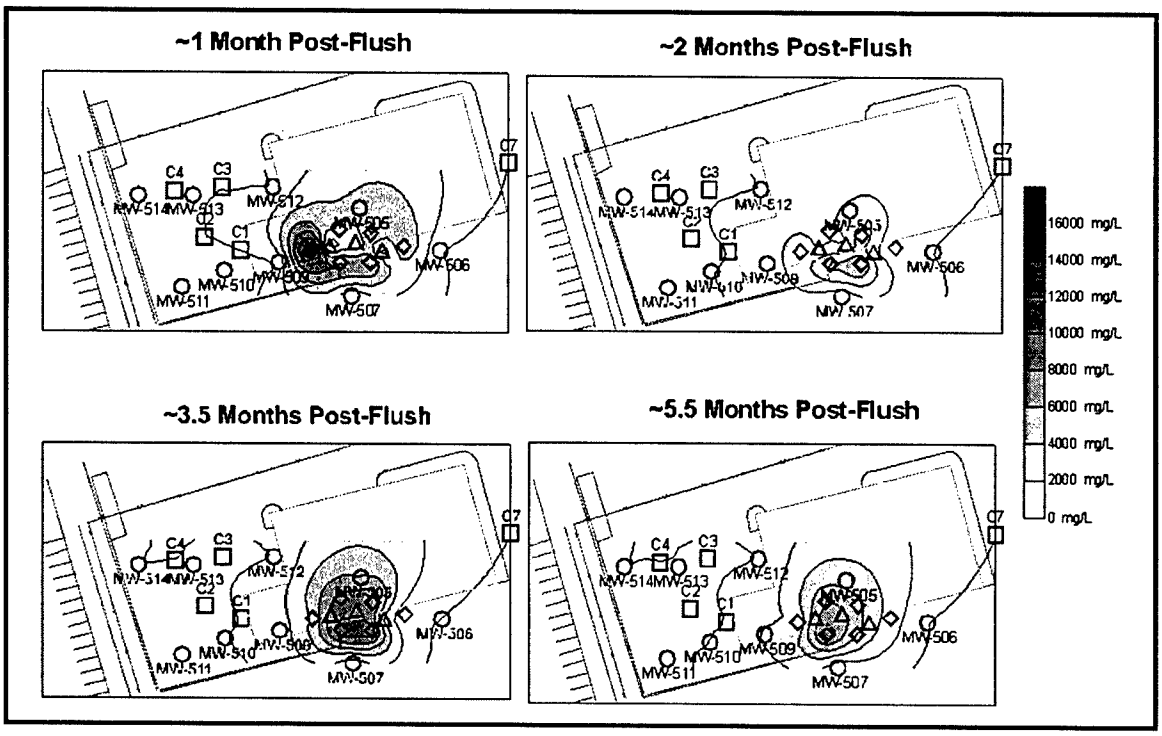


Figure 11. Ethanol contour plot illustrating the change in concentration and movement downgradient during study months 1 to 5.5

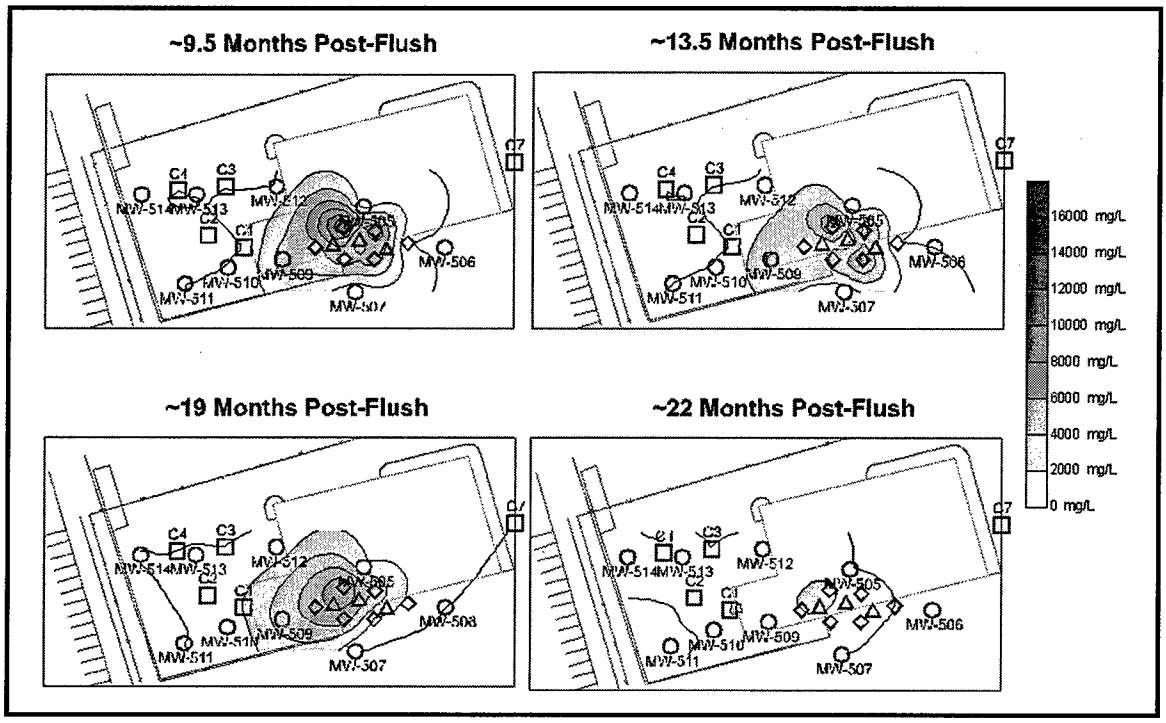


Figure 12. Ethanol contour plot illustrating the change in concentration and movement downgradient during study months 9.5 to 22

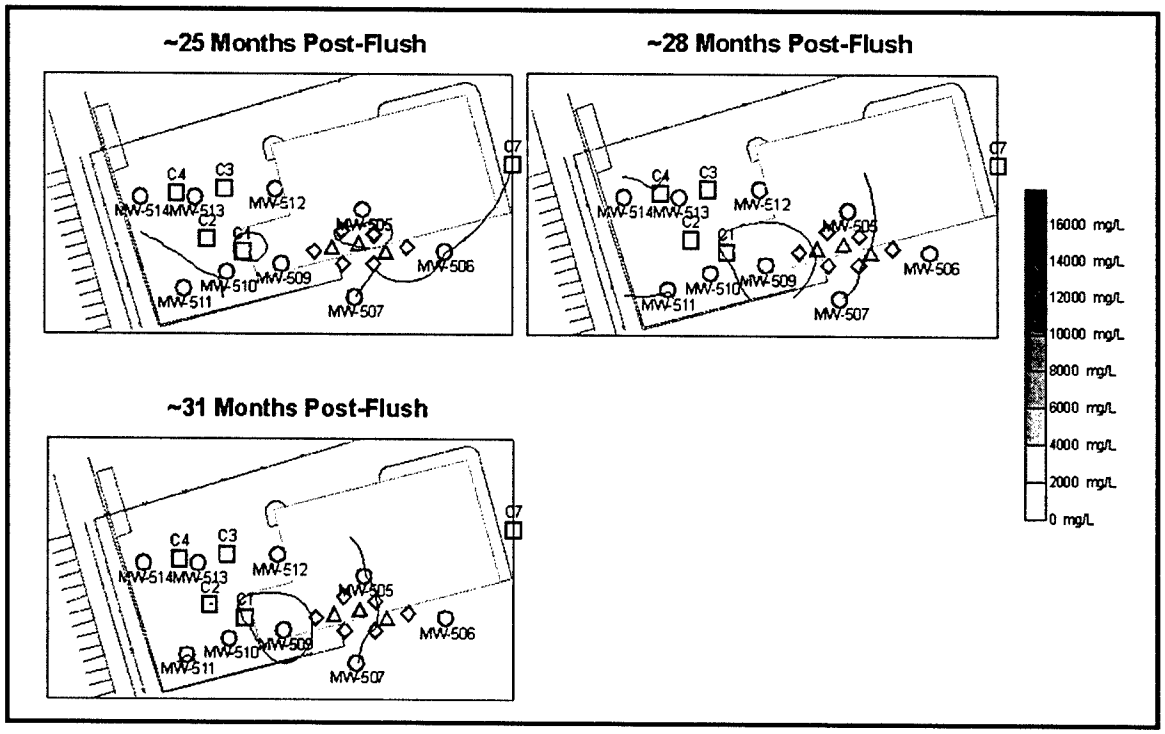


Figure 13. Ethanol contour plot illustrating the change in concentration and movement downgradient during study months 25 to 31

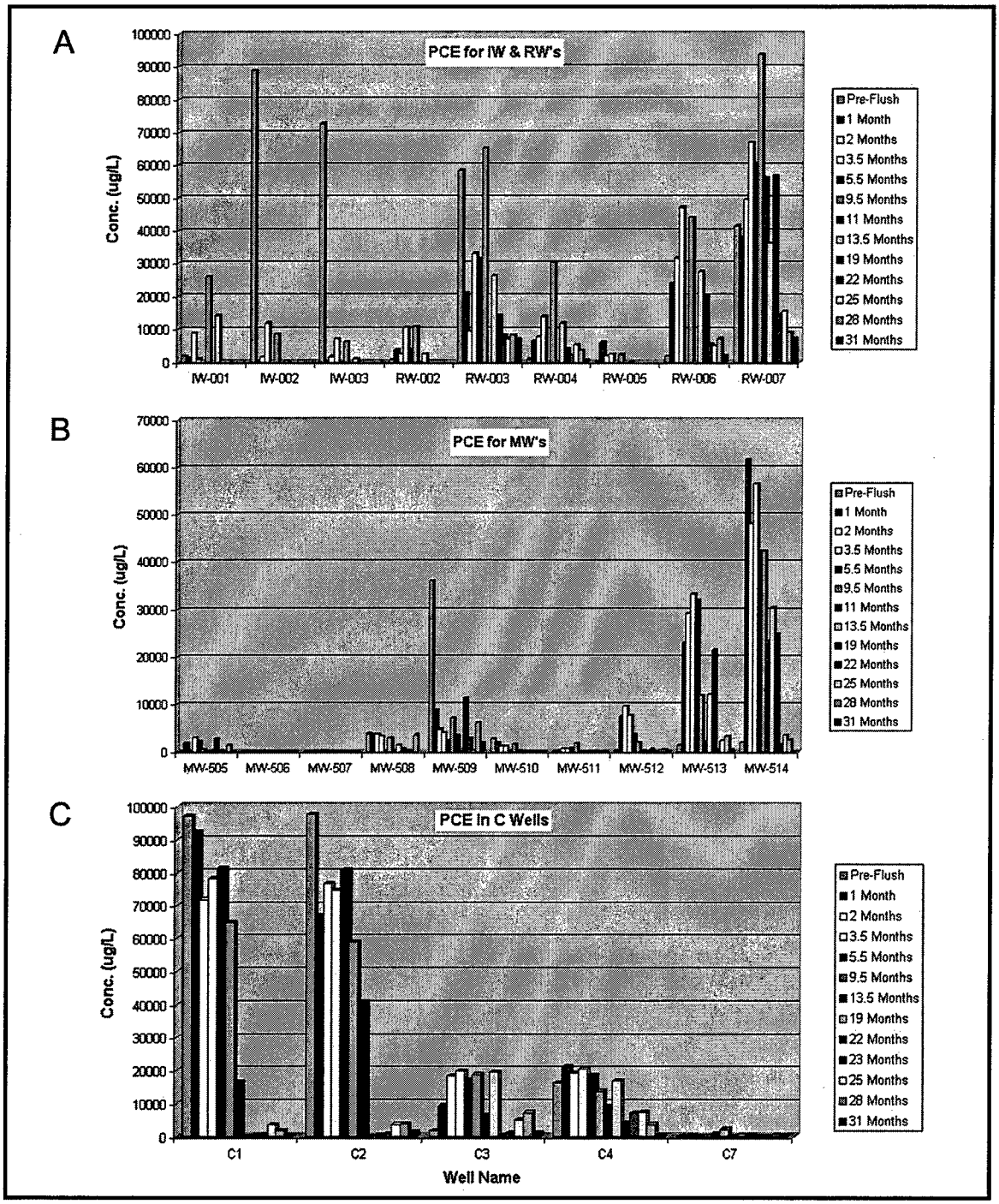


Figure 14. PCE concentrations by well during ground water monitoring

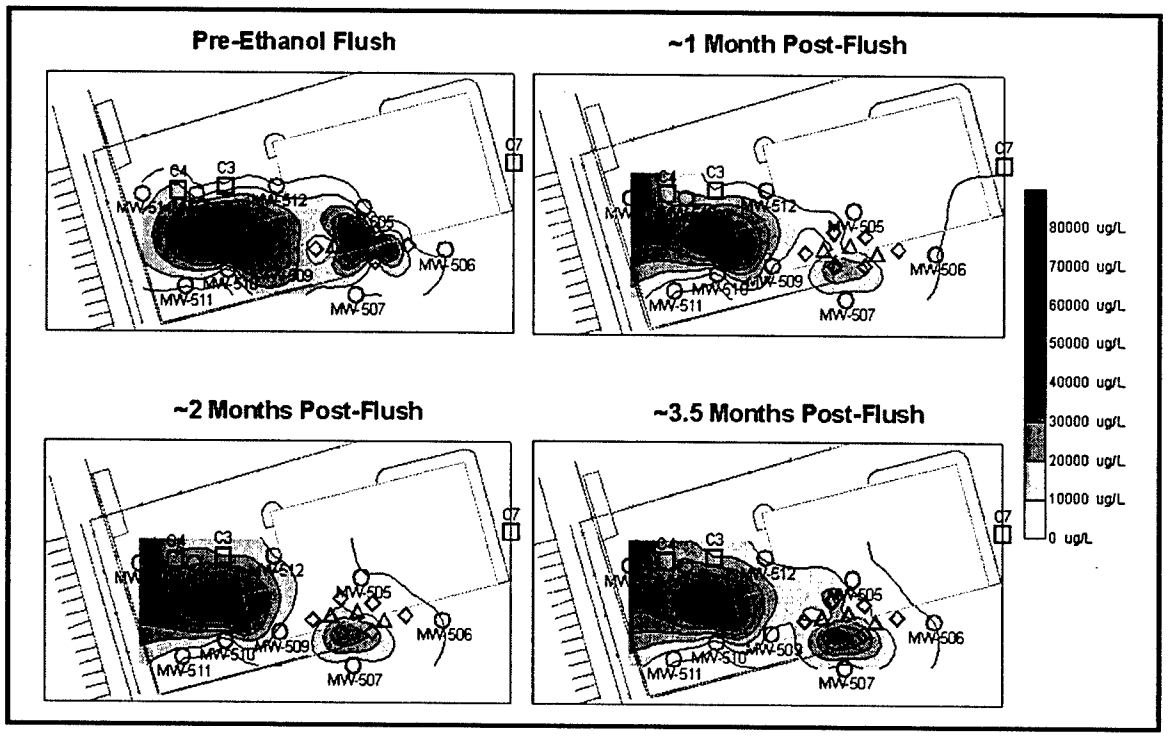


Figure 15. PCE contour plots illustrating the change in concentration and the movement over the monitoring period initial to 3.5 months

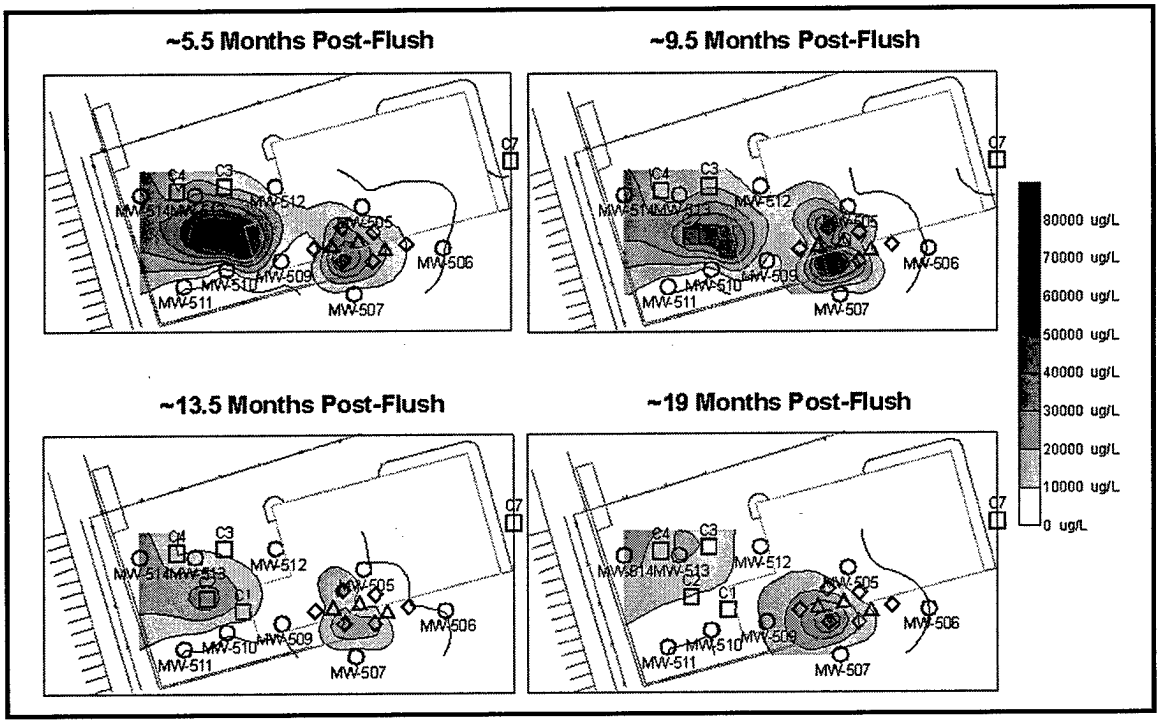


Figure 16. PCE contour plots illustrating the change in concentration and the movement over the monitoring period 5.5 to 19 months

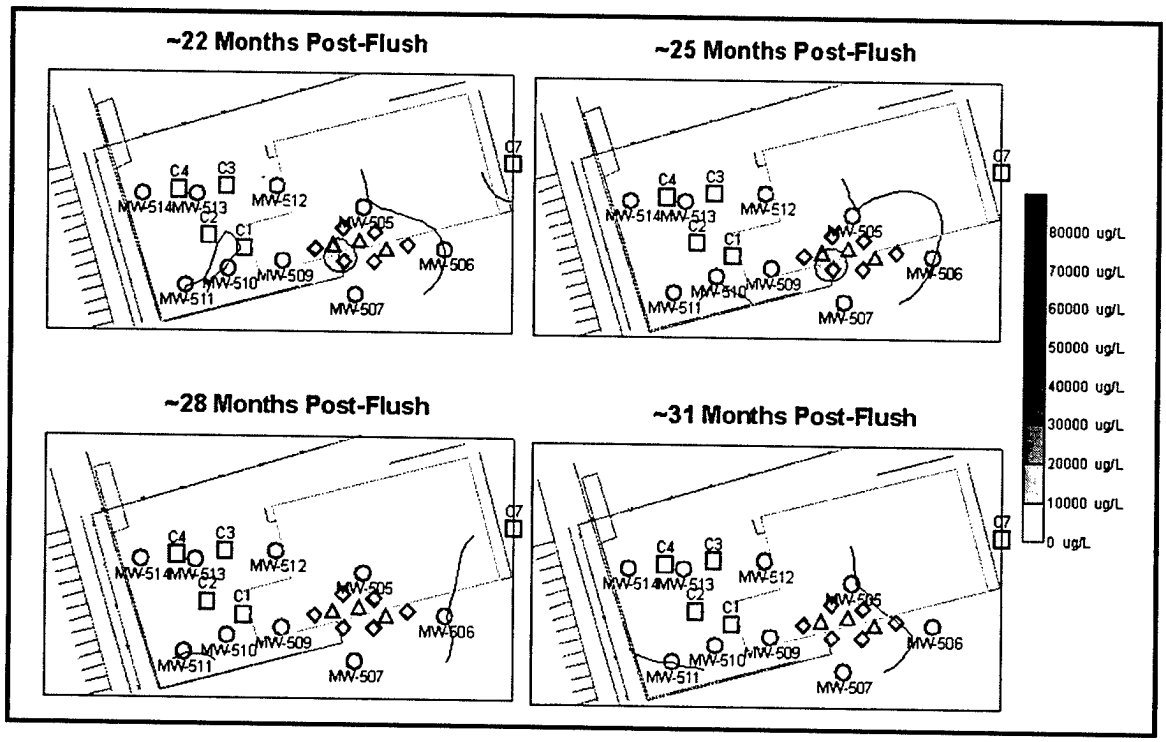


Figure 17. PCE contour plots illustrating the change in concentration and the movement over the monitoring period 22 to 31 months

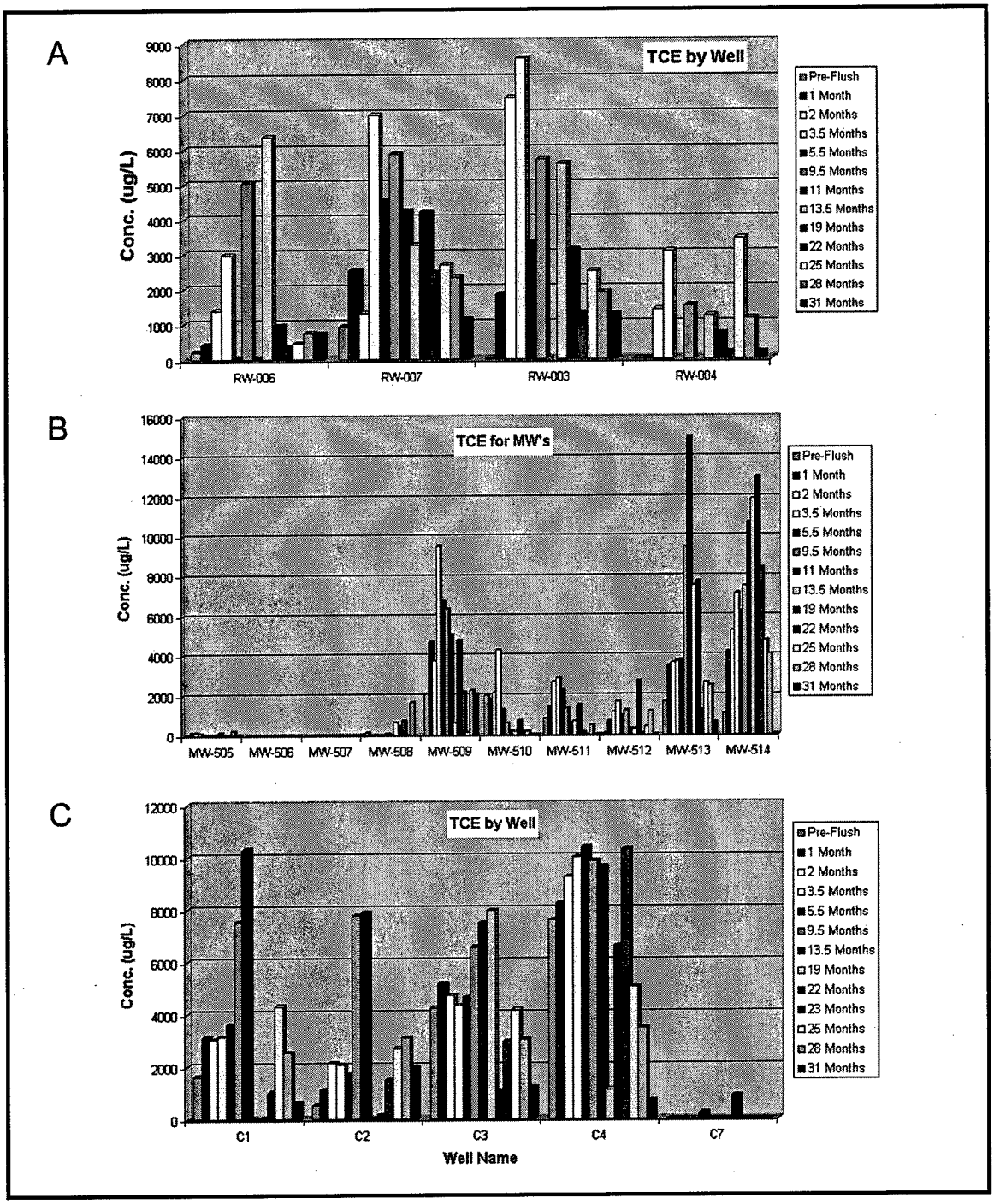


Figure 18. TCE concentrations by well during ground water monitoring

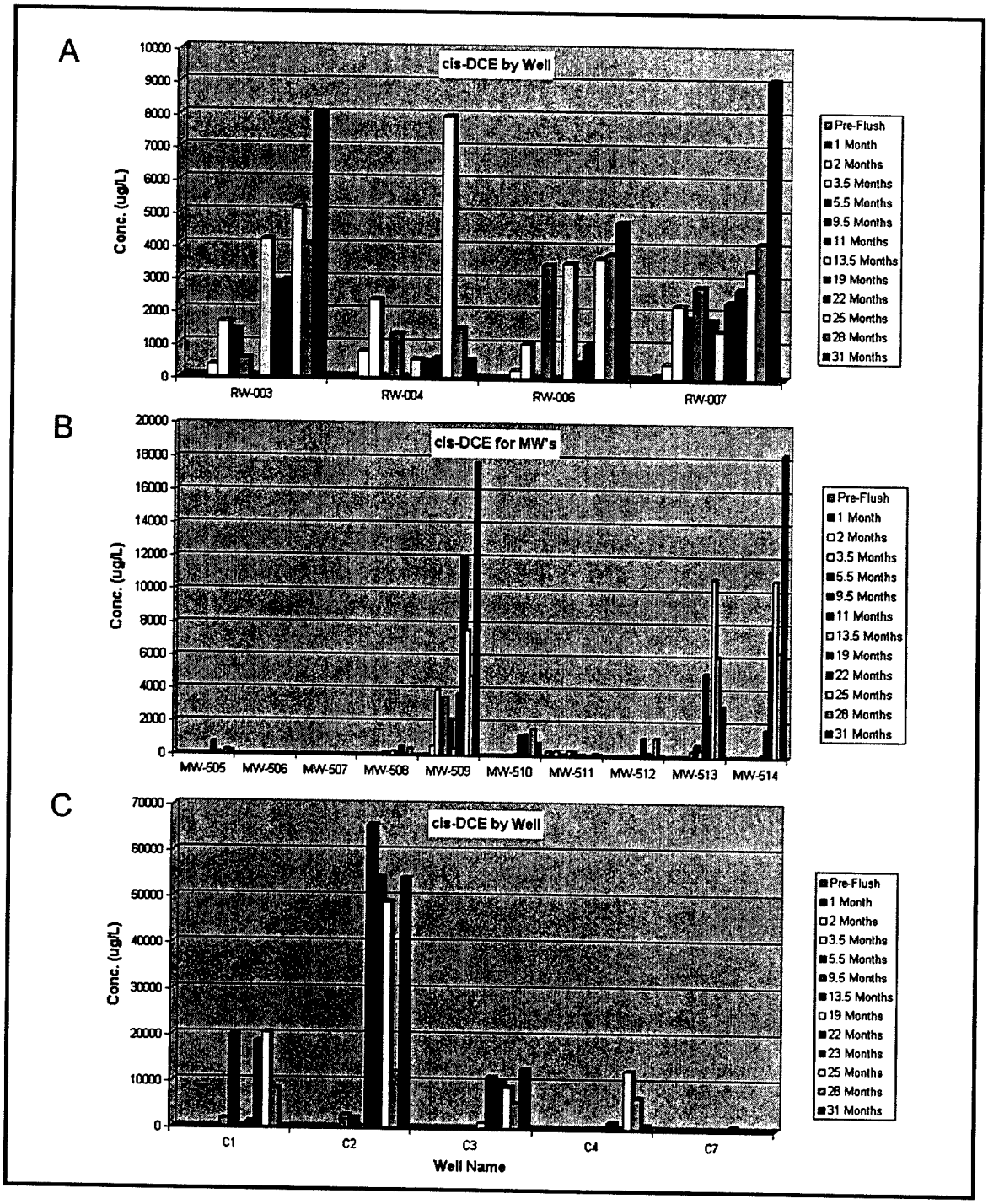


Figure 19. *cis*-DCE concentrations by well during ground water monitoring

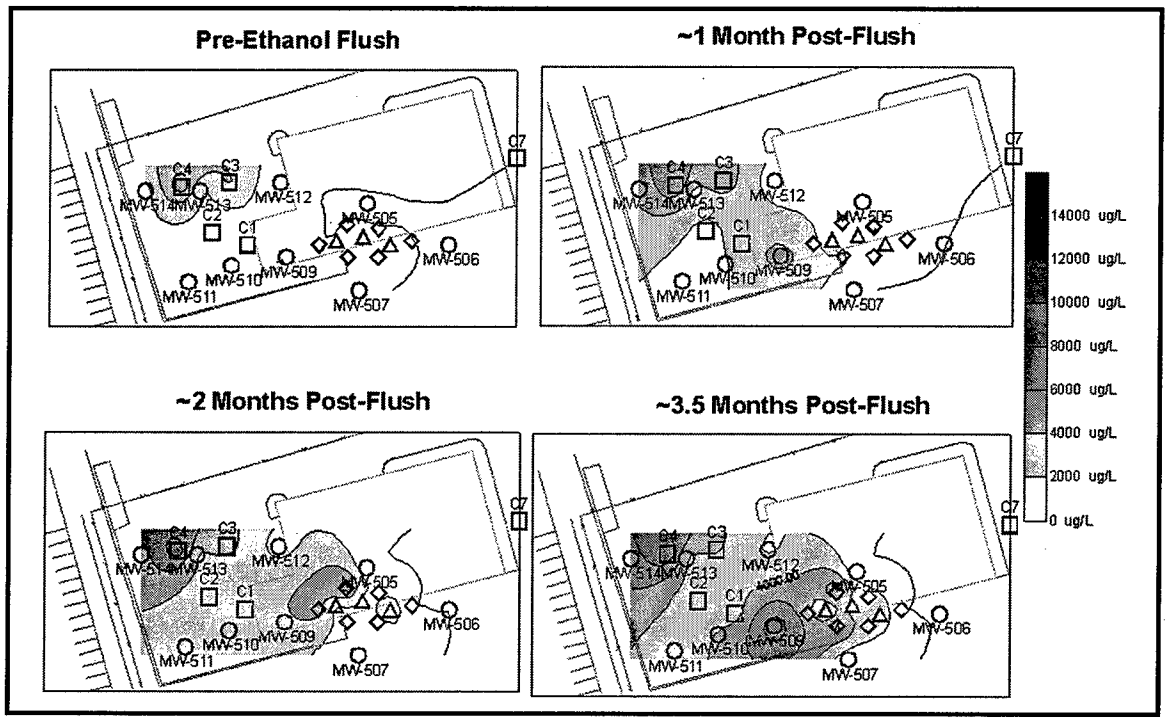


Figure 20. TCE contour plots illustrating the change in concentration and the movement over the monitoring period initial to 3.5 months

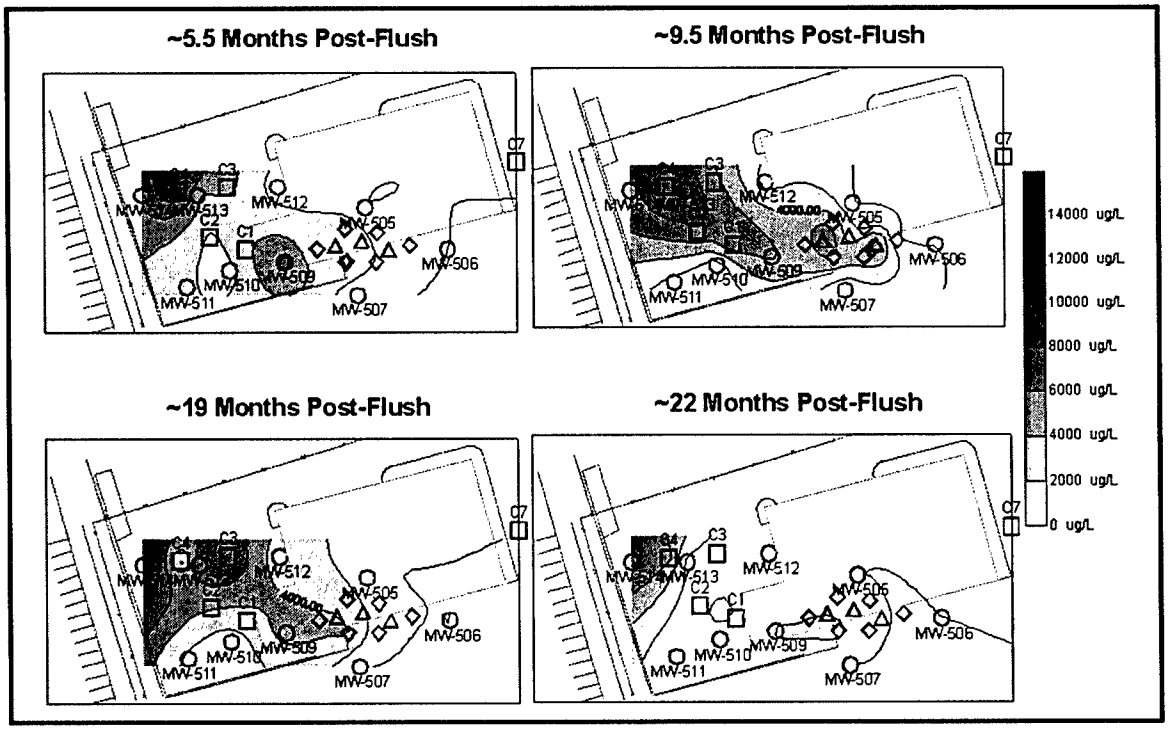


Figure 21. TCE contour plots illustrating the change in concentration and the movement over the monitoring period 5.5 to 19 months

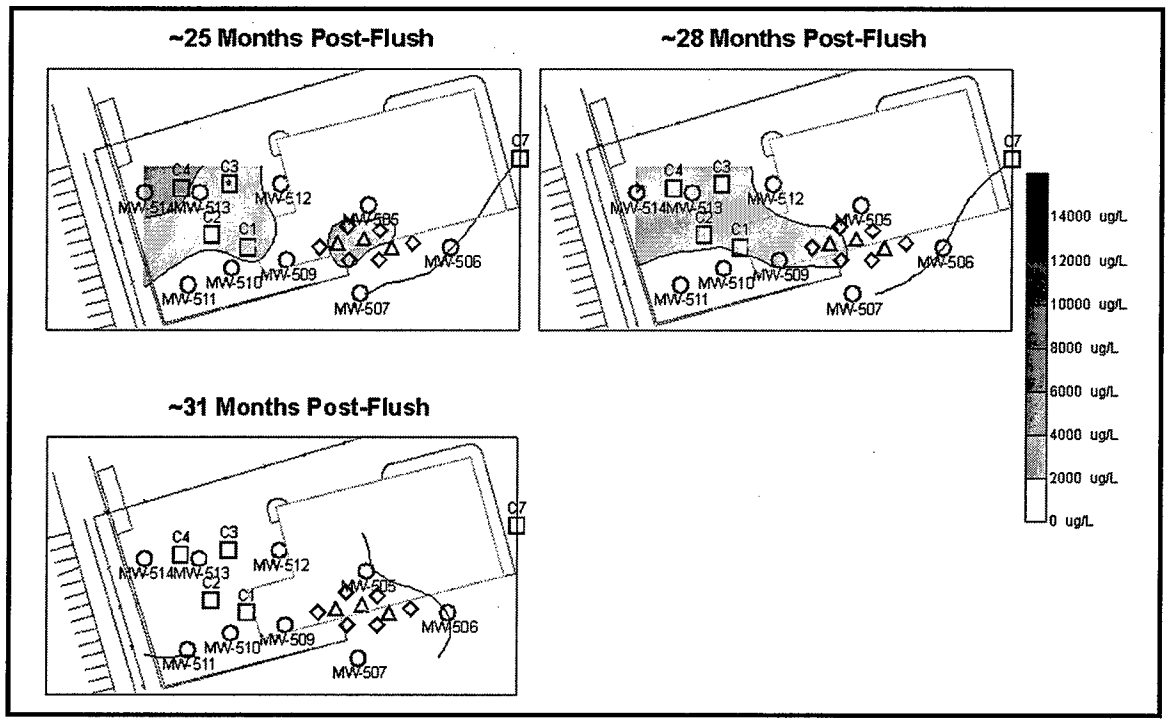


Figure 22. TCE contour plots illustrating the change in concentration and the movement over the monitoring period 22 to 31 months

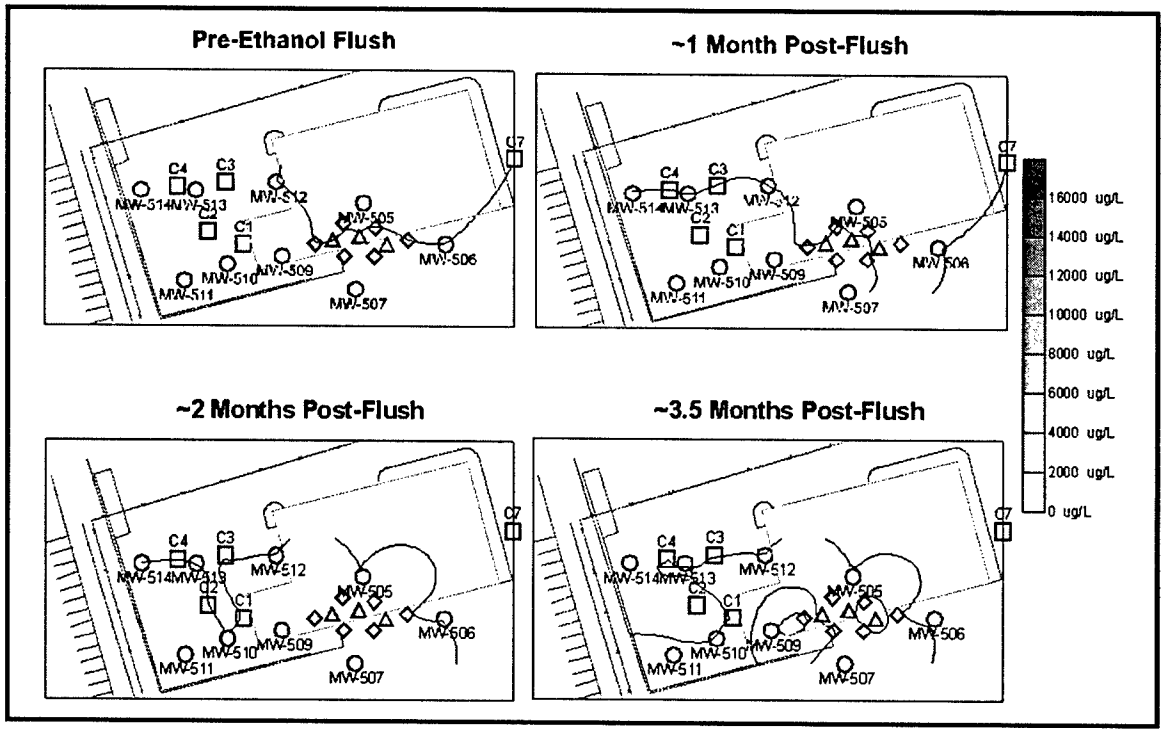


Figure 23. *cis*-DCE contour plots illustrating the change in concentration and the movement over the monitoring period initial to 3.5 months

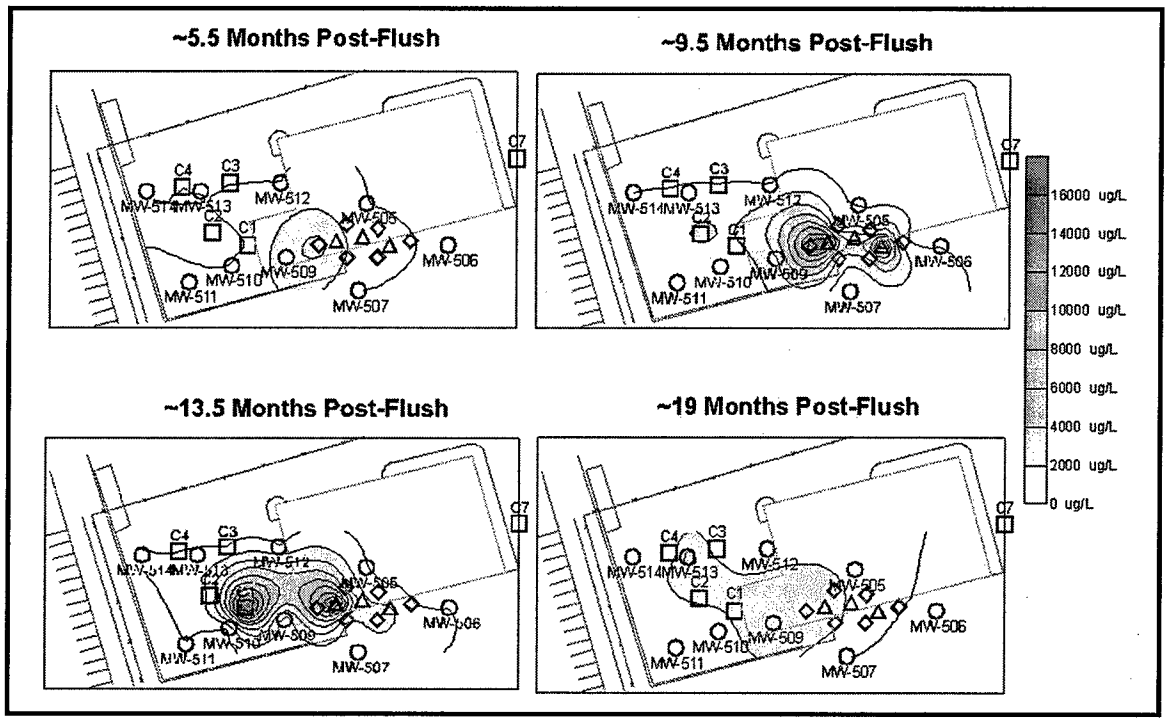


Figure 24. *cis*-DCE contour plots illustrating the change in concentration and the movement over the monitoring period 5.5 to 19 months

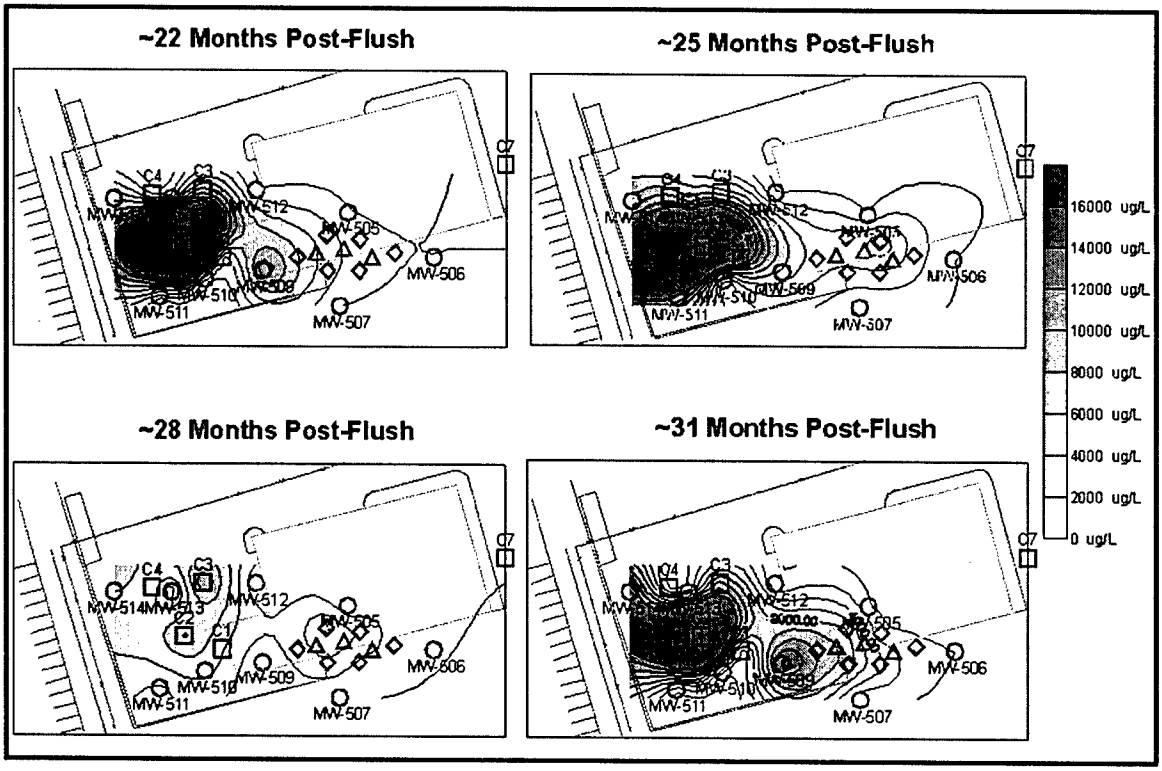


Figure 25. *cis*-DCE contour plots illustrating the change in concentration and the movement over the monitoring period 22 to 31 months

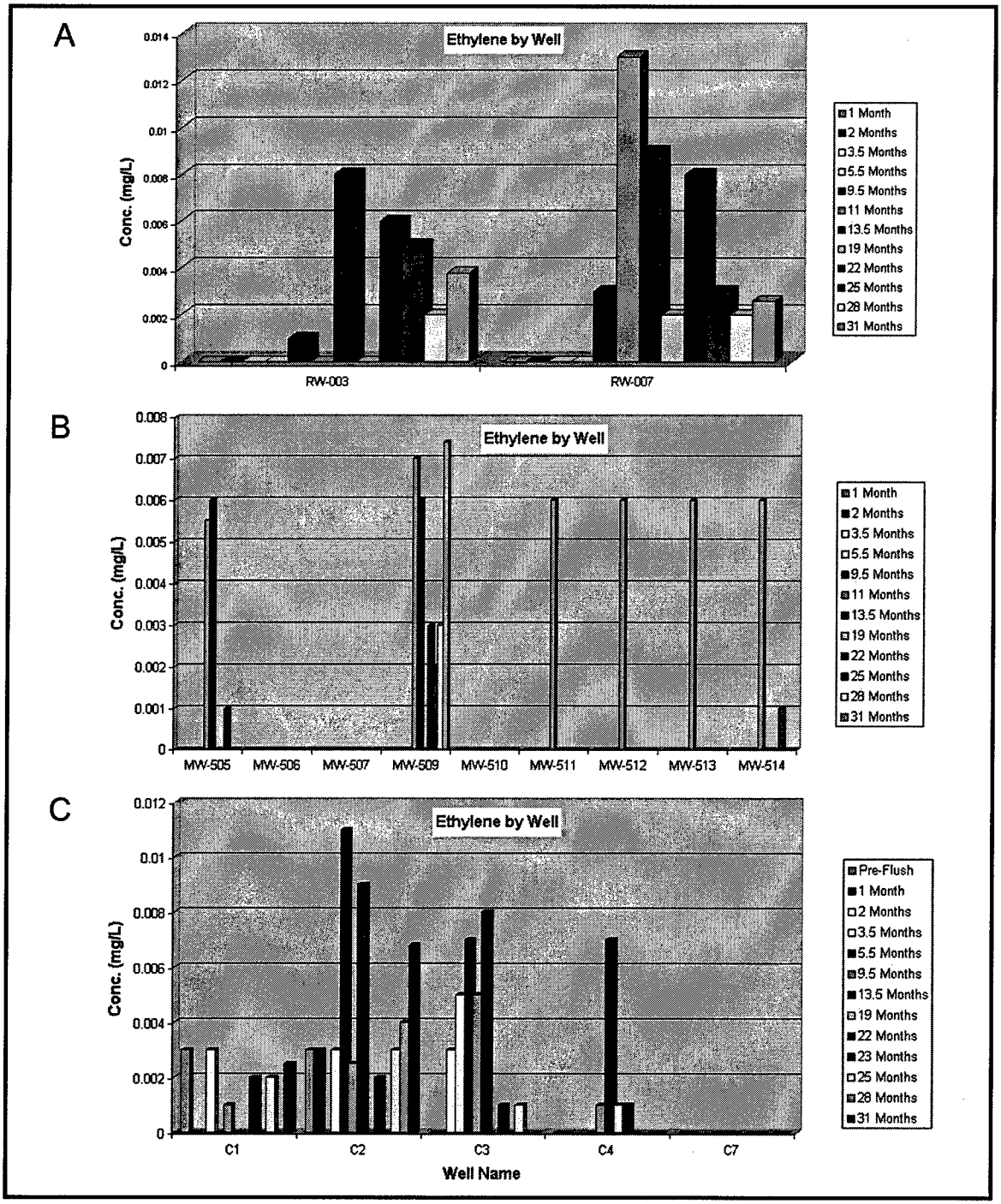


Figure 26. Ethylene concentrations by well during ground water monitoring

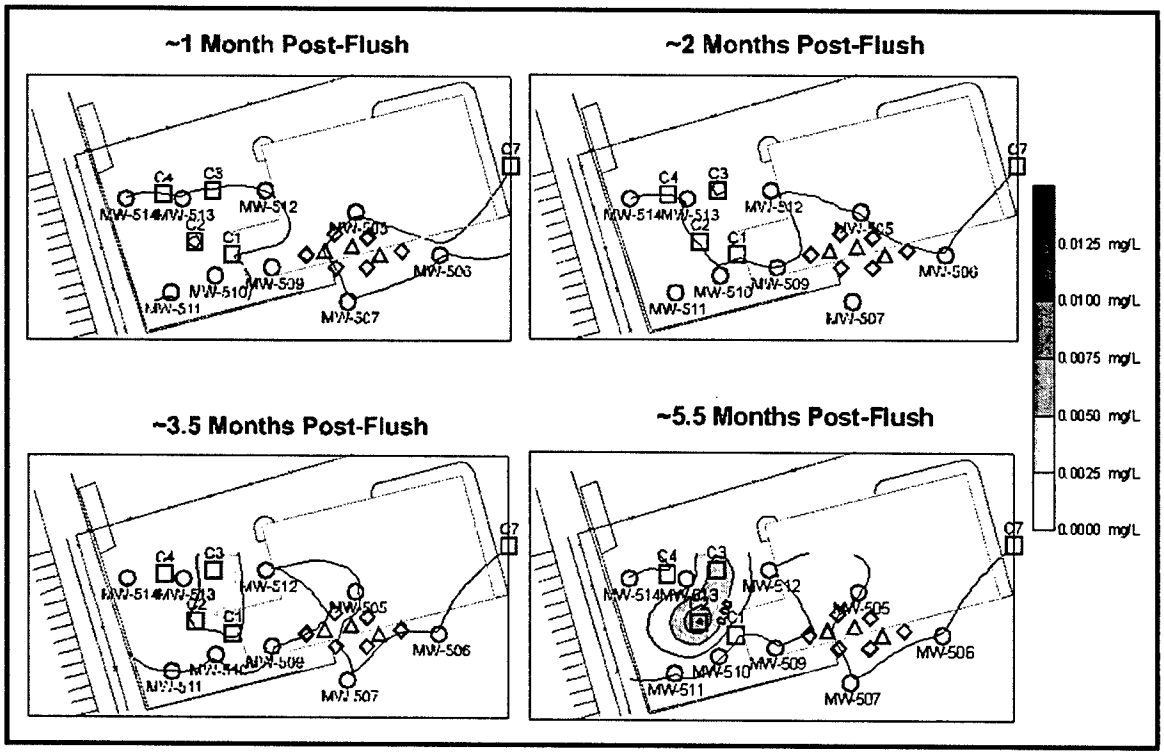


Figure 27. Ethylene contour plots illustrating the change in concentration and the movement over the monitoring period initial to 5.5 months

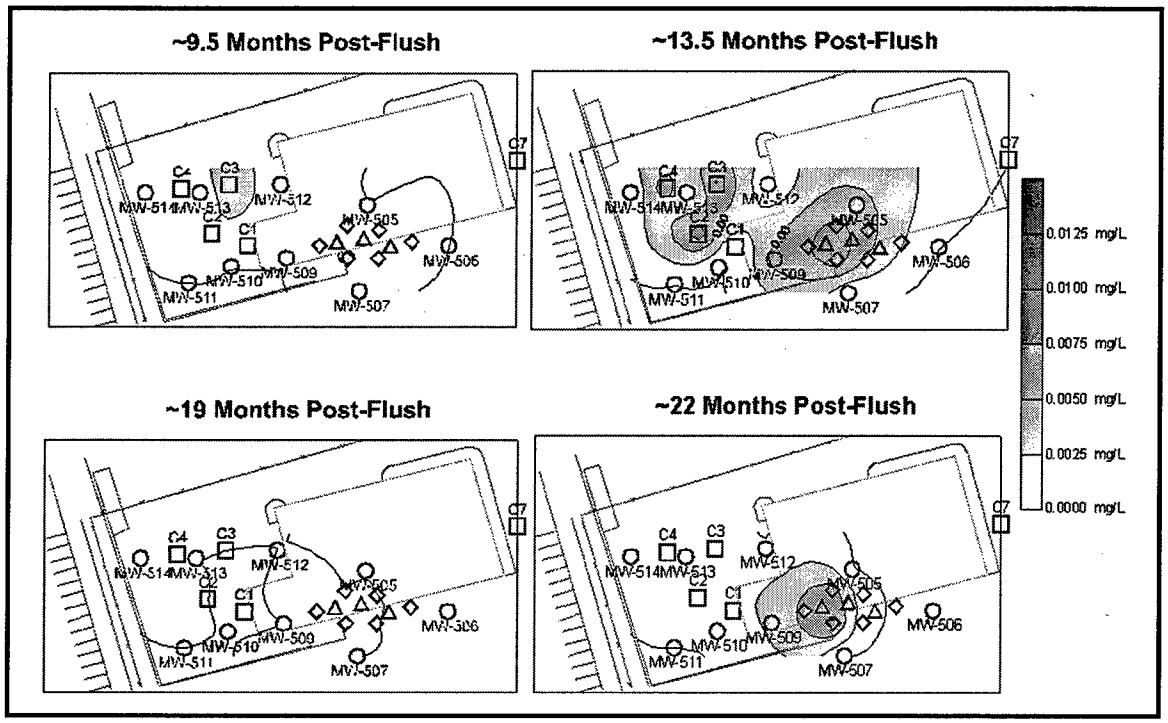


Figure 28. Ethylene contour plots illustrating the change in concentration and the movement over the monitoring period 9.5 to 22 months

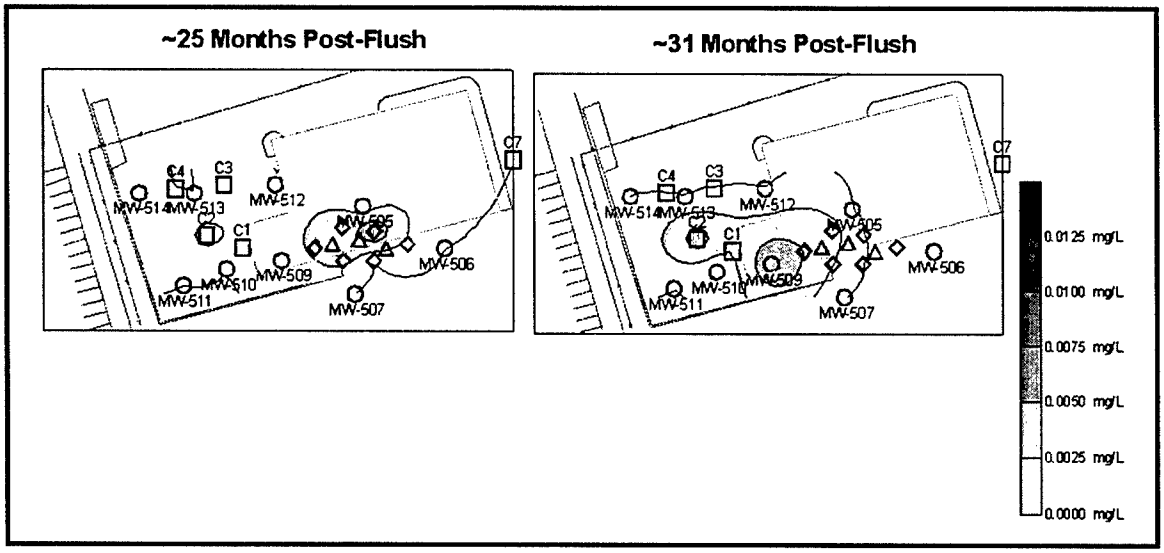


Figure 29. Ethylene contour plots illustrating the change in concentration and the movement over the monitoring period 25 to 31 months

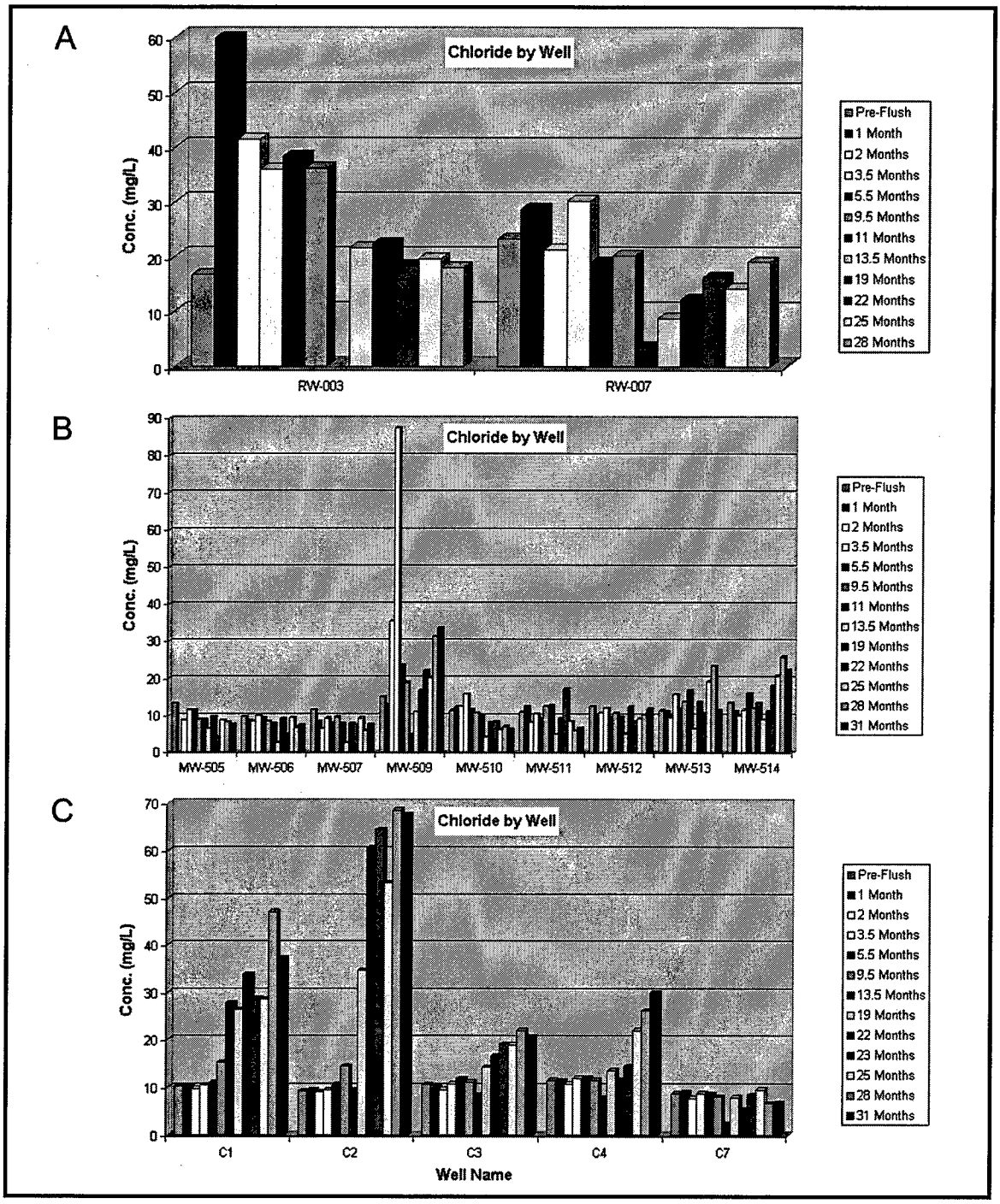


Figure 30. Chloride concentrations by well during ground water monitoring

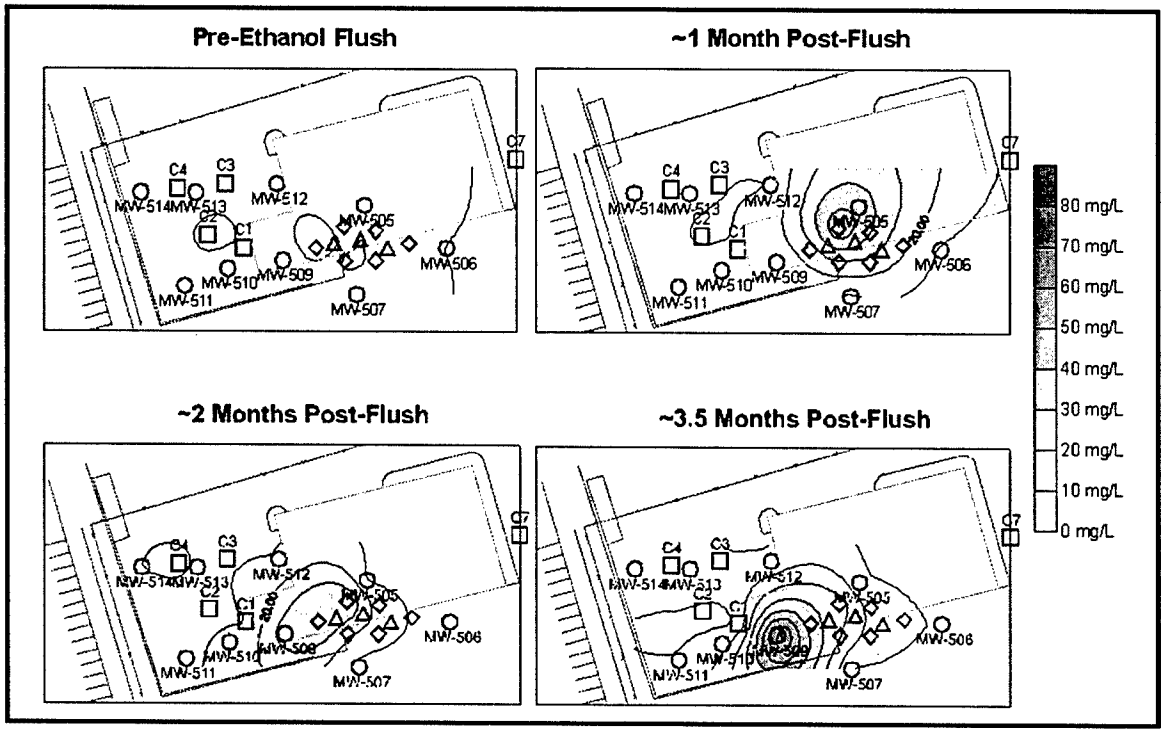


Figure 31. Chloride contour plots illustrating the change in concentration and the movement over the monitoring period initial to 3.5 months

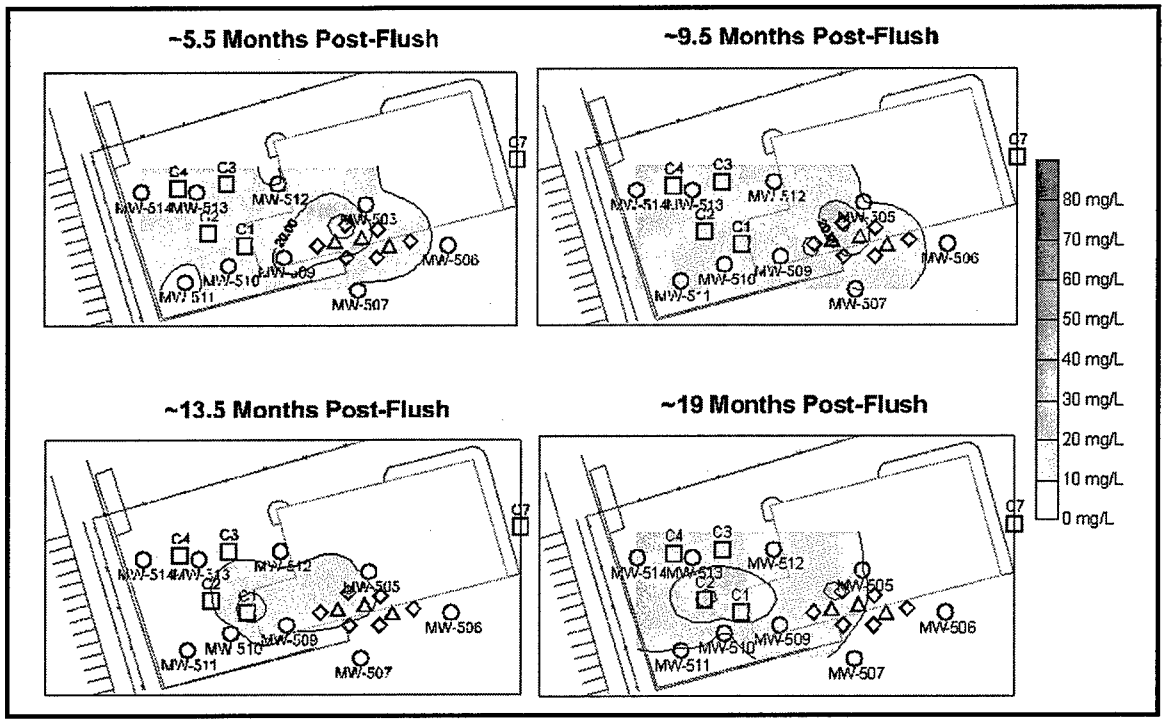


Figure 32. Chloride contour plots illustrating the change in concentration and the movement over the monitoring period 5.5 to 19 months

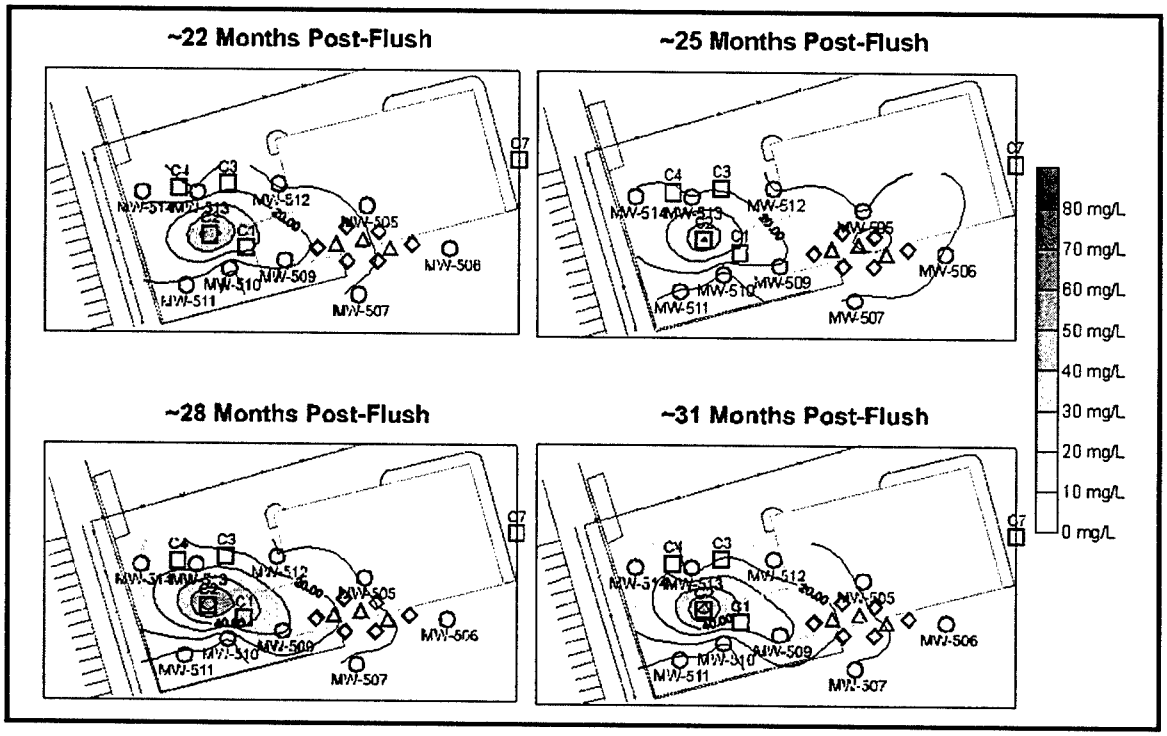


Figure 33. Chloride contour plots illustrating the change in concentration and the movement over the monitoring period 22 to 31 months

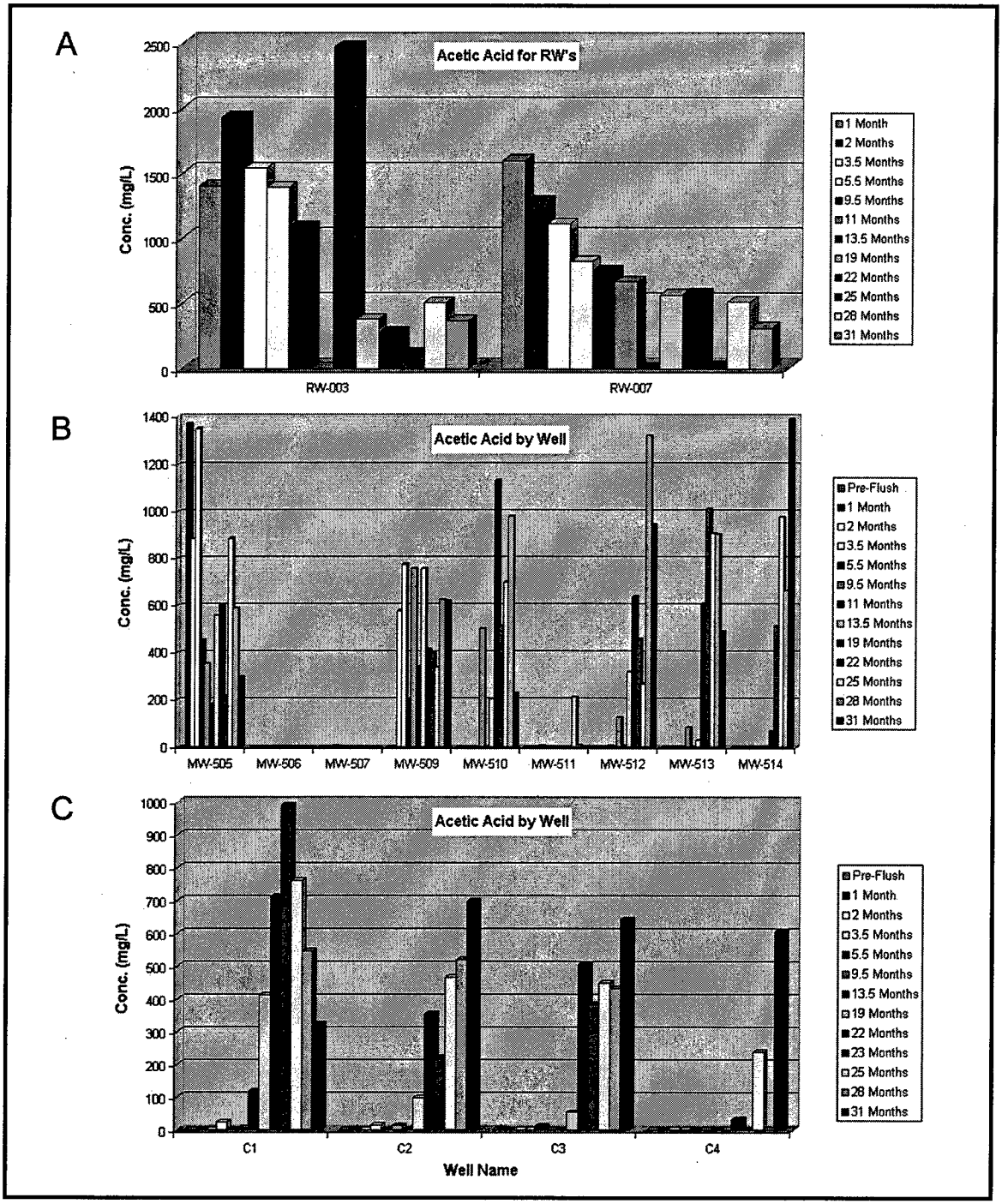


Figure 34. Acetic acid concentrations by well during ground water monitoring

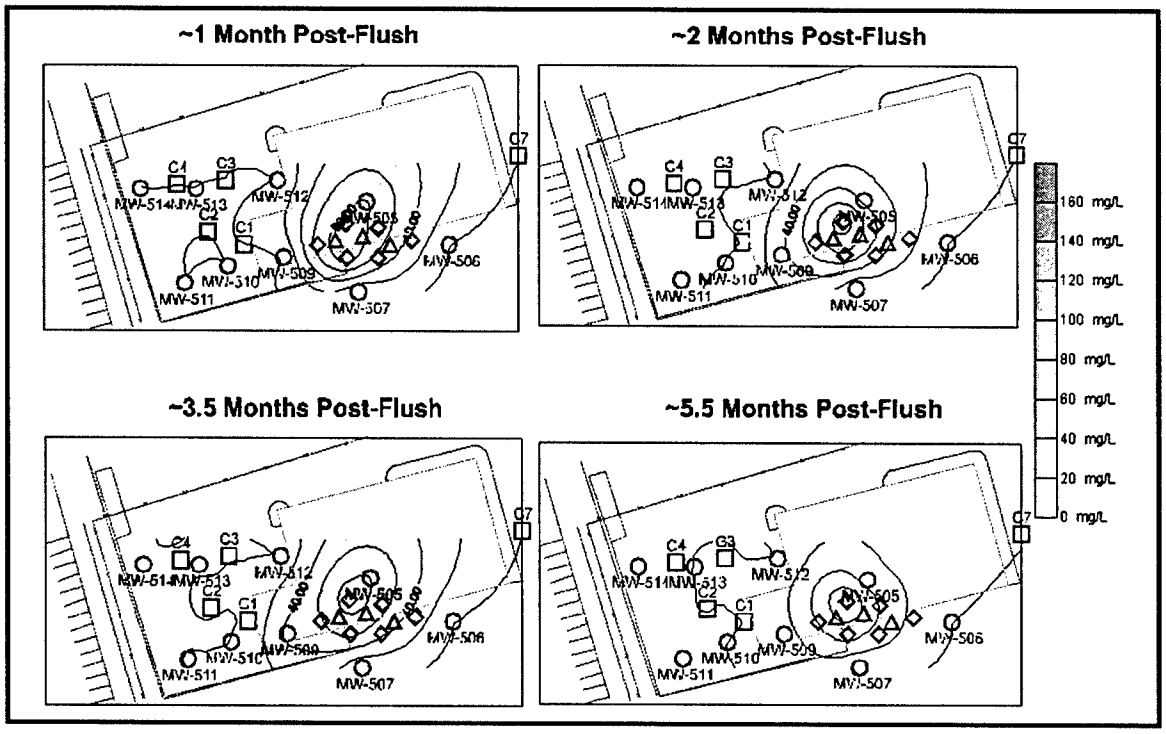


Figure 35. Acetic acid contour plots illustrating the change in concentration and the movement over the monitoring period 1 to 5.5 months

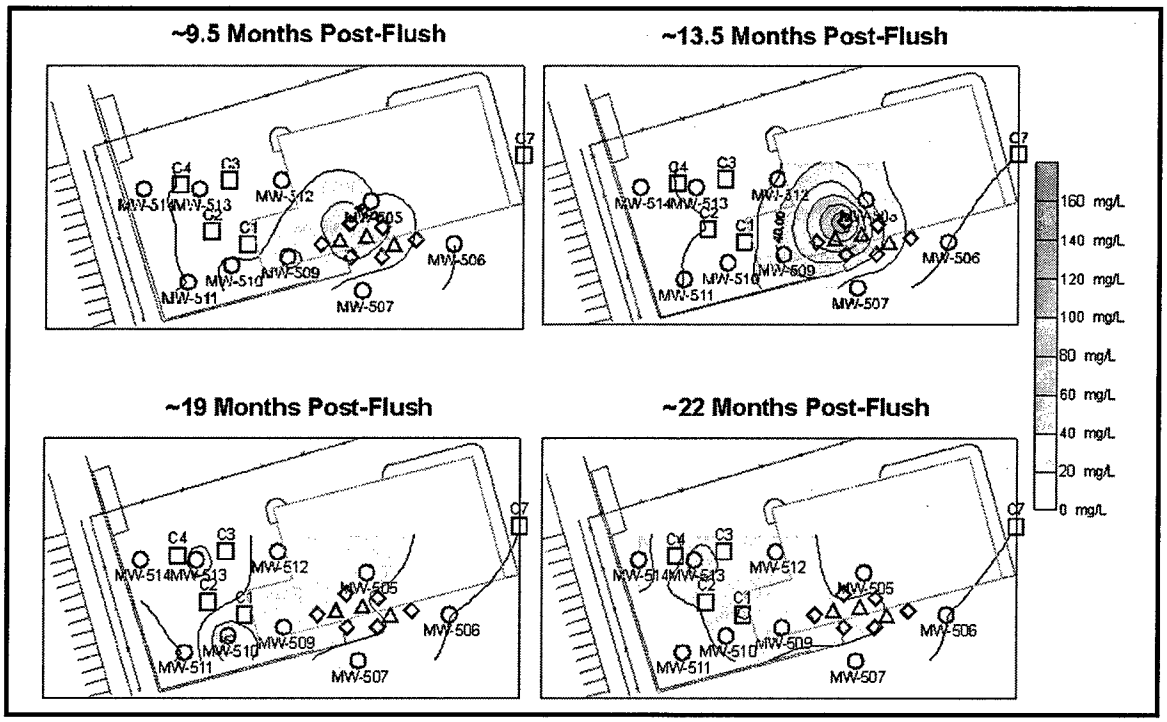


Figure 36. Acetic acid contour plots illustrating the change in concentration and the movement over the monitoring period 9.5 to 22 months

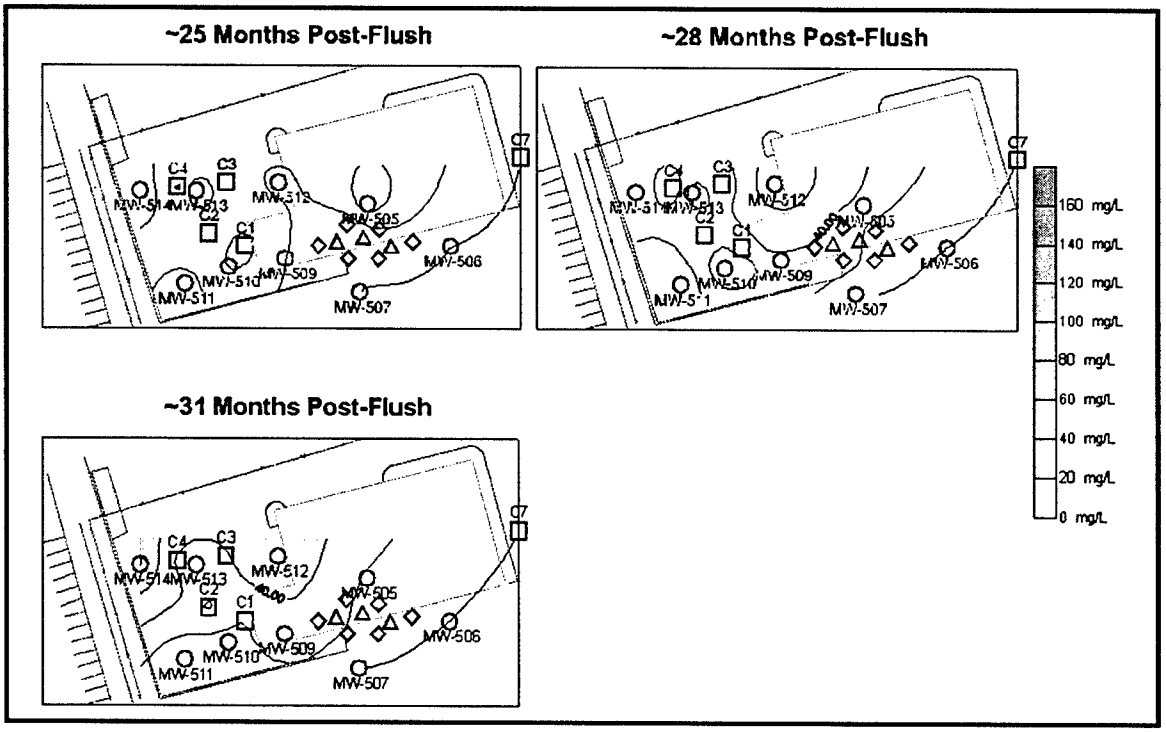


Figure 37. Acetic acid contour plots illustrating the change in concentration and the movement over the monitoring period 25 to 31 months

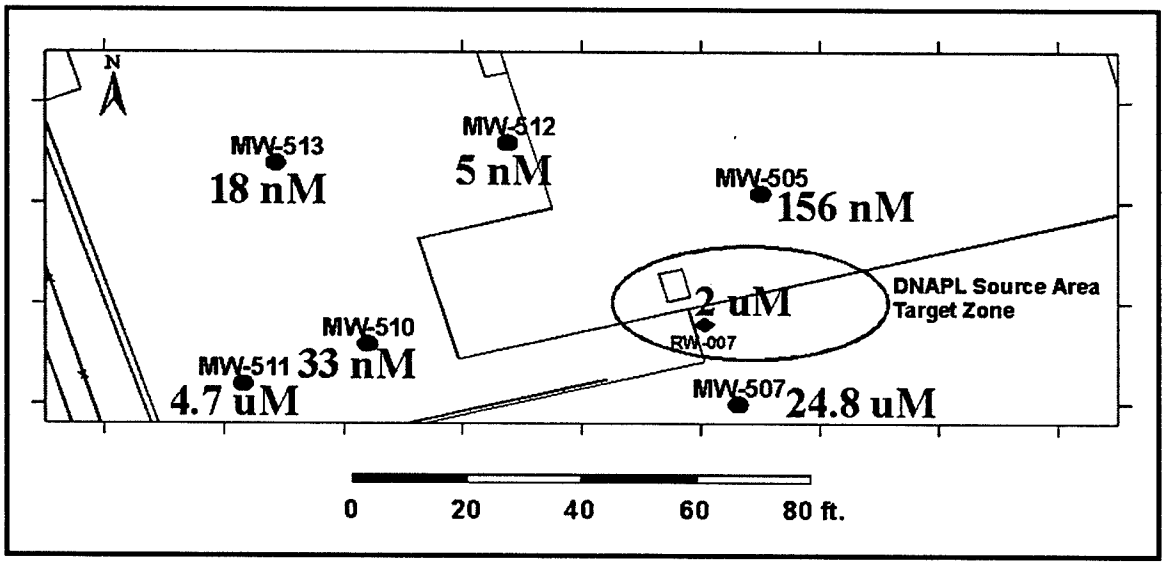


Figure 38. Hydrogen gas concentrations measured on year following the cosolvent flushing test

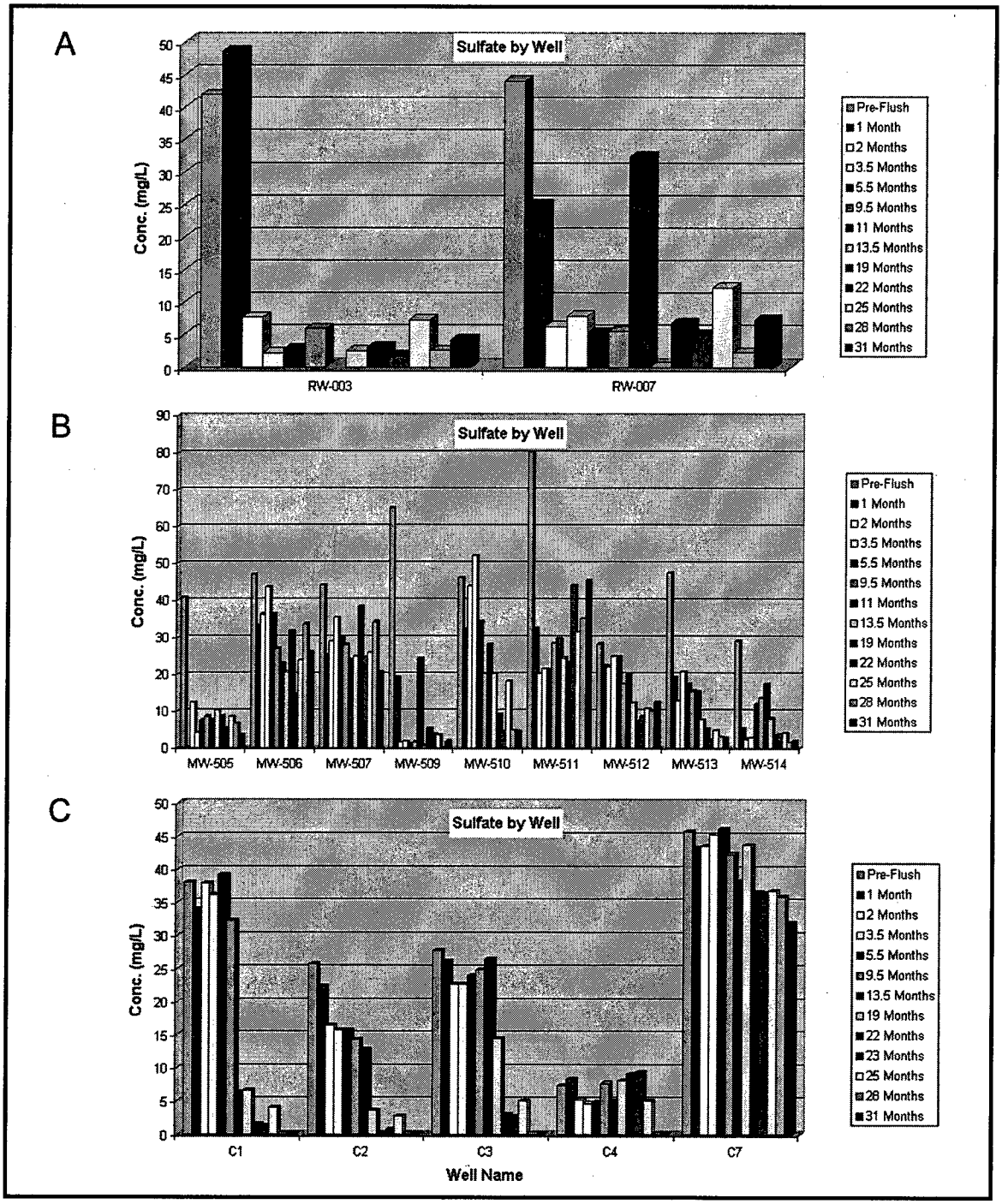


Figure 39. Sulfate concentrations by well during ground water monitoring

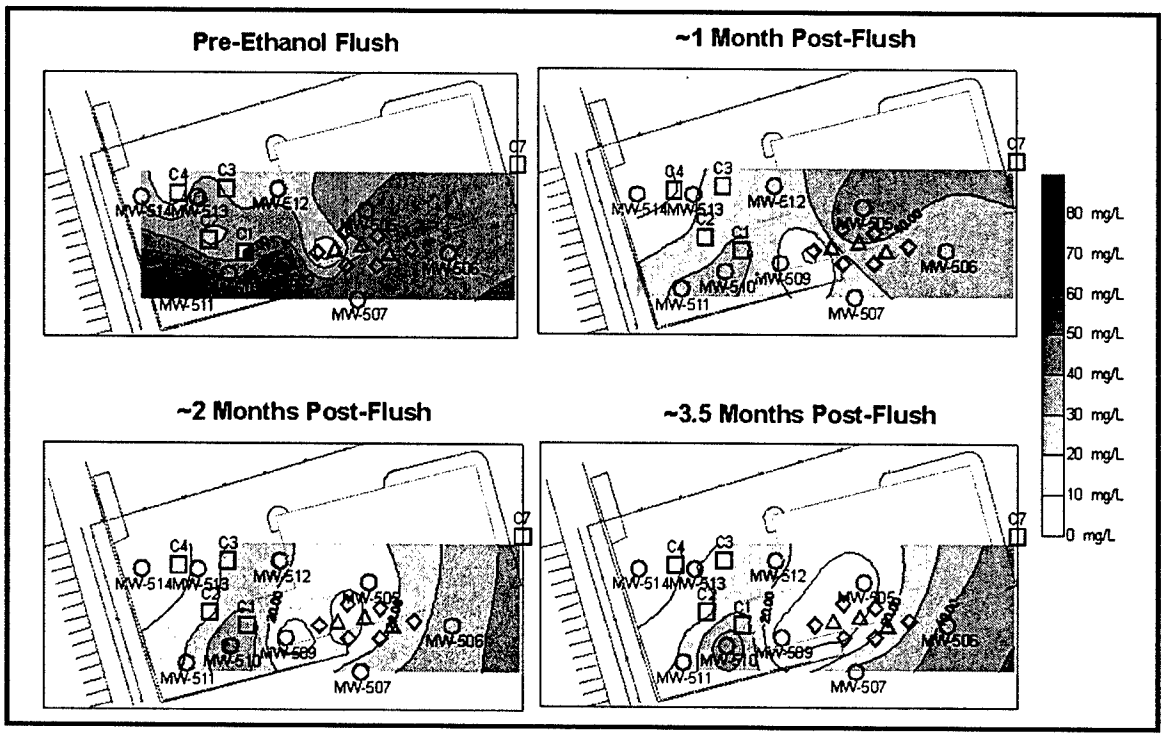


Figure 40. Sulfate contour plots illustrating the change in concentration and the movement over the monitoring period initial to 3.5 months

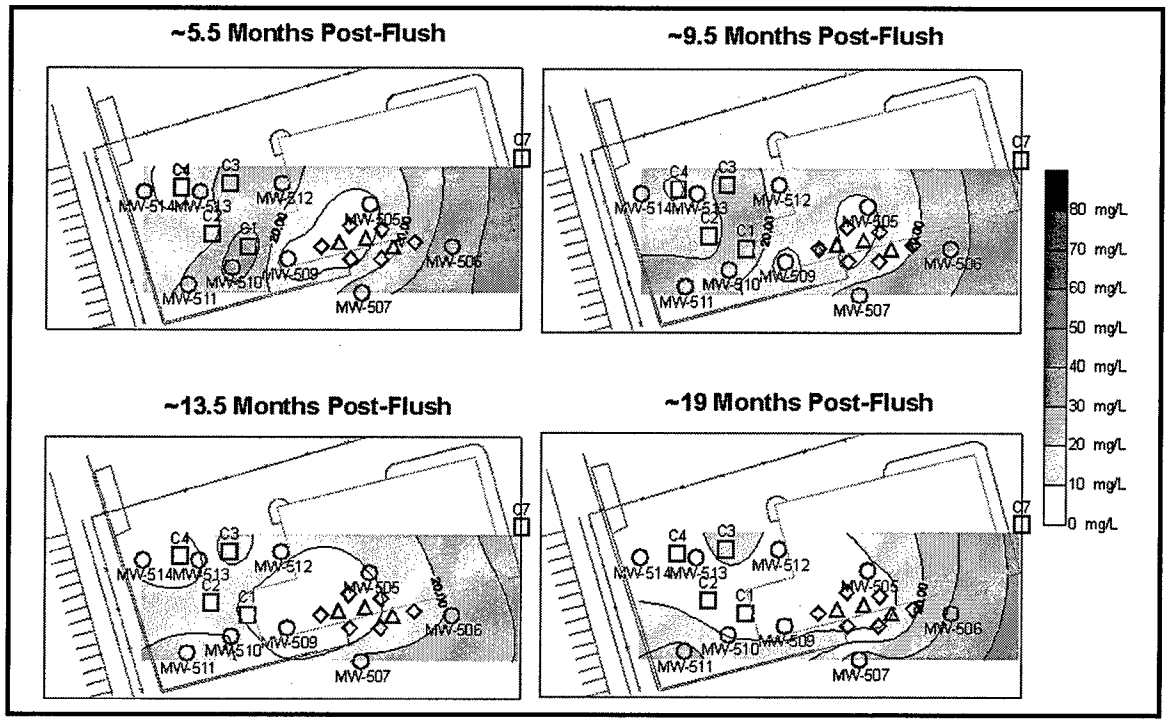


Figure 41. Sulfate contour plots illustrating the change in concentration and the movement over the monitoring period 5.5 to 19 months

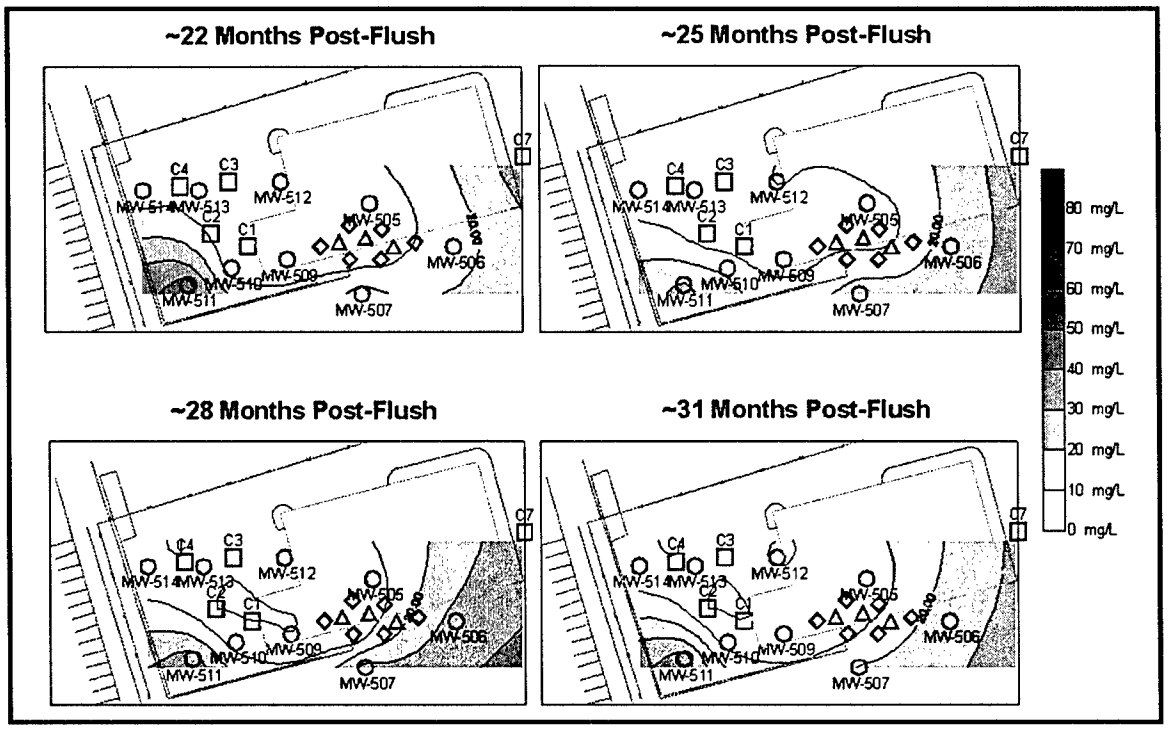


Figure 42. Sulfate contour plots illustrating the change in concentration and the movement over the monitoring period 22 to 31 months

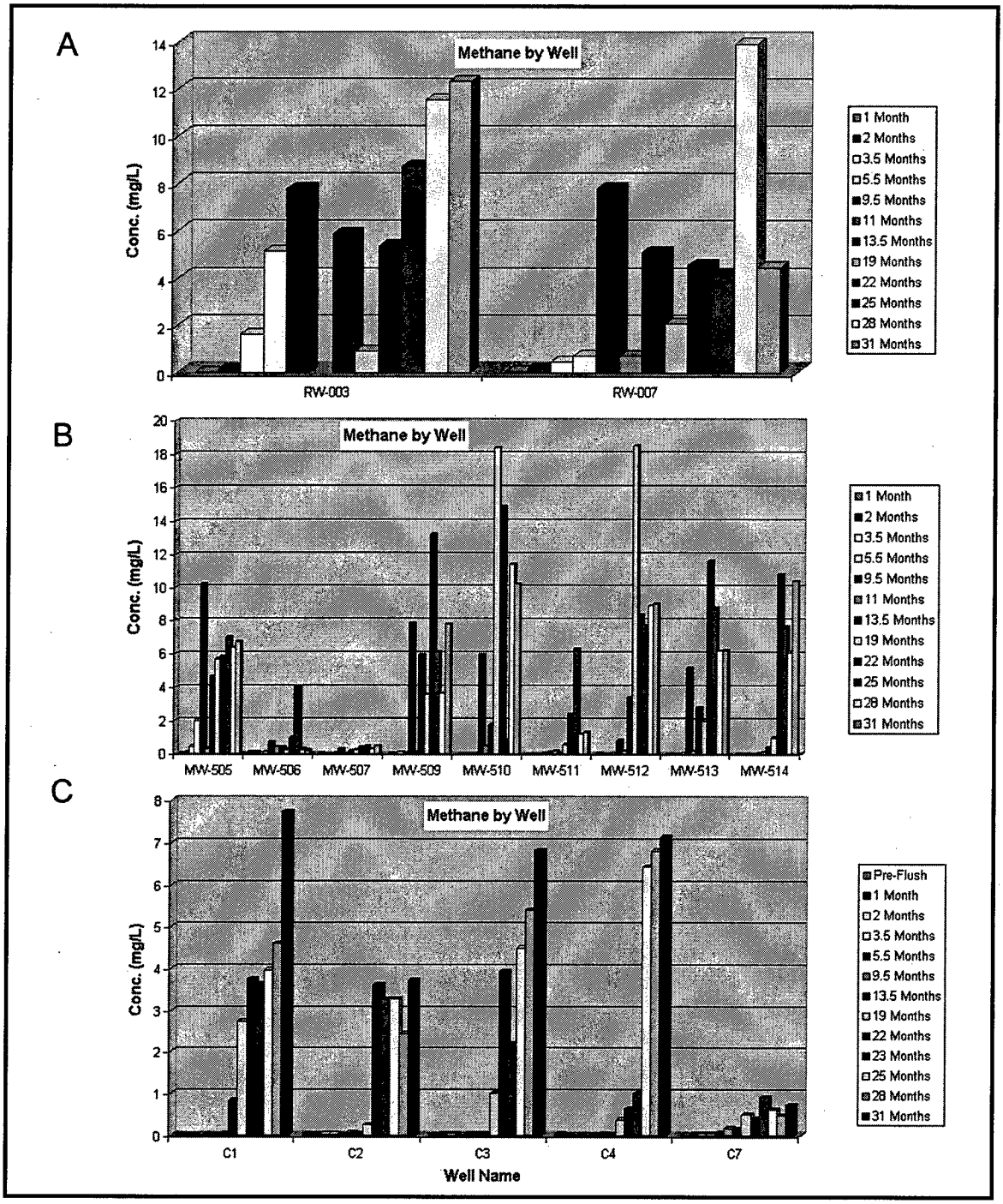


Figure 43. Methane concentrations by well during ground water monitoring

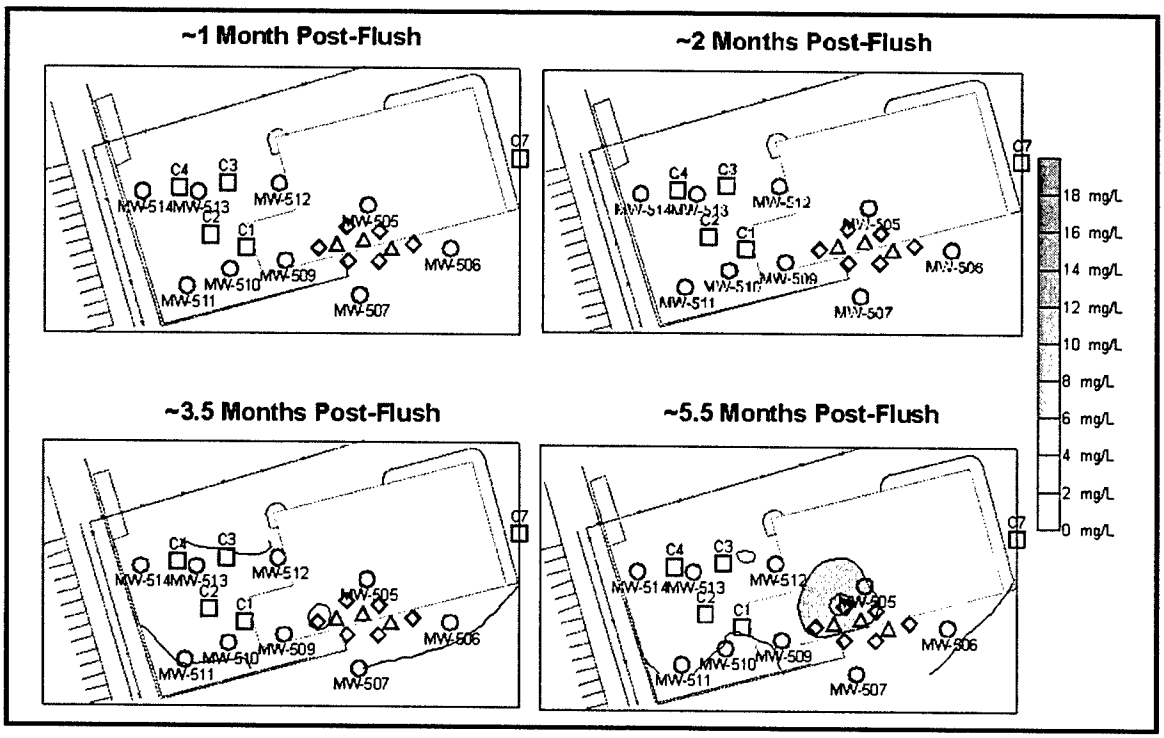


Figure 44. Methane contour plots illustrating the change in concentration and the movement over the monitoring period 1 to 5.5 months

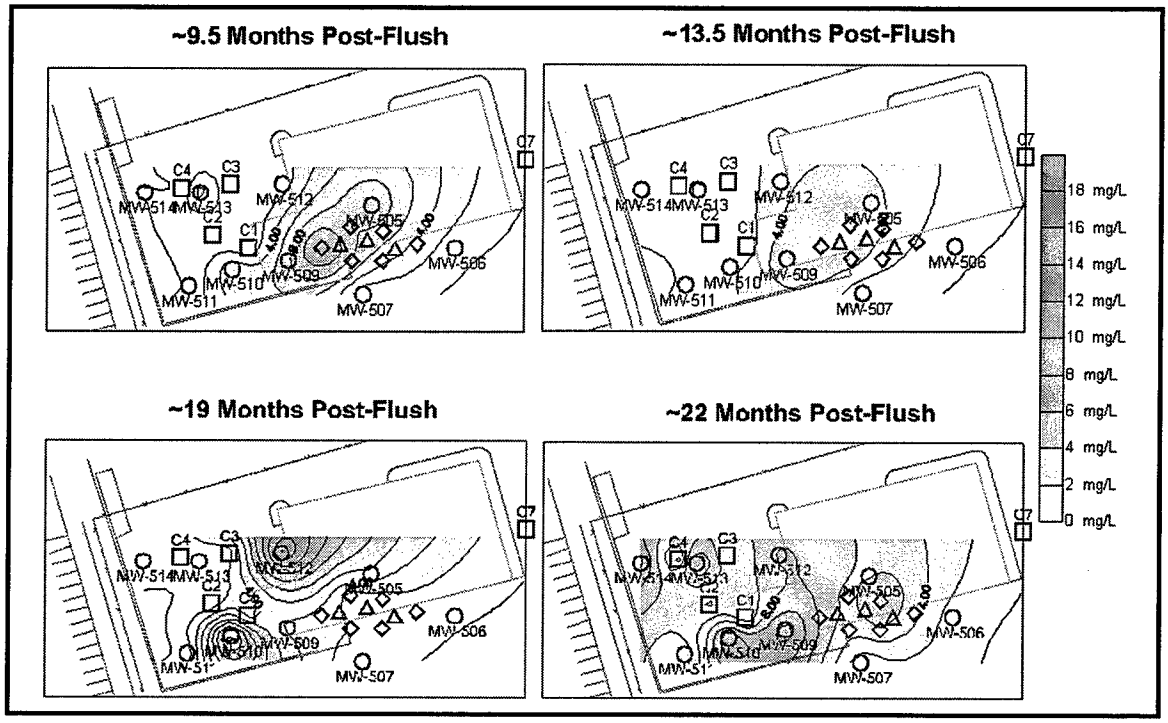


Figure 45. Methane contour plots illustrating the change in concentration and the movement over the monitoring period 9.5 to 22 months

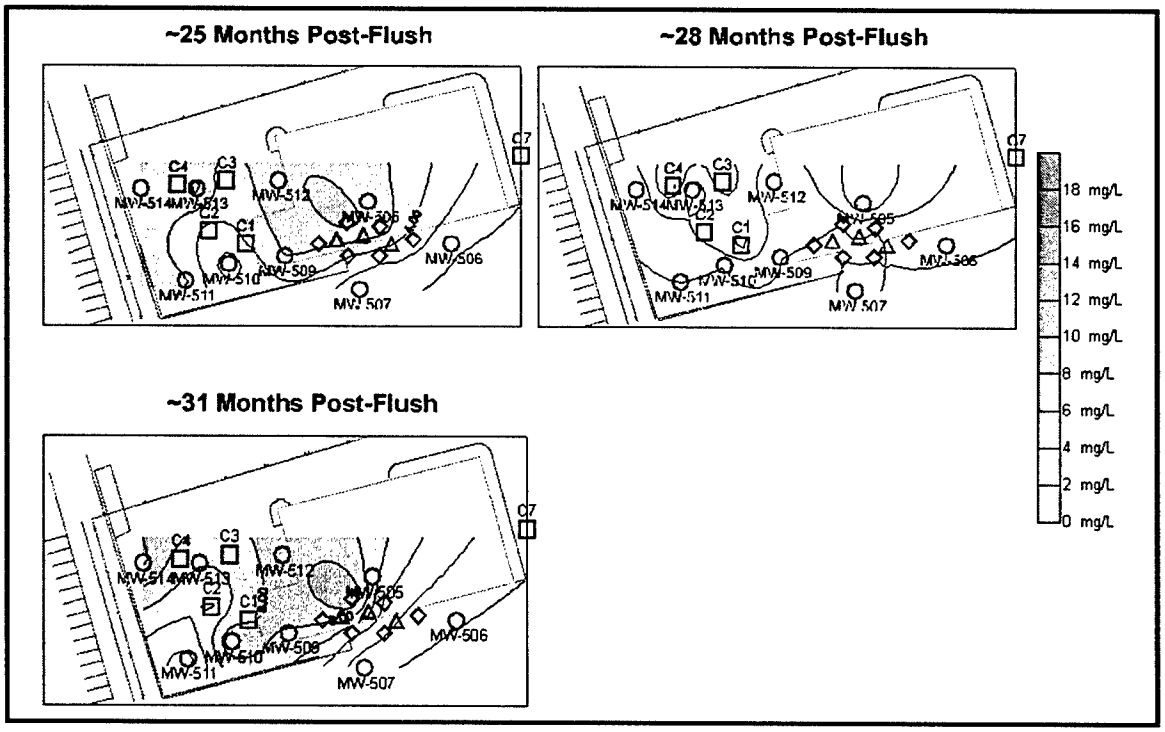


Figure 46. Methane contour plots illustrating the change in concentration and the movement over the monitoring period 25 to 31 months

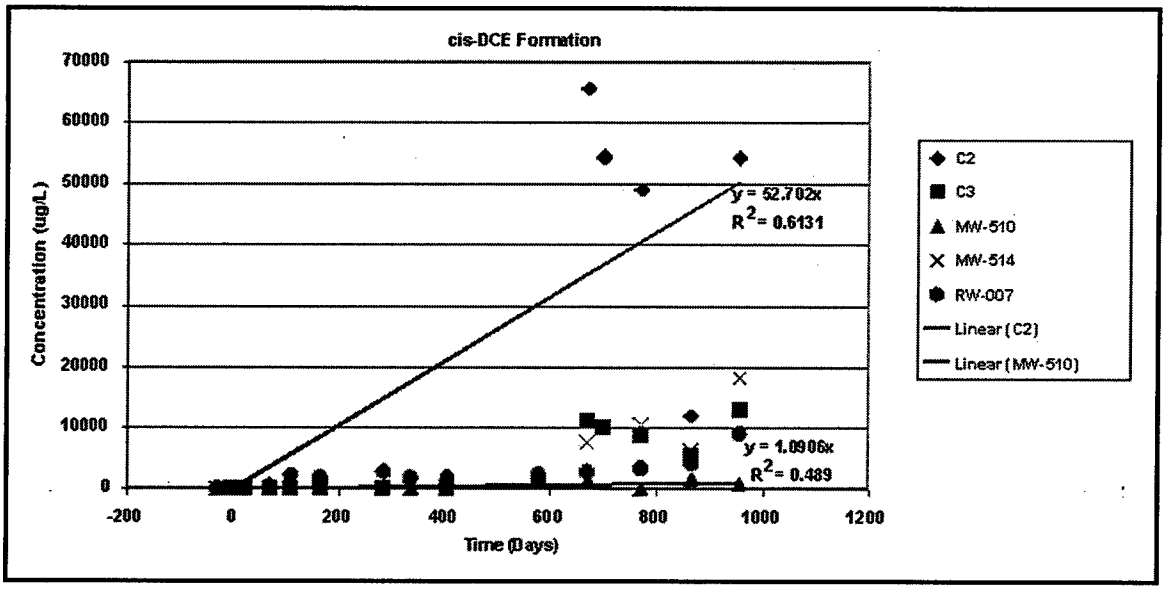


Figure 47. Production of cis-DCE from PCE by microbial reductive dechlorination supported by the residual ethanol from the cosolvent flush at the Sage's site

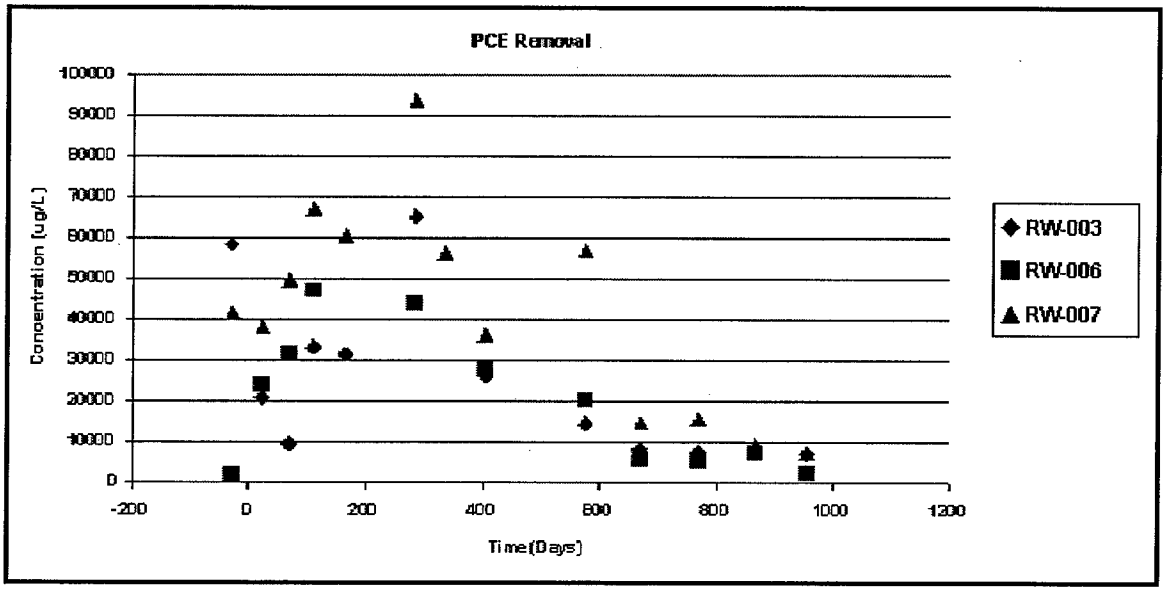


Figure 48. Removal of PCE by microbial reductive dechlorination supported by the residual ethanol from the cosolvent flush at the Sage's site (Y = -37.9 X + 44778)

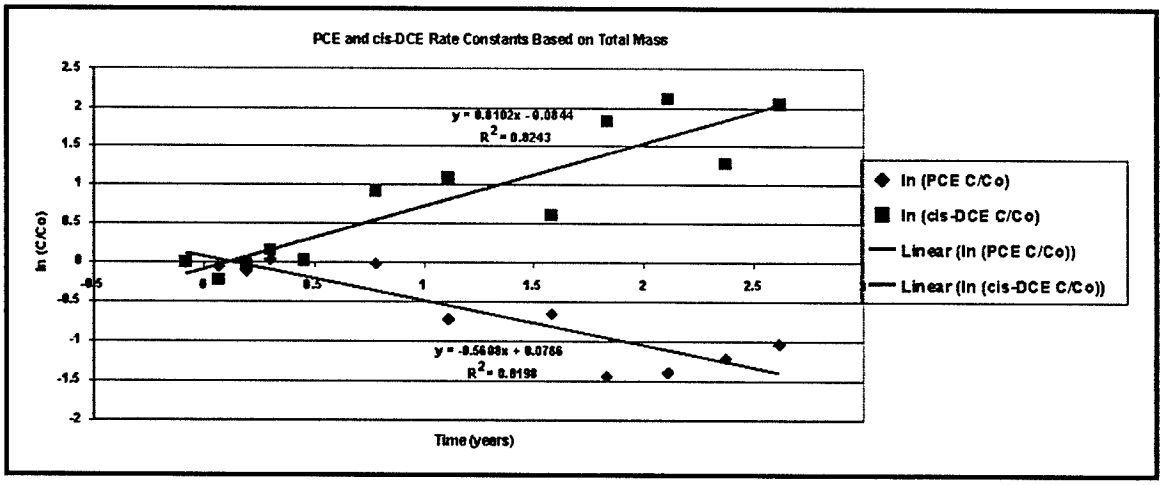


Figure 49. Rate constants from *cis*-DCE and PCE calculated using total mass estimated from contour plots of data for individual sampling events

Table 1
Natural Attenuation Parameters and Scores for Wells at Sage's Site

Analyte	Concentration Criteria	Interpretation
Oxygen	< 0.5 mg/L	Tolerated; suppresses reductive dechlorination at higher concentrations
Oxygen	> 1 mg/L	Vinyl chloride may be oxidized aerobically, but reductive dechlorination will not occur
Nitrate	< 1 mg/L	May compete with reductive pathway at higher concentration
Iron (II)	> 1 mg/L	Reductive pathway possible
Sulfate	< 20 mg/L	May compete with reductive pathway at higher concentration
Sulfide	> 1 mg/L	Reductive pathway possible
Methane	> 0.1 mg/L	Ultimate reductive daughter product
Methane	> 1 mg/L	Vinyl chloride accumulates
Methane	< 1 mg/L	Vinyl chloride oxidizes
Oxidation reduction potential	< 50	Reductive pathway possible
pH	5 < pH < 9	Tolerated range for reductive pathway
Total Organic Carbon	> 20 mg/L	Carbon and energy source; drives dechlorination; can be natural or anthropogenic
Temperature	> 20 C	At T > 20 C, biochemical process is accelerated
Alkalinity	> 2x background	Results from interaction of carbon dioxide with aquifer minerals
Chloride	> 2x background	Daughter product of organic chlorine; compare chloride in plume to background conditions
Perchloroethene		Material released
Trichloroethene		Material released or daughter product of perchloroethene
Dichloroethene		Material released or daughter product of perchloroethene; if amount of cis-1,2-dichloroethene is greater than 80% of total dichloroethene, it is likely a daughter product of trichloroethene
Vinyl chloride		Material released or daughter product of dichloroethenes
Ethene/Ethane		Daughter product of vinyl chloride/ethene
Chloroethane		Daughter product of vinyl chloride under reducing conditions
1,1,1-trichloroethane		Material released
1,1-dichloroethene		Daughter product of trichloroethene or chemical reaction of 1,1,1-trichloroethane

Total Points Awarded

Score	Interpretation
0 to 5	Inadequate evidence for biodegradation of chlorinated compounds
6 to 14	Limited evidence for biodegradation of chlorinated compounds
15 to 20	Adequate evidence for biodegradation of chlorinated compounds
> 20	Strong evidence for biodegradation of chlorinated compounds

(Continued)

Table 1 (Concluded)

Criteria Points Awarded	RW-001 25-35 ft	MW-001 10-20 ft	MW-002 8-18 ft	MW-004 23-33 ft	MW-009 2-10 ft	MW-010 Not Recorded
3	3	NA	NA	NA	NA	NA
-3	NA	-3	-3	-3	-3	-3
2	2	2	2	2	0	2
3	3	0	0	3	0	0
2	2	2	2	2	2	2
3	3	3	3	0	3	3
2	0	0	0	2	0	0
3	NA	NA	NA	NA	NA	NA
/	NA	NA	NA	NA	NA	NA
<50 = 1; <-100 = 2	1	0	0	0	0	1
/	0	0	0	0	0	0
2	2	2	2	2	2	0
1	1	1	1	1	1	1
1	0	0	0	0	0	0
/	0	0	0	2	0	0
2	0	0	0	0	0	0
2	2	0	2	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
>0.01 = 2; > 0.1 = 3	0	0	0	0	0	0
2	0	0	0	0	0	0
/	NA	NA	NA	NA	NA	NA
/	0	0	0	0	0	0
	19	7	9	11	5	6

Table 2
Core Sample Location for Direct Molecular Analysis (16S and FAME)

Location	Pre-Test Core (1998)	1 Yr. Post-Test Core (1999)	2 Yr. Post-Test Core (2000)
DNAPL Target Area, Between RW-6 & RW-7	C6	C10, C19	C36
Background Near MW-2	C5	C11	C35
Upgradient, Well C7	C7	C20	C37
Downgradient, Well C1	C1	C16, C17, C18	C31
Downgradient, Well C2	C2	C14	C32
Downgradient, Well C3	C3	C15	C33
Downgradient, Well C4	C4	C12, C13	C34

Table 3
PCE Removal Effectiveness Calculated by Different Techniques

Technique	Pre-Flushing (mg/L)	Post-Flushing (mg/L)	Removal Effectiveness
RW (n=6)	17.2 +/- 25.9	16.5 +/- 13.5	0.04
IW (n=3)	54.5 +/- 46.3	0.6 +/- 0.5	0.99
RW & IW (n=9)	29.6 +/- 36.1	11.2 +/- 13.2	0.62
Core Material			0.65
Partitioning Tracer			0.62-0.63

Table 4
Sampling Dates and Activities at the Sage's Site

Date	Event
July 16, 1998	Pre-Ethanol Flush Ground Water Monitoring, Site Characterization, Partitioning tracer test
Aug. 11, 1998	C-Wells sampled (included with Pre-Ethanol Flush)
Aug. 9-15, 1998	Cosolvent Flushing Test
Aug. 15, 1998	Partitioning Tracer Test, Hydraulic Containment
Aug. 25, 1998	End Hydraulic Containment
Sept. 8, 1998	1 Month Ground Water Monitoring
Oct. 20, 1998	2 Month Ground Water Monitoring
Dec. 3, 1998	3.5 Month Ground Water Monitoring
Jan. 26, 1999	5.5 Month Ground Water Monitoring
May 26, 1999	9.5 Month Ground Water Monitoring
July 14, 1999	11 Month Ground Water Monitoring Hydrogen Gas Survey, Core Sampling
Sept. 23, 1999	13.5 Month Ground Water Monitoring
Mar. 14, 2000	19 Month Ground Water Monitoring
June 14, 2000	22 Month Ground Water Monitoring
July 13, 2000	23 Month Ground Water Sampling (partial) Core Sampling
Sept. 23, 2000	25 Month Ground Water Monitoring
Dec. 29, 2000	28 Month Ground Water Monitoring
Mar. 20, 2000	31 Month Ground Water Monitoring

Table 5
Sage's Site Data

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
C1	-4	97100	1633.67	57.85	0.003	ND	38	10.2	0.02	ND
	24	92300	3130	134	ND	ND	33.9	10.2	0.02	ND
	70	71800	3030	<1	<0.003	ND	37.9	9.67	0.018	ND
	110	78100	3150	<1	0.003	ND	36.2	10.4	0.019	23.4
	165	81400	3610	ND	<0.003	<1.7	39.2	10.9	0.018	ND
	285	64900	7530	1800	0.001	1	32.2	15.3	0.038	7.99
	405	16500	10300	20000	ND	4	6.24	27.7	0.826	119
	577				ND	2160	6.65	26.3	2.73	414
	670	25.3	34.3	1060	0.002	2249	1.5	33.8	3.75	712
	700	436	1020	18600	ND	2440	1.16	28.8	3.63	991
	770	3390	4310	20400	0.002	2900	4.03	28.6	3.964	761
	865	1590	2550	8330	<0.002	2310	<1.00	47	4.6	547
	948	267	627	156	0.0025	2200	<0.50	37.2	7.73	324
C2	-4	97700	552.5	9.7	0.003	ND	25.7	9.25	0.03	ND
	24	66900	1120	<1	0.003	ND	22.4	9.41	0.03	ND
	70	76600	2150	<1	<0.003	ND	16.5	8.97	0.025	ND
	110	74700	2070	<1	0.003	ND	15.7	9.39	0.026	16.5
	165	80900	1710	ND	0.011	ND	15.7	10.5	0.016	1.7
	285	59000	7780	2610	0.0025	1	14.3	14.4	0.045	13.8
	405	40900	7870	2100	0.009	1	12.8	9.35	0.035	ND
	577				ND	400	3.54	34.55	0.2445	97.6
	670	174	175	65600	0.002	126	<1.00	60.6	3.6	355
	700	816	1500	54300	ND	127	0.7	64.5	3.26	221
	770	3610	2700	49000	0.003	408	2.65	53.2	3.261	467
	865	3860	3110	12100	0.004	1820	<1.00	68.6	2.43	521
	948	1400	1950	54200	0.0068	1850	<0.50	67.7	3.71	700

(Sheet 1 of 12)

Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
C3	-4	1700	4235	30.25	<0.003	ND	27.8	10.6	0.02	ND
	24	9220	5180	<1	ND	ND	26.2	10.4	0.03	ND
	70	18200	4710	<1	0.003	ND	22.7	9.47	0.031	ND
	110	19800	4330	<1	0.005	ND	22.7	10.7	0.014	ND
	165	17000	4650	ND	0.007	ND	24	11.8	0.024	11.5
	285	18700	6550	<1.0	0.005	1	24.9	11.1	0.043	ND
	405	6480	7510	<1.0	0.008	ND	26.4	8.45	0.032	ND
	577	19500	7950	1220	ND	ND	14.4	14.2	1	53.9
	670	165	1070	11100	0.001	2	3.02	16.6	3.93	506
	700	1040	2970	10200	ND	8	2.66	19.1	2.18	385
	770	4910	4160	8890	0.001	301	5.02	19	4.489	449
	865	7200	3040	5510	ND	291	<1.00	22	5.4	432
	948	1140	1200	12850	ND	603	<0.50	20.6	6.82	643
C4	-4	16300	7610	53.5	ND	ND	7.32	11.5	0.03	ND
	24	21100	8240	<1	<0.003	ND	8.35	11.4	0.02	ND
	70	19200	9250	<1	<0.003	ND	5.12	10.7	0.019	ND
	110	20300	9990	<1	<0.003	ND	4.66	11.9	0.016	ND
	165	18600	10400	<1.0	<0.003	ND	4.89	11.9	0.015	ND
	285	13700	9850	<1.0	0.001	1	7.69	11.5	0.033	ND
	405	9050	9640	<1.0	0.007	ND	4.96	7.66	0.016	ND
	577	16800	1130	400	0.001	ND	8.06	13.5	0.371	ND
	670	3940	6610	1450	0.001	ND	9.07	11.6	0.636	31.6
	700	6950	10300	323	ND	ND	9.35	14.4	0.991	<25
	770	7345	5030	12500	ND	<1	5	22	6.433	238
	865	3630	3470	6710	<0.002	1.86	<1.00	26.1	6.81	ND
	948	464	731	1020	<0.0025	<1	<0.50	29.9	7.13	607

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Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
C7	-4	3.1	1.3	ND	ND	ND	45.7	8.7	ND	ND
	24	251	<1	<1	ND	ND	43.2	8.9	0.03	ND
	70	<1	<1	<1	ND	ND	43.5	7.65	0.0415	ND
	110	<1	<1	<1	ND	ND	45.3	8.71	0.031	ND
	165	728	235	ND	ND	ND	46.1	8.63	0.0505	10.6
	285	2090	<1.0	ND	ND	1	42.3	7.92	0.158	
	335				ND	ND	42.6	8.75	0.11	ND
	405	5.36	3.24	<1.0	ND	ND	38.2	2.32	0.147	ND
	577	42.6	8.94	ND	ND	ND	43.7	7.88	0.496	ND
	670	374	879	780	ND	ND	36.5	5.33	0.3875	<25
	700	1.06	<1	<1	ND	ND	36.4	8.36	0.908	<25
	770	1.73	<1	1.72	ND	ND	36.7	9.48	0.611	ND
	865	20.2	7.29	18.8	ND	ND	35.9	6.57	0.476	ND
	948	11.9	4.51	77.7	ND	ND	31.9	6.79	0.726	1.94

(Sheet 3 of 12)

Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
MW-505	-30	84.7	<1	ND			40.8	13.2		ND
	24	1850	116	ND	ND	4620			0.05	1370
	70	ND	128	<1	ND	2330	12.3	8.64	0.105	883
	110	2990	103	<1	<0.003	6000	4.11	11.5	0.453	1350
	165	2150	<1.0	<1.0	ND	5190	7.53	11.3	2.01	456
	285	458	<1.0	ND	ND	618	8.59	8.85	10.2	353
	335	63.1	7.97	6	0.0055	45	7.5	8.81	0.3515	179
	405	169	24.8	ND	0.006	322	10.2	6.32	4.62	558
	577	2740	145	698.5	ND	2150	8.62	9.61	5.675	599
	670	322	16.3	107	ND	17	5.2	4.02	5.82	216
	770	59.8	<1	<1	0.001	302	8.42	8.69	6.97	884
	865	1450	250	228	<0.002	253	6.68	8.21	6.35	588
	948	132	50.9	168	<0.0025	2.9	3.43	7.64	6.71	298
MW-506	-30	24.5	ND	ND			47	9.65		ND
	24	<1	<1	ND	ND	0.74	33.2	8.71	0.135	ND
	70	ND	<1	<1	ND	ND	36.3	8.425	0.1413	ND
	110	<1	<1	ND	ND	<1.7	43.6	9.97	0.0565	ND
	165	<1.0	ND	ND	ND	<1.7	36.4	9.31	0.131	ND
	285	ND	ND	ND	ND	1	27	8.4	0.7425	ND
	335	<1.0	ND	<1.0	ND	ND	23	7.94	0.433	ND
	405	<1.0	5.06	ND	ND	ND	20.6	2.58	0.435	ND
	577	1.24	20	1.18	ND	ND	31.7	9.22	0.2925	ND
	670	<1	<1	ND	ND	ND	14.6	4.75	0.997	ND
	770	1.9	1.38	<1	ND	ND	23.8	9.37	3.97	ND
	865	10.6	1.94	2.3	ND	ND	33.7	6.74	0.286	ND
	948	3.33	ND	<1	ND	ND	26.1	7.33	0.217	ND

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Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
MW-507	-30	129	1.2	ND			44.1	11.4		ND
	24	ND	<1	ND	ND	ND	25.4	8.47	0.05	ND
	70	ND	<1	<1	ND	ND	28.9	6.41	0.023	ND
	110	<1	<1	ND	ND	ND	35.3	9.29	ND	5.74
	165	185	ND	ND	ND	ND	29.9	7.75	0.089	ND
	285	<1.0	<1.0	ND	ND	ND	28.1	9.52	0.301	ND
	335	3.6	3.12	<1.0		ND	24.1	7.73		ND
	405	<1.0	<1.0	ND	ND	ND	24.8	2.53	0.213	ND
	577	1.8	24.8	1.8	ND	ND	38.4	7.82	0.259	ND
	670	1.24	1.98	ND	ND	ND	24.6	7.31	0.451	ND
	770	18.6	2.64	2.92	ND	ND	25.8	9.24	0.457	ND
	865	15.6	3.68	8.23	ND	ND	34.2	5.75	0.302	ND
	948	1.14	1.33	<1	ND	ND	20.8	7.54	0.504	ND
MW-508	-30	3800	148	2.4			12.2	9.21		ND
	24	3640	<1	<1		ND				
	70	3720	<1	<1		ND	1.44	35.5		ND
	110	3320	<1	<1		ND				
	285	2940	92.9	134	ND	ND	3.99	8.14	ND	ND
	405	1390	658	221	0.006	ND	<0.5	3.725	0.021	ND
	577	635	463	145	ND	ND	3.66	8.27	0.022	ND
	670	299	746	474	0.015	ND	2.47	8.09	0.054	ND
	865	3650	1670	347	ND	ND	<1.00	7.03	0.032	ND

(Sheet 5 of 12)

Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
MW-509	-30	36000	2060	4.8			65.1	15		ND
	24	8770	4680	<1	ND	ND	19.15	13.05	0.04	ND
	70	4760	3740	518	ND	518	1.44	35.5	0.011	578
	110	4050	9520	3940	ND	1100	1.8	87.5	0.152	774
	165	2390	6750	3420	ND	2330	1.26	23.6	0.094	195
	285	7190	6370	3480	ND	5140	1.6	19	7.82	758
	335	3440	5090	2190	0.007	632	24.3	4.73	0.14	343
	405	377	597	367	0.006	470	0.57	10.9	5.94	757
	577	11400	4800	3720	ND	5000	5.36	16.8	3.57	416
	670	2980	2160	12000	0.003	1702	3.87	22.1	13.2	405
	770	234	138	7590	0.002	1410	3.52	20.1	6.117	337
	865	6240	2270	4810	0.003	4150	<1.00	31.3	3.63	624
	948	2030	2080	17700	0.0074	3780	1.87	33.7	7.77	620
MW-510	-30	2720	1980	15			46.1	11.35		ND
	24	2020	1800	<1		ND	32.3	12.3		ND
	70	1270	2110	<1	ND	ND	43.8	12.4	0.017	ND
	110	1240	4280	<1	ND	ND	52	15.8	0.032	ND
	165	221	1300	4.63	ND	ND	34.2	11.7	ND	ND
	285	1600	583	ND	ND	35	20.3	10.6	5.95	503.5
	335	212	233	36.6	ND	ND	28.2	9.76	0.49	ND
	405	25	210	91.3	ND	63	20.3	4.12	1.77	205
	577	52.6	723	1240	ND	213	9.2	8.06	18.4	1130
	670	15.9	204	1270	ND	83	4.43	8.43	14.9	513
	770	<1	193	34.9	<0.003	104	18.1	6.21	0.855	700
	865	42.1	37.7	1610	<0.002	39.8	4.79	7.02	11.4	979
	948	21.4	17.9	748	<0.0025	<1	4.64	6.61	10.2	229

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Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
MW-511	-30	147	848	246			80.2	11		ND
	24	317	1420	191	ND	ND	32.6	12.4	0.02	ND
	70	730	2660	280	ND	ND	20.3	8.165	0.0325	ND
	110	683	2870	87.1	ND	ND	21.55	10.5	0.008	6.15
	165	832	2320	101	ND	ND	21.1	9.2	0.066	ND
	285	1810	1340	292	ND	ND	28.4	12.5	0.1005	ND
	335	47.4	600	265	0.006	ND	29.8	12.6	0.189	ND
	405	18.8	684	58.2	ND	ND	24.5	4.74	0.076	ND
	577	112	1500	66	ND	ND	23.4	9.14	0.591	ND
	670	9.49	136	23.2	ND	ND	44.1	17.2	2.4	ND
	770				ND	<1	31.5	8.47	6.315	212
	865	27.3	514	135	<0.002	ND	35.1	5.78	1.19	9.52
	948	6.57	23.8	64	<0.0025	ND	45.4	6.6	1.32	ND
MW-512	-30	212	101	<1			28.3	12.5		ND
	24	7560	697	<1	ND	ND	22.6	10	0.06	ND
	70	9630	1160	<1	ND	ND	22.2	10.6	0.025	ND
	110	7700	1650	<1	<0.003	ND	24.9	11.9	0.0215	3.9
	165	3680	978	ND	ND	ND	24.7	9.48	0.041	ND
	285	1940	1250	<1.0	ND	1	17.35	10.7	0.817	126
	335	352	305	86	0.006	ND	19.85	9.605	0.188	4.59
	405	149	304	49.7	ND	133	12.3	4.95	3.39	318
	577	548	2725	1095	ND	469	7.24	12.3	18.5	637
	670	99	95.1	167	ND	194	8.76	8.22	8.39	458
	770	200	423	67.2	ND	344	10.7	9.21	7.732	269
	865	442	1190	1100	ND	371	10.1	10.2	8.89	1320
	948	56.6	28.5	66	ND	200	12.5	11.8	9.02	942

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Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
MW-513	-30	1410	1660	7.2			47.4	11.3		ND
	24	22950	3500	<1	ND	ND	19.1	11.2	0.02	ND
	70	29200	3660	<1	ND	ND	12.8	9.52	0.026	ND
	110	33300	3710	<1	ND	ND	20.8	15.7	0.015	ND
	165	32100	3780	ND	ND	ND	17.3	11.6	0.048	ND
	285	12000	9440	328	ND	ND	15.5	13.7	5.16	83.7
	335	2170	15000	688	0.006	ND	15.3	16.8	0.188	ND
	405	12100	7500	400	ND	2	7.72	6.31	2.81	29
	577	21400	7720	5060	ND	155	5.27	13.7	2.01	609
	670	376	1230	2540	ND	395	2.12	10.7	11.6	1010
	770	2370	2630	10800	ND	492	4.79	19.2	8.766	905
	865	3290	2500	6080	<0.002	697	3	23.4	6.17	902
	948	441	651	3080	<0.0025	1395	2.77	11.4	6.21	489
MW-514	-30	1960	1080	9.5			29.1	13.55		ND
	24	61600	4210	<1	ND	ND	5.255	11.1	0.02	ND
	70	48100	5250	<1	ND	ND	2.42	10	0.03	ND
	110	56500	7125	<1	ND	ND	2.705	11.6	0.0235	ND
	165	42400	6220	ND	ND	ND	12	16	0.014	ND
	285	42400	7460	<1.0	ND	ND	13.5	11.9	0.045	ND
	335	23500	10700	45.45	0.006	ND	17.5	13.5	0.122	ND
	405	30400	11900	116	ND	ND	8.04	8.89	0.4255	ND
	577	25000	13000	1670	ND	26	3.46	11.2	1	69.1
	670	1650	8370	7630	ND	119	3.84	18.1	10.8	514
	770	3530	4740	10700	0.001	146	4.15	20.8	7.631	975
	865	2550	4050	6350	<0.002	208	1.43	25.9	6.09	664
	948	18	55.1	18300	<0.0025	467	1.9	22.4	10.4	1390

Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
IW-001	-30	1770	58.55	ND			9.49	5.09		ND
	24	1140	ND	ND		ND				
	70	8890	1390	2290		1670				
	110	813	836	794		8600				
	285	25900	8260	15800		8170				
	405	14200	7860	18500		220				
IW-002	-30	88900	108	ND			39.3	20.4		ND
	24	422	3.52	1.27		1560				
	70	1490	1317	<1		1240				
	110	11850	2960	1485		7900				
	285	8560	2970	6290		3130				
	405	482	509	2530		801				
IW-003	-30	72700	94.1	1.2			42.1	12.9		ND
	24	183	7.31			ND				
	70	1690	3810	2110		2080				
	110	7140	3810	4550		3100				
	285	6280	8180	15200		5660				
	405	890	4680	2270		434				

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Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
RW-002	-30	726.5	20.6	ND			16.4	5.99		ND
	24	3750	2755	<1	<0.003	16250	5.48	25.4	0.01	1155
	70	2503	4850	921	ND	3720	13.4	40.6	0.213	1210
	110	10600	4880	4640	ND	4400	6.62	43.8	2.66	973
	165	3720	1953	4870	ND	2160	9.53	22.6	2.83	681
	285	10800	4280	15100	0.002	4940	22.8	5.75	13.4	560
	405	2350	2870	11500		1920				
RW-003	-30	58400	31.45	ND			41.9	16.7		ND
	24	20900	1850	<1	ND	3660	48.6	59.9	0.002	1400
	70	9360	7450	347	ND	1840	7.67	41.5	0.1105	1930
	110	33100	8570	1670	ND	8600	2.22	35.95	1.6	1540
	165	31600	3310	1440	ND	8040	2.76	38.4	5.13	1390
	285	65200	5710	532	0.001	15500	5.91	36.2	7.79	1090
	405	26200	5600	4170	0.008	9330	2.49	21.6	5.88	2470
	577	14300	3120	2860	ND	10200	3.07	22.3	0.902	377
	670	8200	1310	2950	0.006	2578	1.73	18.4	5.365	274
	770	7260	2510	5140	0.005	2260	7.24	19.7	8.733	112
	865	8350	1910	4020	0.002	1300	2.52	18	11.5	503
	948	7110	1300	8040	0.0038	732	4.22	18	12.3	366

(Sheet 10 of 12)

Table 5 (Continued)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
RW-004	-30	848	7.2	ND			51	18.1		ND
	24	6430	<1	<1		6940				
	70	7740	1420	784		5820				
	110	13900	3080	2350		11000				
	285	30300	1520	1310		11100				758
	405	11900	1230	531		7490				790
	577	4120	732	493	ND	5950	3.38	7.63	1.06	235
	670	2060	185	605	0.001	517	4.42	6.55	8.36	368
	770	5260	3430	7950	0.007	3640	3.55	26.4	7.398	301
	865	3640	1150	1490	0.002	184	9.44	9.22	6.01	193
	948	557	179	542	<0.0025	8.78	8.11	7.68	3.59	221
RW-005	-30	67.7	1.1	ND			42.4	16.7		ND
	24	6090	599	<1		6200				
	70	1820	81	<1		369				
	110	2520	110	<1		300				
	285	2330	<1.0	ND		3				
	405	15.9	3.4	ND		ND				

(Sheet 11 of 12)

Table 5 (Concluded)

Well Name	Days Post-Flush	PCE (ug/L)	TCE (ug/L)	cis-DCE (ug/L)	Ethylene (mg/L)	Ethanol (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Methane (mg/L)	Acetic Acid (uM)
RW-006	-30	1770	221	1			42.8	18.3		ND
	24	24050	408.5	<1		9145				
	70	31700	1380	224		8890				
	110	47100	2980	1040		12000				
	285	44000	5050	3460		7480				
	405	27600	6350	3490		973				
	577	20400	957	475	ND	1640	8.23	6.59	1.48	414
	670	5490	334	1010	0.001	251	7	9.54	5.84	365
	770	5010	456	3640	ND	42.4	13.6	13.6	2.73	83.1
	865	7220	761	3780	<0.002	10.1	15.9	14.2	1.39	227
	948	2230	733	4750	<0.0025	1.75	14.5	13.1	1.25	140
RW-007	-30	41600	939	1.5			44	23.25		ND
	24	37900	2540	121	ND	8930	24.95	28.75	0.03	1600
	70	49500	1320	447	ND	6885	6.25	21.267	0.178	1285
	110	67100	6970	2220	<0.003	11500	7.86	30.15	0.4575	1110
	165	60500	4550	1850	<0.003	11600	5.33	18.9	0.714	822
	285	93800	5880	2800	0.003	7100	5.57	20.1	7.805	749
	335	56200	4230	1810	0.013	1170	32.3	3.52	0.69	666
	405	36200	3260	1460	0.009	517	<0.5	8.68	5.09	
	577	56800	4210	2360	0.002	6920	6.77	12.1	2.06	564
	670	14600	2470	2760	0.008	1633	5	16.1	4.555	569
	770	15500	2700	3320	0.003	1050	12.3	14.1	4.119	14.3
	865	9050	2340	4160	0.002	1600	2.23	19	13.9	512
	948	7220	1130	9135	0.0026	180	7.25	19	4.45	310

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Table 6
Estimates of PCE and Ethanol Mass Remaining in Subsurface by Various Methods

	PCE		Ethanol		Ratio
	Volume (L)	Mass (moles)	Volume (L)	Mass (moles)	Moles Ethanol: Moles PCE
Extraction Wells & Partitioning Tracers	26[a]	254[a]	2720[b]	47,234[b]	186:1
Dissolved Phase – after Solvent Extraction	1.1[c]	11[c]	295[c]	5,119[c]	465:1
Dissolved Phase – after Residual Biotreatment (2.5 years)	0.4[c]	4[c]	110[c]	1,926[c]	482:1
Total System PCE, Ethanol from Extraction Well Concentration		265		47,234	178:1
Total System PCE, Ethanol from Dissolved Phase – after S.E.		265		5,119	19:1

[a] estimated by partitioning tracer data (Jawitz et al. 2000)
[b] estimated from recovery well concentrations (Jawitz et al. 2000)
[c] calculated from Surfer™ contour plots

Table 7
Rate Constants for *cis*-DCE Formation for Individual Wells

Well Name	k (ug/L/day)	Correlation Coefficient
C1	14.65	0.25
C2	57.29	0.59
C3	10.27	0.68
C4	4.67	0.27
MW-505	0.24	0.19
MW-506	0.0008	0.20
MW-507	0.003	0.28
MW-509	11.64	0.59
MW-510	1.09	0.49
MW-511	0.14	-1.47
MW-512	0.54	0.25
MW-513	6.29	0.54
MW-514	10.86	0.63
RW-3	6.32	0.79
RW-4	2.89	0.08
RW-6	4.27	0.50
RW-7	6.01	0.66
Avg. RW-3,6,&7	5.54	0.64

Table 8
Rate Constants for PCE Removal for Individual Wells

Well Name	k (ug/L/day)	R^2
C1	**-110.8**	**0.90**
C2	**-102.46**	**0.92**
C3	-11.48	0.25
C4	**-18.87**	**0.82**
MW-505	-0.60	0.03
MW-506	-0.0021	0.01
MW-507	-0.062 X	0.12
MW-509	-11.94	0.19
MW-510	**-2.17**	**0.58**
MW-511	-0.70	0.18
MW-512	-6.65	0.41
MW-513	-22.84	0.36
MW-514	-47.98	0.51
RW-3	-34.59	0.37
RW-4	-7.70	0.10
RW-6	-25.77	0.31
RW-7	-50.47	0.44
Avg RW-3,6,&7	**-37.89**	**0.31**

Attachment C-1: Technology Transfer Documents

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Books/Book Chapters/Manuals

Implementation Manual for the Solvent Extraction Residual Biotreatment (SERB) Technology. 2001. (Table of Contents as Attachment C-2.)

Attachment C-2: Implementation Manual Table of Contents

Implementation Manual for the Solvent Extraction Residual Biotreatment (SERB) Technology.

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Enhancing PCB Bioremediation

Strategic Environmental Research and Development Program

Enhancing PCB Bioremediation

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Draft Report

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Preface

Project Title

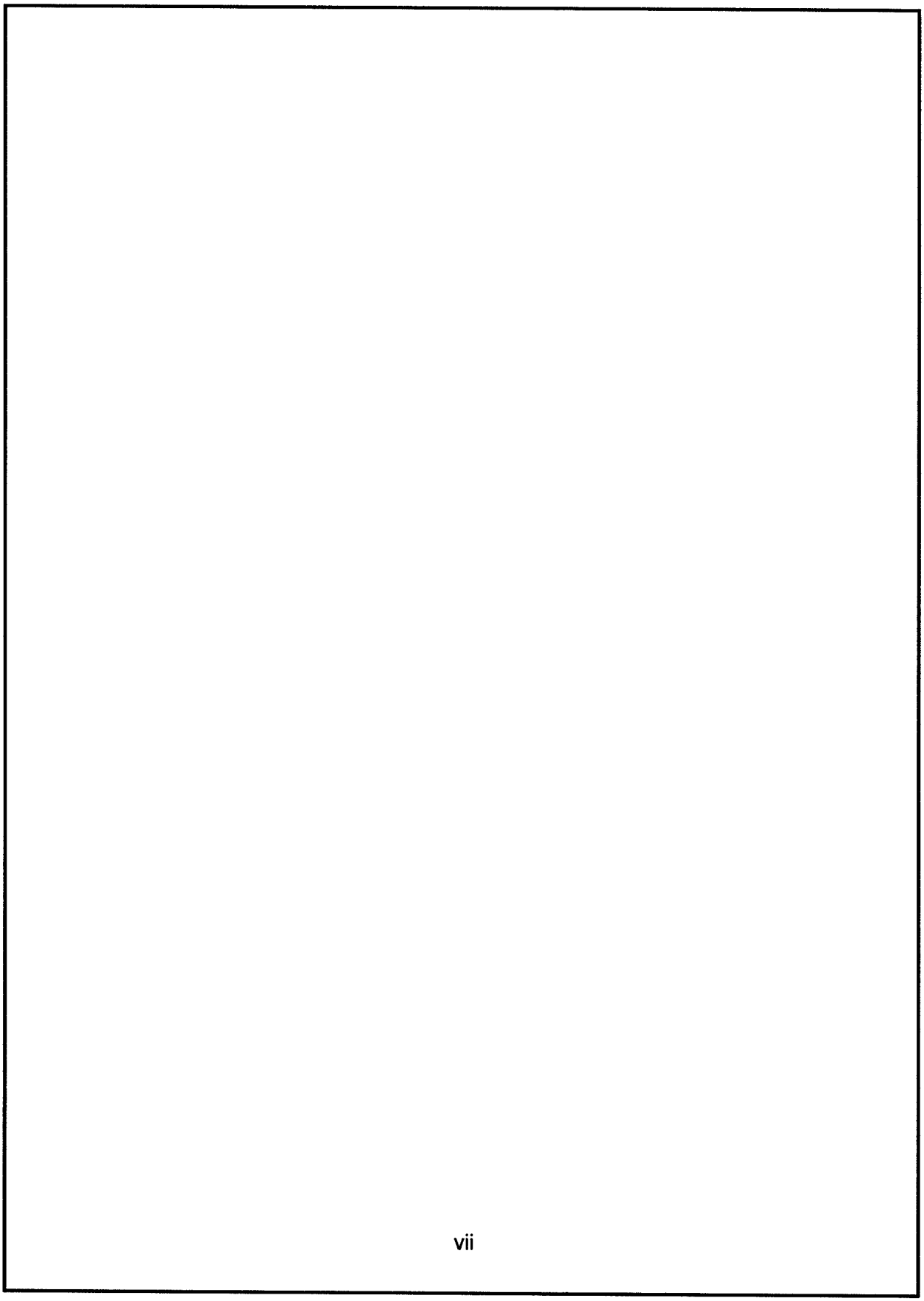
Enhancing PCB Bioremediation

Performers

The lead Principal Investigator (PI) is Dr. James M. Tiedje of Michigan State University. The Co-Principal Investigators are Dr. Tamara V. Tsoi, (Michigan State University), Dr. Kurt D. Pennell, (Georgia Institute of Technology), Mr. Lance Hansen and Dr. Altaf Wani, (U.S. Army Engineer Research and Development Center).

Performing Organizations

The project was performed jointly by Michigan State University, Georgia Institute of Technology, and the US Army Corps of Engineers, Engineering Research and Development Center, Environmental Laboratory, Vicksburg, MS.



1 Project Background and Rationale

This project addresses key barriers to bioremediating PCBs, which are:

developing microorganisms that will grow on the major congeners produced by anaerobic dechlorination of PCBs,

1) improving bioavailability of PCBs through use of surfactants,
2) optimizing field delivery of anaerobic/aerobic PCB bioremediation technologies, and
3) validating the new two-phase anaerobic-aerobic bioremediation strategy in a pilot-scale test.

Polychlorinated biphenyls (PCBs) remain among the most expensive hazardous waste cleanup problems facing the country. By use of existing technology, principally incineration, the cleanup cost is estimated to exceed $20 billion. If PCB concentrations could be reduced *in situ* by using biotechnological approaches, the cost should be substantially reduced. The research on microbial degradation of PCBs has a 20-year history (Ahmed and Focht, 1973), and many field trials of PCB bioremediation have taken place. This research has shown that bioremediation requires a more sophisticated technology than the simplistic attempts that have been tried so far. The 20 years of PCB research, however, defined the barriers that must be overcome for successful bioremediation and the discoveries in basic biochemistry and molecular biology have now provided the feasible approaches to overcome these barriers.

The fundamental barriers to bioremediation of PCBs are:

1) the absence from nature of organisms that will grow on PCBs,
2) the slow rate of reductive dechlorination and the fact that it is usually incomplete, and
3) the low solubility of PCBs and hence its poor bioavailability.
4) practical barriers including a microbial delivery technology that insures high survivability of introduced microorganisms in soils, and appropriate "in field" remediation technologies.

Polychlorinated biphenyls (PCBs) are a class of chlorinated compounds, the general structure for which is given in Figure 1. Each of the numbered positions may or may not be chlorinated, resulting in 209 different congeners. For industrial purposes, PCBs were manufactured as complex mixtures containing from 60 to 90 congeners by the catalytic chlorination of biphenyl (Shulz et al., 1989). Depending on the amount of chlorine added, these mixtures were mobile oils, viscous liquids, or sticky resins, but all were non-flammable, thermally stabile, chemically inert, and excellent electrical insulators. Because of these properties, they were widely used as dielectric fluids in electrical capacitors and transformers and as plasticizers. Lesser but still significant amounts were used as lubricants, hydraulic fluids, heat transfer fluids, cutting oils, as extenders in waxes, pesticides, and inks, and in carbonless copy paper (Hutzinger et al., 1974).

In the United States and Great Britain, nearly all PCBs were manufactured by Monsanto under the trade name Aroclor and given a four-digit number. The first two digits were 12 for PCBs, 54 for polychlorinated terphenyls, and 25 or 44 for blends of PCBs and polychlorinated terphenyls. The last two digits indicated the percent chlorine by weight. Thus Aroclor 1242, for example, is a PCB mixture that is 42% chlorine by weight and averages 3.1 chlorines per molecule. Aroclor 1260 contains 60% chlorine and averages about 6 chlorines per molecule. Aroclor 1016 is an exception to this scheme; it contains 41% chlorine by weight, and appears to be a fractional distillation product from Aroclor 1242 with marked reduction in the amount of congeners with 5 or more chlorines. In other countries, PCB mixtures were manufactured under the trade names Fenclor, Pheneclor, Pyralene, Clophen, and Kanechlor, to name a few (Hutzinger et al., 1974).

As the result of manufacturing processes and spills, several hundred million pounds of PCBs have been released into the environment (Hutzinger and Veercamp, 1981), and the same properties that made them so industrially useful make them environmentally persistent. Because they are sparingly soluble in water, they have a limited potential for migration through soil, and even the bulk of PCBs deposited in sediments may remain in place for decades. At the same time, they are lipid soluble and therefore bioaccumulate, increasing risks associated with exposure through the food-chain. A variety of adverse biological effects have been ascribed to them. Perhaps the most notable eco-toxicological effect of PCBs concerns poor reproductive success and deformities in some fish and fish-eating birds (Ludwig et al, 1993). Also, PCBs are suspected carcinogens, and there is epidemiological evidence that they can cause abnormal neurological development in infants and children and alter immunological responses (ATSDR). Thus they are recognized as one of the most problematic environmental contaminants.

The remediation of PCB-contaminated soils and sediments typically involves excavation of the contaminated material followed by landfill disposal or incineration. The high costs, long-term liability and regulatory issues associated with this approach have reduced the attractiveness of excavation as an ultimate remediation option. In addition, excavation and off-site transport of PCB-contaminated wastes may actually increase the potential for human exposure. Recognition of the potential economic and health implications associated with traditional PCB treatment methods has led to a renewed interest in the development of in-situ and on-site treatment technologies, including enhanced bioremediation processes. Due to their low solubility in water (or hydrophobicity) and low vapor pressures, PCB congeners are not effectively removed from soil/sediment systems by conventional abiotic remediation technologies such as soil vapor extraction or solvent flushing. Thus, the current state-of-the-art for PCB remediation typically involves the excavation of PCB-contaminated soil/sediment, followed by incineration. Estimated costs for incineration are on the order of $300-$600/ton of soil, including transportation and excavation costs. This remediation method frequently involves increased risk of human exposure resulting due to the excavation and transport of PCB-contaminated soils. The primary routes of exposure for this scenario are inhalation and dermal contact with soil particles containing sorbed-phase PCBs.

A competing *in-situ* technology that is currently under development by General Electric involves low-temperature thermal desorption/oxidation of PCB-contaminated surface soils (Iben et al., 1996). The technique involves the use of a "thermal blanket" containing resistive tubular heaters spaced at 8-cm intervals. The thermal blanket is placed over the soil surface and covered with a layer of insulation (vermiculite or ceramic fiber) and an impermeable sheet of fiberglass

reinforced silicon rubber. Off gases are pulled through a central tube and passed through a thermal oxidizer operated at about 900°C. A pilot-scale study has been conducted at an abandoned racetrack where PCB-containing oil was applied to the soil surface to reduce dust. PCB concentrations averaged approximately 680 mg/kg from 0-7.5 cm (0-3 in) depth and 100 mg/kg for 7.5-15.0 cm (3-6 in) depth. Below 15 cm (6 in), PCB concentrations were below the 2 mg/kg target level. The thermal blanket was heated to about 900 °C for 21 hours, with temperatures at the 15 cm depth maintained at 250 °C for about 29 hours after the heaters were turned off. In most cases soil PCB concentrations were reduced to well below the 2 mg/kg target. The costs for thermal blanket remediation were estimated at $150-200/ton for a larger site (>6 ha) for a treatment depth of 15 cm (6 in). These costs may be viewed as rather optimistic because they were based on a scaled-up application (not the actual test case) and the depth of treatment was only 6 inches. Limitations and concerns of this technology include:

1) small depth of treatment,
2) potential for downward migration of mobilized PCBs, and
3) potential formation of undesirable low-temperature thermal products near the edge of the treated zone. (A detailed Technology Comparison is presented in Attachment D2).

2 Objectives

Overall Objectives

The primary objectives of this project are:

1) development of genetically engineered organisms that will grow on PCBs;
2) evaluation of surfactants and $FeSO_4$ to enhance PCB dechlorination; and
3) implementation and testing of PCB bioremediation in pilot-scale field tests.

The goal of the first objective is to construct pathways for PCB degradation that should result in bacteria capable of using PCB congeners as a growth substrate, and to use these organisms to remove products of anaerobic reductive PCB dechlorination (i.e, the less chlorinated mono-, di-, and trichlorobiphenyls, predominantly *ortho*-, and *ortho+para*-chlorinated congeners). To achieve this goal, we combined two metabolic capabilities in the same organism, co-metabolism of PCBs to chlorobenzoates, and dechlorination and mineralization of chlorobenzoates as a growth substrate. Our research activities have focused on several biphenyl-degrading, PCB-cometabolic, bacterial strains studied in our laboratory, and the dechlorination genes found, isolated and studied under our core GLMAC HSRC project. Once such strains are constructed, our goal is to characterize the ability of the designed organisms to enhance PCB degradation in soils. The practical effectiveness of constructed bacteria will be tested at the U.S. Army Engineer Waterways Experiment Station.

The second objective of the project is designed to identify and evaluate surfactants capable of enhancing the bioremediation of PCBs in soils and sediments. Research activities have specifically focused on the selection of surfactants that are compatible with the engineered bacteria discussed above, including *Rhodococcus erythreus* NY05, *Rhodococcus* RHA1 and *Comamonas testosteroni* VP44. The experimental approach involves a systematic screening of selected surfactants in microbial batch systems for toxicity or inhibitory effects, prior to the addition of engineered bacteria and surfactant to a contaminated soil or sediment system. The ideal surfactant candidate would not be readily utilized as growth substrate by the bacteria, possibly serving as a preferential growth substrate over PCBs and/or reducing the selective pressure for PCB growth genes. In addition to biological compatibility, the selected surfactants were also tested for sorptive losses to several natural soils, capacity to solubilize PCB congeners, coupled solubilization and microbial transformation, and effects on plasmid stability.

During the past year, the overall direction of PCB project has shifted to focus more directly on field implementation using soils from at Lake Ontario Ordinance Works (LOOW) and Picatinny Arsenal, and lately, the GE site in Rome, GA. Using the latter soil, evaluation of the practical effectiveness of the designed two-phase anaerobic-aerobic bioremediation scheme is currently underway in co-operation with engineers in the SERDP Bioconsortium, specifically at Georgia Tech. and Waterways Experiment Station.

Research Objectives to Design PCB-Growing GEMS

1) Develop gene cloning and chromosomal integration technique in *Rhodococcus* strains;
2) Design genetically enhanced *para-* and *ortho*-PCB-growing Gram negative and *Rhodococcus* strains;
3) Evaluate the fate of the designed organisms and their effect on PCBs in soils;
4) Develop methods allowing tracking the introduced organisms and genes *in situ*;
5) Evaluate effect of anaerobic/aerobic shift and FeSO4 and FeS on survivability and PCB degradative activity of the designed organisms in soils;
6) Develop suitable protocol for soil inoculation with the engineered microorganisms;
7) Enhance anaerobic reductive PCB dechlorination in PCB contaminated soil;
8) Evaluate the recombinant PCB remediation two-phase technology on pilot scale using PCB contaminated soil(s) provided by WES (in co-operation with WES and GeorgiaTech).
9) Evaluate feasibility of anaerobic PCB dechlorination in the Picatinny Arsenal and LOOW soil to enrich for the congeners that would be accessible for degradation by aerobic genetically enhanced microorganisms;
10) Establish methods allowing rapid and quantitative detection of genetically engineered PCB-growing bacteria *in situ*;
11) Characterize PCB growing variants of strains RHA1 and LB400 possessing hydrolytic *para*-dechlorination (*fcb*) and oxygenolytic *ortho*-chlorobenzoate
12) (*ohb*) dechlorination genes, respectively, for their substrate range;
13) Evaluate survivability of the recombinant PCB growing organisms RHA1(fcb) and LB400(ohb) and their impact on PCBs in Picatinny Arsenal soil microcosms
14) Evaluate effect of FeSO4 and FeS on the fate and activity of the recombinant strains LB400(ohb) and RHA1(fcb) in soil microcosms;
15) Evaluate survivability of introduced biphenyls degrading bacteria in the LOOW soil;
16) Isolate and evaluate feasibility of genetic enhancement of indigenous biphenyl degrading organisms from the PCB contaminated LOOW soil to increase chances for successful PCB remediation in this environment which has a complex contamination profile.

Research Objectives to Enhance PCB Remediation

1) Investigate the growth of *Comamonas testosteroni* VP44 and *Rhodococcus erythreus* NY05 on biphenyl and 4-chlorobiphenyl (4-CBP) in the presence of selected nonionic surfactants (Tween 80, Tergitol NP-15 and Witconol SN-120).
2) Develop analytical methods (HPLC and GC) to measure the concentration of both surfactant and PCB congeners in the aqueous phase.
3) Measure the rates of micellar solubilization and equilibrium solubilization capacity of the selected surfactant for specific PCB congeners.
4) Measure the sorption and desorption of surfactants on natural soils possessing a range of organic carbon contents (Wurtsmith aquifer material-0.02% OC, Appling soil-0.7% OC, and Webster soil-3.5% OC), and the influence of surfactant sorption on PCB sorption by the solid phase.

5) Quantify the aerobic degradation of both surfactant and PCB congeners in aqueous systems by recombinant variants of *Comamonas testosteroni* VP44 and *Rhodococcus erythreus* NY05.
6) Conduct microcosm experiments (500 ml reactors) to assess the desorption and aerobic dechlorination of sorbed-phase PCB congeners in the presence of Tergitol NP-15 and Tween 80.
7) Develop and test a mathematical model to describe the coupled sorption/desorption, micellar solubilization, and transformation of PCB congeners under sequential anaerobic and aerobic conditions.
8) Investigate the growth of *Comamonas testosteroni* VP44 and *Rhodococcus erythreus* NY05 on biphenyl and 4-chlorobiphenyl (4-CBP) in the presence of selected nonionic surfactants (Tween 80, Tergitol NP-15 and Witconol SN-120).
9) Develop analytical methods (HPLC and GC) to measure the concentration of both surfactant and PCB congeners in the aqueous phase.
10) Measure the rates of micellar solubilization and equilibrium solubilization capacity of the selected surfactant for specific PCB congeners.
11) Measure the sorption and desorption of surfactants on natural soils possessing a range of organic carbon contents (Wurtsmith aquifer material-0.02% OC, Appling soil-0.7% OC, and Webster soil-3.5% OC), and the influence of surfactant sorption on PCB sorption by the solid phase.
12) Quantify the aerobic degradation of both surfactant and PCB congeners in aqueous systems by recombinant variants of *Comamonas testosteroni* VP44 and *Rhodococcus erythreus* NY05.
13) Conduct microcosm experiments (500 ml reactors) to assess the desorption and aerobic dechlorination of sorbed-phase PCB congeners in the presence of Tergitol NP-15 and Tween 80.
14) Develop and test a mathematical model to describe the coupled sorption/desorption, micellar solubilization, and transformation of PCB congeners under sequential anaerobic and aerobic conditions.
15) Design and test mixing capacity of low water content bioreactor (~1/5-scale) for the treatment of PCB-contaminated muck from Lake Ontario Ordinance Works (LOOW).
16) Perform economic analysis of bioreactor system and compare costs to competing technologies based on site-specific information using RACER.
17) Optimize anaerobic-aerobic reactor treatment scheme including materials handling, addition of bulking agents, biocarriers, and nutrients, and disposal procedures.
18) Conduct surfactant performance tests using PCB-contaminated LOOW material and spiked Picatinny soil to test the potential for surfactant sorption losses and PCB phase distribution and PCB desorption rates.
19) Assist in scale-up of the bioreactor and implementation at the LOOW field site.
20) Assist in regulatory compliance with regard to PCB and GEMs handling and disposal procedures.
21) Coordinate interaction among PCB Thrust Area PI's and incorporate laboratory results into field-scale reactor design and operation.
22) Evaluate mixing performance and materials addition of full-scale reactor during operation.

Field-Test Phase Objectives

1) Evaluate effect of vermiculite and Fe(II) on survival and activity of GEMs in soil
2) Evaluate survivability and PCB degradative activity of GEMs in Picatinny soil
3) Develop protocol for preparation and delivery of GEMs in soil (field test)
4) Conduct laboratory-scale experiments to evaluate the effects of surfactant additions on PCB desorption and biodegradation under mixing regimes similar to those utilized in the pilot-scale tests.
5) Develop and evaluate mathematical models designed to simulate PCB desorption and biodegradation in the presence and absence of surfactants.
6) Validate design and strategy for two-phase anaerobic-aerobic full-scale bioremediation of PCB contaminated soils (laboratory scale soil microcosms)
7) Evaluate the use of PCB-growing GEMs in combination with enhanced anaerobic dechlorination and surfactants for bioremediating PCB contaminated soils
8) Design treatment systems, including slurry and land farming reactors, to pilot-scale test PCB remediation technologies developed within the PCB Thrust Area
9) Develop cost estimates of pilot-scale treatment systems and perform economic analysis of full-scale implementation with comparisons to conventional PCB treatment technologies.
10) Evaluate the effects of different solids loading rates (moisture contents) on the uniform application of amendments/GEMs, and the bioremediation of PCBs
11) Determine the maximum solids loading rate optimum for the activity of GEMs, in order to offset subsequent dewatering costs for the disposal/reuse of stabilized soils
12) Identify PCB-contaminated sites available for the field-test and evaluate feasibility of their PCB treatment
13) Conduct ex-situ two-phase anaerobic-aerobic PCB remediation field-test and evaluate efficiency of the PCB removal and performance of GEMs
14) Prepare final report over the project life-time

3 Technical Approach

Summary

This project addresses key barriers to bioremediating PCBs, which are:

1) developing microorganism that will grow on the major congeners produced by anaerobic dechlorination of PCBs,
2) improving bioavailability of PCBs through use of surfactants, and
3) optimizing field delivery of anaerobic/aerobic PCB bioremediation technology.

Three central parts of the current SERDP funded work are the use of a combination of genetically engineered organisms that will grow on PCBs, the use of surfactants to enhance the bioavailability, and bioslurry experiments as a first stage in a "flask-to-field" transfer technology (Figure 2). The lack of organisms that grow on PCBs results from the fact that organisms with PCB (biphenyl moiety) cometabolizing activity do not seem to have the ability to use the chlorobenzoate product for growth, and those few that can would attempt to metabolize it via the chlorocatechol pathway forming an acyl halide which is immediately toxic. Hence if any organism in nature does have the capacity to grow on PCBs, it would be suicidal. Through the use of dechlorination genes we have devised a scheme to remove chlorines before chlorocatechols are formed hence, providing an energy (growth) product and avoiding toxicity. This approach should be a more desired solution for PCB remediation since it would avoid the need to manage co-metabolism, which can be difficult *in situ*.

One of the biological barriers to an effective PCB remediation is slow rates of intrinsic anaerobic dechlorination. The first step in optimizing a sequential anaerobic/aerobic biotreatment process for PCBs is to maximize the extent of dechlorination. Several dechlorination processes, generally believed to be due to the actions of different species of microorganisms, have been recognized on the basis of their congener specificities (Table 1).

In Table 1, M, for example, removes both flanked and unflanked *meta* chlorines. A flanked *meta* chlorine is one adjacent to another (*ortho* or *para*) chlorine. An unflanked chlorine has no other chlorine next to it. Not fully captured in Table 1 is the fact that these processes also vary in their abilities to attack more heavily chlorinated congeners. Processes M and Q, for example, are less effective in dechlorinating Aroclor 1260 than is process N. Thus the extent of PCB dechlorination that occurs is dependent on which microorganisms are present and active, and more extensive dechlorination can be achieved by combining dechlorination processes with complementary activities. This has occurred naturally in the upper Hudson River where PCB dechlorination was first recognized. In several locations there the combined activities of at least the first four processes listed in Table 1 have removed most of the meta and para chlorines from Aroclor 1242 with the result that about 90% of the remaining PCBs are mono- and di-chlorinated

congeners substituted only in the *ortho* positions. These congeners comprise less than 8 % of Aroclor 1242.

In laboratory experiments, we have obtained PCB dechlorination by complementary processes using two different approaches. We discovered that the addition of $FeSO_4$ fosters simultaneous dechlorination by processes M and Q (Zwiernik et al, 1998), and we achieved enhanced dechlorination of Aroclor 1260 using sequential inoculations of process N and process Q microorganisms (Quensen et al., 1990).

$FeSO_4$ Amendment

It has been our experience that *meta* dechlorination is more readily achieved than *para* dechlorination, and that process Q dechlorination is not reliably achievable. While investigating the use of ferrous sulfate as a way of precipitating heavy metals, which can inhibit dechlorination, we discovered a way of "rescuing" process Q activity. Figure 3 depicts the extent of Aroclor 1242 dechlorination during the course of his experiment, and it can be seen that dechlorination was more extensive with the addition of 10 mM $FeSO_4$. Dechlorination occurred only subsequent to sulfate depletion, and our thinking is that the dechlorinating microorganisms are sulfate reducers (i.e., they dechlorinate PCBs in the absence of sulfate), that addition of a small amount of sulfate allowed them to increase in numbers, and that the iron precipitated the sulfide formed, to which the *para* dechlorinating microorganisms are sensitive. Evidence for this last point comes from the fact that precipitation of sulfides with $FeCl_2$ or $PbCl_2$ also enhanced dechlorination, but to a lesser extent.

The differences in congener profile achieved with and without the ferrous sulfate amendment are shown in Figure 4 in which the mole percent of the congeners in each chromatographic peak are plotted by peak elution order. For a complete list of congeners associated with each peak number see Quensen et al. (1990b). Without addition of $FeSO_4$, Pattern M resulting from *meta* dechlorination alone was achieved. With ferrous sulfate, Pattern C resulting from the combined *meta* and *para* dechlorinating activities of Processes M and Q was achieved. Thus, addition of $FeSO_4$ is one way of achieving enhanced PCB dechlorination through the combined activities of complementary dechlorination processes.

Sequential Inoculations

A second way of obtaining a combination of complementary dechlorination activities is to use inocula from different sources. The results from such an experiment are depicted in Figure 5. The progress of Aroclor 1260 dechlorination in various treatments is compared by plotting the average number of chlorines remaining in the meta or para positions versus time. The most extensive dechlorination occurred with sequential inoculations with Silver Lake followed by Hudson River microorganisms.

Process N microorganisms in Silver Lake sediments are much more effective in dechlorinating the heavily chlorinated congeners in Aroclor 1260 than are the microorganisms in Hudson River sediments, which are mainly process M and Q microorganisms. The extent of dechlorination

achievable by Silver Lake microorganisms alone is limited because process N removes only flanked meta chlorines. Hudson River microorganisms (M and Q together), however, have the potential to remove all *meta* and *para* chlorines, whether or not there is an adjacent chlorine.

Combined activities could not be achieved by simply mixing microorganisms from the two sediments together, apparently because of some incompatibility, but we were able to achieve enhanced activity through sequential inoculations, first with Hudson River microorganisms and then with Silver Lake microorganisms. A possible explanation can be gleaned from examining the chromatographic profiles of the PCBs generated in the various treatments (Figure 6). The profiles for Hudson River microorganisms alone and mixed with Silver Lake microorganisms were similar and indicate that dechlorination was limited to the *meta* positions. In other words, process Q or *para* dechlorination activity was lost, and even N activity was diminished. The profile for Silver Lake microorganisms alone indicates that high levels of *ortho* and *para* substituted congeners, notably 24-26-CB peak 21), 24-24-CB (peak 26) and 246-24-CB (peak 34), were formed. These congeners could serve as a substrate for process Q microorganisms in the subsequent inoculation with Hudson River microorganisms. It is possible that the accumulation of these congeners even selected for, or primed, this *para* dechlorination activity. In any event, the result was that *ortho-* only substituted congeners increased from less than 1% in Aroclor 1260 to 39% with sequential inoculations at the end of the experiment.

Another biological barrier to PCB bioremediation is that there are no bacteria that have been found in nature that can grow on important PCB congeners as a growth substrate. Anaerobic co-metabolic reductive dechlorination of highly chlorinated PCBs produces less chlorinated congeners (mono-, di-, and tri-chlorinated biphenyls), preferentially *ortho*-chlorobiphenyls and *ortho-+para*-derivatives. Aerobic biphenyl degrading bacteria can non-specifically co-oxidize less chlorinated PCBs, but this process requires biphenyl or related other source as a growth substrate. The major product of this dead-end co-metabolism is the respective chlorobenzoate. If the barrier of growth on PCBs can be overcome, then a natural enrichment by growth on PCBs should occur, increasing the rate of PCB removal. This approach should be a more desired solution for PCB remediation since it would avoid the need to manage co-metabolism which can be difficult and costly.

Previously, we obtained a collection of bacterial isolates that aerobically degrade biphenyl and co-metabolize certain PCB congeners. Under our core HSRC research project, we have cloned and characterized the genes specifying anoxic hydrolytic *para*-dechlorination (*fcb*-operon from *Arthrobacter globiformis* KZT1), oxygenolytic *ortho*-dechlorination (*ohb*-operon from *Pseudomonas aeruginosa* 142), and lately reductive *ortho*-dechlorination (*rod* genes from *Corynebacterium sepedonicum* KZ4). These genes encode enzymes that remove chlorine from chlorobenzoates funneling non-chlorinated products into common degradative pathways for non-chlorinated aromatics. Combining these dehalogenase genes and biphenyl oxidation pathways should result in engineered pathways for *ortho-*, *para-*, and *ortho+para*-chlorinated PCB congeners that are of a major concern in the proposed anaerobic-aerobic bioremediation scheme (Figure 7). In a difference from approaches employed by other groups, using the specific chlorobenzoate dehalogenases not only allows the choice of a desirable host (for example, PCB-tolerant bacteria, Gram positive or Gram negative), but also prevents accumulation of toxic aromatic ring *meta*-cleavage products that would be produced from using broad specificity benzoate oxygenases that produce chlorocatechol from chlorobenzoate.

Another potential problem we envision for bioremediation *in situ* is that combining the anaerobic phase for reductive dechlorination of highly chlorinated PCBs and the aerobic phase for oxidation of less chlorinated PCB congeners in the same remediation scheme might be too complicated to manage if the common aerobe such as Gram-negative bacteria were used for the aerobic phase. To overcome this barrier, we propose to use not only Gram-negative but Gram-positive bacteria as well, for construction of PCB degradation pathways. The well-known ability of Gram-positive micro-aerophilic bacteria such as bacilli, corynebacteria, rhodococcaceae, and others to persist in harsh environments and survive anaerobic conditions should reduce the cost of site management.

Although microbial transformation of PCBs has been the subject of intense study over the past 25 years, it is now apparent that the use of simplistic remediation approaches, based on conventional bioreactor and landfarming strategies, will not be successful. One of the primary barriers to effective PCB bioremediation is the limited availability of PCBs to microbial populations. PCBs are extremely hydrophobic compounds, which results in their low equilibrium solubilities and slow rates of desorption from solid phases. These physical/chemical barriers may contribute to incomplete bioremediation of PCB-contaminated sites and the inability to reach target PCB concentrations. To overcome such limitations, we have proposed the use of surfactants to increase the equilibrium solubility and mass transfer rate of PCBs into the aqueous phase. Three commercially available surfactants were selected for study; Tween 80, Witconol SN-120, and Tergitol NP-15. These surfactants cost approximately $1.00/lb, and thus, surfactant costs for a 5,000 mg/L solution would be approximately $3.78 per ton of soil, assuming a water-filled porosity of 0.3.

4 Accomplishments of the Flask Evaluation

Designing and Testing PCB-Growing GEMS

Characterization of aerobic PCB metabolism by biphenyl degrading organisms

Analysis of the dechlorination patterns in anaerobic sediments resulted in identification of 8 *ortho*- and *ortho+para*-chlorinated PCB congeners that account up to 80 mole% of the total Aroclor 1242 products in anaerobically dechlorinated sediments and are primary targets for aerobic phase of PCB bioremediation scheme. Correspondingly, we have concentrated on studying aerobic metabolism of these 8 PCB congeners, both individual and given as defined mixtures M and C (Figure 8).

We have conducted characterization of PCB co-metabolism by biphenyl-degrading Gram positive *Rhodococcus erythreus* NY05, *Rhodococcus* sp. RHA1, and Gram negative *Comamonas testosteroni* VP44, and *Burkholderia* sp. LB400. Metabolism of PCBs by these strains has been studied in detail for the range of substrates, rates of PCB oxidation, intermediates, dead-end products, and specificity of biphenyl ring oxidation. 2-, 4-, and 2,4-CB were easily degraded by all four strains. Equimolar accumulations of the corresponding chlorobenzoic acids were detected in all cases with the exception of the transformation of 2-CB by *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1, which yielded only 55 to 75% of the expected 2-CBA (Table 2). Accumulation of high concentrations of chlorobenzoic acids from monochlorinated CBs and 2,4-CB showed that the non-chlorinated ring of these CBs was preferentially oxidized by all four strains. The appearance of yellow color with λ_{max} 394 nm during incubation of 2-CB with RHA1 indicated incomplete transformation and accumulation of HOPDA intermediate which could account for the difference in concentrations between consumed 2-CB and accumulated 2-CBA. Some fading of the yellow color and a shift towards shorter wavelengths was detected during the next two days of incubation indicating further transformation of the *meta*-cleavage product; however, no increase of 2-CBA concentration was noted (<5%). Accumulation of the 2-chlorobiphenyl *meta*-cleavage product was also detected during incubation of 2-CB with *R. erythreus* sp. NY05, but in contrast to the strain RHA1, this intermediate completely disappeared at 24 h of incubation followed by equimolar production of 2-CBA.

The dynamics of accumulation of 2-CBA by *Burkholderia* sp. LB400 suggested that it could further degrade this acid. Furthermore, subsequent resting cell experiments using 2-, 4-, and 2,4-CBA as substrates showed that this strain degraded from 40 to 60% of 2-CBA during 24 h of incubation, while no disappearance of 4-CBA and 2,4-CBA was detected. No metabolites of 2-CBA degradation were found by HPLC. The three other strains showed no further metabolism of 2-, 4- or 2,4-CBA.

2,6-CB proved to be the congener most resistant to microbial attack (Table 2). Less than 5% of it was depleted by strains *R. erythreus* NY05 and *C. testosteroni* VP44 with subsequent accumulation of trace amounts of 2,6-CBA, and only 5-10% was degraded by strains *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1 with recovery of only 5% of it as 2,6-CBA. Formation of some yellow colored product was detected during incubation of RHA1 with 4-CB, 2,4-CB and 2,6-CB, indicating that some of the HOPDA formed from these congeners did not undergo complete transformation into the corresponding chlorobenzoic acids and pentadienes.

Two congeners with one ring containing one chlorine in the *para* position (2-4' and 2,4-4') were efficiently degraded by *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1 (Table 3). Formation of 4-CBA as the major product from both of these congeners suggested that their *ortho*-substituted rings were preferentially oxidized. Transient appearance of yellow color with a λ_{max} of 434 nm occurred during the degradation of 2,4-4'-CB by *Burkholderia* sp. LB400, also indicating that this strain primarily oxidized the *ortho*-substituted ring (Seeger et al., 1995). Interestingly, following a decrease in absorption of HOPDA (434 nm), during a longer (24 h) incubation of LB400 with 2,4-4'-CB, a very small peak of HOPDA with λ_{max} of 398 nm was observed. These data suggest that the 4'-chlorinated ring of 2,4-4'-CB could be also oxidized by LB400. Accumulation of low amounts of 2,4-CBA among the degradation products of 2,4-4'-CB, which was verified by GC/MS, confirmed that LB400 is capable of some oxidation of the *para*-chlorinated ring of this congener.

Complete depletion of 2-4'- and 2,4-4'-CB was also observed with *R. erythreus* NY05 and with *C. testosteroni* VP44; however, amounts of chlorobenzoates formed did not exceed 10-12% of the expected (Table 3). Disappearance of these congeners was accompanied by formation of very intense yellow color. The metabolite of 2-4'-CB was characterized by molecular ion at m/z 430, low abundance M^+-15 and M^+-35 ions and by a prominent ion M^+-117 arising from the loss of CH_3, Cl, and COOTMS from the molecular ion, respectively. The same fragmentation pattern was obtained for the metabolite of 2,4-4'-CB (molecular ion 464). The mass spectral features of these compounds are in good agreement with previously published mass spectra of the TMS derivatives of chlorobiphenyl *meta*-cleavage products (HOPDAs) produced by some bacterial strains (Furukawa et al., 1979a, 1979b; Masse et al., 1989). Absorption maxima of these HOPDAs at λ_{max} 398 nm indicated that they have the chlorine substituent at the *ortho*-position (Seeger et al., 1995) and therefore were formed by the oxidation of the *para*-chlorinated ring. Further transformation of the *meta*-cleavage products formed from 2-4'-, and 2,4-4'-CB should result in accumulation of 2-CBA and 2,4-CBA, respectively. Both strains yielded some 2,4-CBA from 2,4-4'-CB, but only NY05 produced traces of 2-CBA from 2-4'-CB. No significant decrease of these HOPDAs nor a corresponding increase of the chlorobenzoic acids were detected during another 72 h of incubation suggesting that *meta*-cleavage products formed by *R. erythreus* NY05 and *C. testosteroni* VP44 were stable. Recovery of about 5% of 2-4'-CB as 4-CBA by both strains showed that they could also oxidize the *ortho*-chlorinated ring.

Efficient degradation (80-100%) of 2-2'- and 2,4-2', containing chlorine in *ortho*-position on both rings was observed with *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1 (Table 3). Both strains transformed 2,4-2'-CB into the equimolar amounts of 2,4-CBA, and 60-80% of 2-2'-CB was recovered as 2-CBA. The strain RHA1 accumulated a yellow *meta*-cleavage product (λ_{max} 394) from 2-2'-CB which could account for the difference in the depleted CB and accumulated 2-CBA. No fading of yellow color or shift of λ_{max} was detected even after the reaction mixture was

left for one month suggesting that this HOPDA was very stable. Non-equimolar accumulation of 2-CBA by strain LB400 could be due to the capability of this strain to further transform this compound as noted above.

Activities of strains *R. erythreus* NY05 and *C. testosteroni* VP44 towards 2-2'- and 2,4-2'-CB were much lower, 5-30% (Table 3). Depleted 2-2'- and 2,4-2'-CB were transformed by these strains into equimolar amounts of 2-CBA and 2,4-CBA.

Only transient appearance of yellow *meta*-cleavage products was detected with *Burkholderia* sp. LB400, while strain RHA1 accumulated HOPDA from 2-CB and 2-2'-CB. This difference between two strains with very similar CB degradative abilities might be due to the different substrate specificity of their respective HOPDA hydrolases which have only about 30% sequence identity (Hofer et al., 1993; Masai et al., 1996). We could not quantitate HOPDAs because standards are not available and there are no data on their molar extinction coefficients; however, the differences between consumed CBs and produced chlorobenzoates, which were 35% and 10% for 2-CB and 2-2'-CB respectively, are likely attributable to the formation of HOPDAs. Thus, poor turnover of HOPDAs can exclude some CB from further productive metabolism.

Two principal and complementary modes of PCB metabolism (preferential oxygenation of *ortho*- or *para*-chlorinated ring of biphenyl moiety) were demonstrated by *Rhodococcus* strains NY05 and RHA1 and have been found most attractive for use in bioremediation. Consumption rates and products of metabolism of the 8 PCB congeners by strains NY05, VP44, and LB400 were determined. These findings were summarized in Pellizari et al., 1997; Hrywna et al., 1999; Maltseva et al., 1999. We have studied cometabolism of the targeted 8 PCB congeners supplied as defined mixtures simulating most extensive (pattern C) and average (pattern M) anaerobic dechlorination products accumulating in PCB contaminated soils/sediments. Three strains (NY05, RHA1 and LB400) were used in resting cell experiments. From 40 to 80% of PCBs supplied in Mix M were depleted after 24 h of incubation with these strains and from 65 to 95% of the expected amounts were recovered as chlorobenzoates (Figure 9). 2-CBA and 4-CBA were the major products of degradation of Mix M. From 60 to 75% of PCBs supplied in Mix C were depleted after 24 h of incubation with the three strains and from 70 to 90% of the expected amounts were recovered as chlorobenzoates (Figure 10). 2-CBA was formed by all strains as the major degradation product of MixC. Rates of degradation of some congeners were affected when they were supplied in mixtures. 2-2'-CB, 2,6-CB, 2,4-2'-CB and 2,4-4'-CB were the most resistant to microbial attack when supplied in PCB mixtures M and C. Accumulation of yellow color was detected during degradation of mixtures M and C by *Rhodococcus* strains NY05 and RHA1, which accumulated HOPDA during the transformation of several individual congeners.

Strains RHA1 and LB400 that directed their primary attack towards *ortho*-chlorinated rings were capable of degrading 65-80% of PCBs in mixture M and 70-75% in mixture C, respectively. Degradation rates of PCB congeners containing chlorine on both biphenyl rings were affected when they were supplied in mixtures, possibly due to the presence of easily degradable congeners with one non-chlorinated ring. From 70 to 95% of depleted chlorobiphenyls were recovered as chlorobenzoates. *Ortho*-directed strains were equally efficient in depletion of PCBs not only from the most extensive anaerobic dechlorination product C, but also from the most common product M.

Lower rates of degradation were observed with strain NY05, 40 and 60% for Mix M and C, respectively). Thus, similar to the results with individual congeners, degradation of PCBs in defined mixtures M and C and production of chlorobenzoates was also more efficient with the *ortho*-directed strains LB400 and RHA1. In the only comparable investigation *Alcaligenes eutrophus* strain H850, also an *ortho*-directed strain (Bedard et al., 1987a) was reported to deplete 81% of Aroclor 1242 anaerobic dechlorination products in Hudson River sediments (10 ppm, pattern C), production of chlorobenzoates was not monitored it that work (Bedard et al., 1987b).

These experiments showed that degradation of several congeners is affected under these conditions, including less efficient degradation of 2,4-4-CB, 2-2-CB, 2,6-CB in mixture M. *Ortho*-directed strains LB400 and RHA1 appeared more efficient PCB degraders than *para*-directed NY05. We have identified several intermediate products of aerobic PCB oxidation with potential biotoxic effect, among them meta-cleavage products – chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDAs), and dihydrodiols, mono- and dihydroxybiphenyls. The amount of Cl-HOPDA produced by para-directed strains NY05 and VP44 was especially significant from 2-4-CB and 2,4-4-CB. Biochemical assay suggested that these chlorinated intermediates reversibly inhibit hydrolase activity preventing complete transformation of the HOPDAs to the respective chlorobenzoates. These results were summarized in our recent paper by Maltseva et al., 1999.

Aerobic degradation of PCBs by biphenyl-growing bacteria is usually cometabolic and results in partial degradation, particularly the accumulation of chlorobenzoates (Abramowicz, 1990; Unterman, 1996). Introduction of genes for dehalogenases that control removal of chlorine from *ortho*- and *para*-chlorinated benzoates into PCB cometabolizing strains should result in growth of the recombinant bacteria on the targeted *ortho*- and *ortho+para*-substituted congeners. The ideal host for constructing genetically modified microorganisms that would grow on the anaerobic Aroclor dechlorination products should:

1) completely deplete all eight targeted congeners,
2) accumulate no early intermediates of CB degradation, and
3) not funnel produced chlorobenzoates into unproductive pathways.

Based on these results, we proposed that while none of the investigated bacteria completely meets all these criteria, the *ortho*-directed strains RHA1 and LB400 catalyzed more efficient oxidation of a wider range of CBs with higher yield of chlorobenzoates than do the *para*-directed strains VP44 and NY05. Thus, the strains preferentially oxidizing *ortho*-chlorinated CB rings are still more suitable for genetically engineering bacteria capable of growth on Aroclor anaerobic dechlorination products.

Conceptual proof of designing PCB growth pathway

In contrast to the *in vivo* construction, the use of sequenced and well characterized genes permits targeting of specific compounds for degradation, and better prediction of expected intermediates and products with minimal effects on existing cellular metabolism. In a two-phase anaerobic-aerobic PCB bioremediation scheme, a limited number of *ortho*-, 2,6-, 2,4'-, and 2,4-chlorobiphenyls, are major targets for the aerobic degradation of PCBs (Ouensen et al., 1990a;b). These congeners upon oxidation by *bph* pathway enzymes produce the respective *ortho*-, *para*-,

and *ortho+para*-chlorobenzoates. Therefore, introduction of specific *ortho*-, and *para*-dechlorination genes into biphenyl-degrading bacteria should result in growth on and mineralization of the targeted PCB congeners by the recombinant organism. We have recently published the first results on construction of the recombinant variants using the PCB-cometabolizing *Comamonas testosteroni* strain VP44 as the host (Figure 11). The dehalogenation genes used were the *fcb*-operon for hydrolytic dechlorination of *para*-chlorobenzoate we have previously and *ortho+para*-chlorinated biphenyls such as 2-, 2,2'-, 2,4,2'-, 2,4,4'-, isolated from *Arthrobacter globiformis* strain KZT1 (Plotnikova et al., 1991; Tsoi et al., 1991), and the *ohb*-operon for oxygenolytic dechlorination of *ortho*-halobenzoate we have recently isolated from *Pseudomonas aeruginosa* strain 142 under the related HSRC project (Tsoi et al., 1999).

Recombinant strain VP44(pE43) was constructed by introduction of the recombinant broad host range plasmid pE43 that carries the *ohb* DNA fragment from *ortho*-chlorobenzoate degrading bacterium *P. aeruginosa* strain 142. The *ohb* DNA region contains structural genes *ohbAB* encoding small and large subunits, respectively, of the terminal oxygenase of the three-component *ortho*-halobenzoate 1,2-dioxygenase, possible regulatory gene *ohbR*, and *ohbC*, the gene of unknown function that overlaps the *ohbB* gene in an opposite transcripitional direction (Tsoi et al., 1999). Prior results showed that the expression of the *ohb* genes in *E. coli* and *P. putida* cells results in dechlorination and transformation of 2-CBA to catechol and, in case of *P. putida*, a recombinant pathway for growth on the chlorobenzoate (Tsoi et al., 1999). Strain *C. testosteroni* VP44 was transformed with DNA of plasmid pE43 and transformants grown on LB medium containing tetracycline were transferred onto K1 plates with 1.24 mM and 2.5 mM 2-CBA as a sole source of carbon. After an extended 8-week incubation colonies presumptive for containing plasmid pE43 were visible on the 2-CBA plates.

Recombinant strain VP44(pPC3) was constructed by introduction of the recombinant plasmid pPC3 carrying 4.36 Kb *fcb* DNA fragment from *A. globiformis* KZT1. The *fcb* DNA fragment contains structural genes *fcbABC* encoding ATP-dependent CoA-ligase, hydratase/dehalogenase, and thioesterase, respectively. Prior results showed that expression of the *fcb* operon in *E. coli* and *P. putida* results in dechlorination and conversion of 4-CBA to 4-hydroxybenzoate (4-HBA) and, in case of *P. putida*, a recombinant pathway for mineralization of 4-CBA (Plotnikova et al., 1991; Tsoi et al., 1991). Strain *C. testosteroni* VP44 was transformed with plasmid pPC3, and transfromants that grew on LB medium with tetracycline were transferred to K1 plates with 4-CBA (5 mM) as a sole source of carbon. Transformants VP44(pPC3) formed visible colonies on 4-CBA plates after 1 week of incubation.

Batch cultures of the recombinant strains VP44(pE43) and VP44(pPC3) grew efficiently on chlorobenzoates and chlorobiphenyls as a sole source of carbon at concentrations of the substrate as high as 10 mM. The growth was measured by increased optical density, chloride release, substrate disappearance, and protein yield. Strain 44(pE43) containing the *ohb* genes, when grown on 2-CBA at concentrations of 5 mM and 10 mM showed a proportional increase in A_{600} absorbency along with substrate disappearance, stoichiometric amounts of chloride released and a proportional increase in protein yield. The protein yield was comparable with that observed from growth on benzoate. Growth of the recombinant strain 44(pPC3) containing the *fcb* genes, on 4-CBA was similar to that of VP44(pE43) on 2-CBA, at concentrations of 4-CBA up to 10 mM, except for a shorter lag-period. As A_{600} increased over a 3-day period, the concentration of

4-CBA in the culture supernatant dropped below detection limits and the concentration of chloride rose to 10.1 mM. Protein yield was proportional to the concentration of substrate and similar to that for benzoate-grown cultures (Table 4).

Recombinant strains VP44(pE43) and VP44(pPC3) efficiently grew on 2-CB and 4-CB, respectively, at concentrations of up to 10 mM. As shown in Figure 12, only transient accumulation of the corresponding chlorobenzoates was observed during growth. Strain 44(pPC3) exhibited rapid growth on 4-CB, with transient production of yellow color (HOPDA) and 4-CBA in the culture supernatant during log phase, with concomitant release of inorganic chloride (Figure 12a). The maximal accumulation of the 4-chlorobenzoate was about 50 μM, or about 5% of the original substrate concentration. Growth of strain 44(pE43) on 2-CB was slightly slower with a greater accumulation of 2-CBA, 125 μM or 12.5% of original substrate concentration, which persisted slightly longer (Figure 12b).

As seen from Table 4, when grown on 2-CB, strain VP44(pE43) yielded approximately the same amount of protein per mmole of the substrate as compared to growth on biphenyl, and released stoichiometric amounts of chloride. Protein production and chloride release were linear up to at least 10 mM 2-CB. When VP44(pE43) was grown on 4-CB, an inappropriate substrate for this recombinant, it produced no chloride, roughly half as much protein compared to the growth on 2-CB and biphenyl, and stoichiometric concentrations of 4-CBA accumulated. Similarly, growth of the recombinant strain VP44(pPC3) on 4-CB was marked by stoichiometric chloride release, and protein yield comparable to that observed on biphenyl. Strain VP44(pPC3) grew on 2-CB without dechlorination, yielding only about half as much protein compared to the growth on 4-CB and biphenyl, and accumulating stoichiometric amounts of 2-CBA in the medium (Table 4). Production of protein and chloride release on 4-CB were linear only at low concentrations of substrate, with maximal production reached at 5 mM 4-CB, suggesting incomplete utilization at higher concentrations of this substrate. These results confirm the ability of parental strain to utilize pentadiene derived from the non-chlorinated ring of the (chloro)biphenyl molecule for growth.

Growth on biphenyls via the *bph* pathway for biphenyl degradation yields a benzoate and pentadiene as key intermediates, either or both of which may be chlorinated, depending on the PCB congener (Hofer et al., 1993). Introduction of the *ohb* (pE43) and *fcb* (pPC3) operons controlling *ortho*-, and *para*-dechlorination of aromatic ring, respectively, resulted in recombinant pathway for degradation of chlorobiphenyls via dechlorination of chlorobenzoates. Both recombinant variants VP44(pE43) and VP44(pPC3) compared favorably to other chlorobiphenyl-degrading recombinant strains previously constructed via intergenic mating. Both of our strains were capable of growth on chlorobenzoates and chlorobiphenyls at concentrations up to 10 mM. Strain VP44(pPC3) grown on 2 mM 4-CB exhibited 90% chloride release, a doubling time of about 4 h, and protein yield of 80 μg/μmol 4-CB (final concentration of 160 μg/ml). Strain VP44(pE43) grown on 2 mM 2-CB exhibited 95% chloride release, a doubling time of about 7 h, and protein yield of 88 μg/μmol 2-CB (final concentration of 177 μg/ml). By comparison, strain M3GY (McGular et al., 1994) grown on 0.89 mM 3-4'-CB, released 67% of the total chlorine, had a doubling time of approximately 20 days, and accumulated protein to a final concentration of 58 μg/ml. Strain JHR22 (Havel and Reineke, 1991) was reported to grow on several chlorobiphenyls including 2-CB and 4-CB, and exhibited doubling times of approximately 16 h and 10 h, as well as 80% (up to 4 mM) and 50% chlorine

release, respectively. Another strain, UCR2 (Hickey et al, 1992), was reported to mineralize both 2-CB and 2,5-CB, with doubling times of 20 h and 48 h, and dechlorination of 90% and 48.9%, respectively. Introduction of specific dechlorination operons can permit growth on otherwise recalcitrant substrates, and may be more easily accomplished than methods previously used to generate recombinant PCB degraders. Each recombinant strain was also tested for growth on other PCB congeners. Strain VP44(pE43) grew on plates with 2-2'-, and 2-4'-dichlorobiphenyls, while VP44(pPC3) grew on plates with 4-4'-, and 2-4'-chlorobiphenyls. Although so far we were unable to achieve a reproducible growth on these substrates in batch cultures, these congeners are expected to yield either 2-CBA or 4-CBA as intermediates that would serve as a growth substrate for the recombinant strains (Pellizari et al., 1996). To our knowledge, none of the previously constructed PCB growing strains grew on congeners with both substituted rings.

The biphenyl-degrading *C. testosteroni* strain VP44 was capable of growth on low concentrations of both 2-, and 4-monochlorobiphenyls, with accumulation of stoichiometric amounts of the corresponding chlorobenzoates in the culture supernatant and without dechlorination. In accordance, 2-CBA growing variant VP44(pE43) grew not only on 2-CB, but 4-CB as well, however in the latter case 4-CBA accumulated in the cultural medium, no dechlorination occurred and only half as much protein yielded as compared to its growth on biphenyl and 2-CB (Table 4). Vice versa, 4-CBA growing recombinant VP44(pPC3) grew on 2-CB with accumulation of 4-CBA and without dechlorination, and the protein yield accounted for about half as much as compared to its growth on biphenyl and 4-CB (Table 4). These results indicate the ability of the strain VP44 to metabolize the non-substituted pentadiene produced by the biphenyl pathway for growth. Thus, the introduction of the dehalogenation genes has resulted in recombinant organisms that are capable of complete mineralization of *ortho*-, and *para*-chlorobiphenyls.

No growth was observed when cells of VP44 were amended with chlorobiphenyls containing halogen atoms in both rings of biphenyl moiety, such as 2,2'- and 2,4'-CB, suggesting the strain does not possess a pathway for oxidation of chlorinated pentadiene. Apparently, this is a common characteristic for natural biphenyl degrading bacteria that can grow on monochlorobiphenyls via oxidation of non-chlorinated ring producing chlorobenzoate as a final product, however do not grow on PCB congeners with both chlorinated aromatic rings (Ahmed and D.D. Focht 1973, Brenner et al., 1994). One of the exceptions is *Pseudomonas* strain MB86 (Barton, R.L.Crawford, 1988) that was isolated on 4-CBA and that grew poorly on 4-CB, probably due to toxicity of 4-chloroacetophenone, which is formed as an intermediate from 4-CB. Chlorinated pentadienes presumably generated from the degradation of PCBs, based on analogy to the biphenyl pathway (Omori , Sigimura, Minoda 1986), are uncharacterized, and therefore their fate remains unknown, although it was suggested that chlorinated pentadiene can be metabolized through formation of chloroacetate followed by dehalogenation of the latter (Brenner et al., 1994). Strain VP44 did not grow on chloroacetate.

Conclusively, the *ohb* and *fcb* operons for *ortho*-, and *para*-dechlorination of chlorobenzoates, respectively, have been successfully expressed in *C. testosteroni* strain VP44. While host strain VP44 was incapable of either growth or dechlorination of chlorobenzoates, the introduced genes encoding dehalogenation of these compounds permitted both. The resulting transgenic strains were capable of growth on, and dechlorination of the respective chlorobenzoates and chlorobiphenyls. Degradation of *ortho*-chlorinated PCB congeners is especially significant given

their predominance among the products of anaerobic PCB dechlorination. Up to 80 molar % of the PCBs present following anaerobic dechlorination of Aroclor 1242 consist strictly of *ortho*-chlorinated congeners; 2-chlorobiphenyl alone may constitute as much as 40 molar % of the total PCBs in anaerobically dechlorinated sediments (Bedard and Quensen, 1995). These results were summarized in our recent paper by Hrywna et al., 1999 and demonstrated an alternative approach for the construction of PCB degrading bacteria, *i.e.* using genes encoding peripheral enzymatic activities for modification of xenobiotics into substrates for the central metabolic pathway for degradation of aromatic compounds. The introduction of specific dechlorination genes and their expression in the biphenyl-degrading bacterium *C. testosteroni* strain VP44 demonstrated the efficacy of this method for extending the substrate range for PCB degradation by biphenyl-degrading bacteria.

Developing gene transfer system for G+/G- PCB degrading bacteria

Because of inefficient rates of degradation of important PCB congeners such as 2-2', 2-4', and 2,4-2'-CBs by strain VP44, other Bph-degraders, particularly Gram-positive RHA1 and NY05, as well as the most active strain LB400 were chosen for subsequent design of PCB-growing GEMs. Gram-positive bacteria, especially *Rhodococcus* strains, offer a number of advantages for environmental use. These include higher growth yields on biphenyl, the presence of multiple PCB metabolic systems allowing co-oxidation of a wider range of PCB congeners (Masai et al., 1997; Seto et al., 1995), and more tolerance to environmental stresses such as drought or exposure to toxic compounds (Warhust, A.W.; Fewson, 199; Tsoi et al., 1991). Genetic engineering of catabolic pathways in *Rhodococcus*, however, is not well developed. We developed a broad host range vector pRT1 suitable for transfering dehalogenase gene cassettes into *Rhodococcus* as well as Gram-negative strains (Figure 13). We have constructed plasmid pRHD34 that now contains the gene cassette carrying 4-chlorobenzoate degradation *fcbABC* operon (Tsoi et al., 1991) (Figure 13). *E. coli lac*-promoter contained in the pRT1 was shown to enhance expression of the cloned *fcb* genes in both. NY05 and RHA1. We summarized these results in our recent paper (Rodrigues et al., 2001). Transformation of the *Rhodococcus* strain RHA1 by electroporation with pRHD34 enabled cells to grow on LB plates containing tetracycline (40 μg/mL). Whole cell PCR amplification with *fcb*A and *fcb*B specific primers confirmed the presence of the two catabolic genes in the transformants (Figure 13b). No PCR product was detected when the wild type RHA1 strain was used as a template, but PCR products were obtained with *E. coli* strain JM109 harboring the plasmid pCH1 used as positive control. The recombinant strain, RHA1(pRHD34), grew exponentially in medium containing 4-CBA as the sole carbon source, and released stoichiometric amounts of chloride (Figure 14). Complete disappearance of 4-CBA was confirmed by HPLC. Controls of the wild type strain did not grow in medium containing 4-CBA. The *fcb* operon in strain RHA1 appeared to be stable under non-selective conditions. All screened cells from the first cycle of growth contained both *fcb* genes and 49 out 50 colonies tested from the second cycle contained the genes. All colonies in which PCR amplification products were observed grew on plates containing 4-CBA.

Degradative capabilities of the recombinant RHA1(*fcb*).

Similarly to VP44, *Rhodococcus* strains RHA1 the *fcb* operon grew on and completely mineralized 4-CB (Figure 15). The recombinant strain RHA1 grew with 4-CB (1 mM nominal

concentration) as the only C source and released nearly stoichiometric amounts of chloride. The molar growth yield of the recombinant strain on biphenyl and 4-CB was 177 ± 6 and 189 ± 9 g dry weight of cells/mol of substrate, respectively, which is similar to the theoretical value of 173 g dry weight of cells/mol for complete 4-CB oxidation. An initial long lag-phase of approximately 50 h was followed by a sharp increase in growth to OD_{600} = 0.46. No transient formation of 4-CBA was detected during growth.

Growth on 4-CB was possible only when cells were previously grown on 4-CBA. Our failure in trying to grow recombinant cells in 4-CB after growth in biphenyl is believed to be due to the rapid turnover of 4-CB with accumulation of intermediate compounds such as HOPDA when the *bph* pathway is fully induced. Seto et al. (1996) have suggested that chlorinated HOPDA or its metabolites inhibited growth of wild type RHA1. In support of this interpretation, we observed that when our strain was inoculated into 4-CB-containing medium from biphenyl cultures, formation of HOPDA was rapid and intense, but no growth was detected. In contrast, the OD_{600} and protein concentration increased over time when inoculation was done with recombinant cultures grown in 4-CBA and only a light yellow color was seen indicating a low concentration of HOPDA. This result suggests that the RHA1 inoculum for a soil application should be grown on 4-CBA.

Resting cells of the recombinant strain RHA1(*fcb*), which were previously grown on biphenyl, completely degraded of 2-, 4-, and 2,4-CB after 24 h of incubation. No 4-CBA was detected, whereas accumulation of equimolar amounts of 2- and 2,4-CBA was observed. Wild-type RHA1 accumulated stoichiometric quantities of 4-CBA from 4-CB. Complete utilization of 4-CBA occurred in all treatments in which 4-CB was degraded, suggesting activity of the enzymes encoded by the *fcb* operon in the recombinant strain.

Because the wild type strain RHA1 could degrade the major products from pattern M anaerobic dechlorination with a theoretical recovery of 50% of *para*-chlorobenzoates (Maltseva et al., 2000), the eight PCB congeners that most accumulated in this pattern were used to test the activity of the recombinant strain. Similar degradation rates for mixture M congeners by the wild type RHA1 and its recombinant were obtained (Figure 16). Sixty percent of PCBs present in the mixture M were degraded by the wild-type strain with accumulation of 2-, 4- and 2,4-CBAs. In contrast, strain RHA1 containing the *fcb* genes accumulated only trace concentrations of 4-CBA. HOPDA concentrations were 38% less for the recombinant strain. Final concentrations of 2- and 2,4-CBA were similar for both wild type and transformant strain, with no utilization of these metabolites in either case.

Survival and activity of GEM RHA1(*fcb*) in non-sterile soil

To determine the stability of this construct, survivability and degradative activity of GEMs in soil using model system of RHA1(*fcb*), microcosm experiments were conducted. Soil samples were obtained from a non-contaminated area adjacent to PCB contaminated soils at Picatinny Arsenal, New Jersey (28 miles northwest of New York City). The soil, a loamy sand (84.6 % sand, 12.1 % silt, and 3.4 % clay) with 3.5 % organic matter, pH 7.6, was passed through a 4-mm mesh sieve and stored at 4 °C until used. Total soil bacterial counts before inoculation were performed by staining with 5-(4,6-dichlorotriazine-2-yl) aminofluoroscein (DTAF) followed by epifluorescence microscopy (Bloem 1995). Naturally occurring rifampicin (Rif^+) mutants from

the recombinant RHA1 were isolated from K1 medium amended with biphenyl (Smith, Tiedje, 1980).

Rif-resistant recombinant cells grown on 4-CBA-containing medium were washed twice with 50 mM phosphate buffer (pH 7.0), resuspended in K1 medium, diluted, and added to 20 g of Picatinny soil previously amended with 100 ppm of 4-CB, giving a density of 10^4 cells/g of soil (Figure 17). Sterile treatment was obtained by autoclaving soil for 1 h during 3 consecutive days. A non-contaminated soil control was inoculated at the same cell density. Soil was brought to 30% moisture content with sterile K1 medium, mixed well, and incubated in triplicate at 30 °C. Cells were recovered from soil by vortexing 1 g of soil with phosphate buffer (9 mL) for 10 min. Appropriate dilutions were spread on LB plates containing rifampicin (50 μg/mL). Colony forming units (CFUs) were counted after one week of incubation. Fifty randomly selected colonies from each sampling time were used for PCR with the *fcb*B specific primers and plated on K1 medium containing 4-CBA or biphenyl as the only C source.

Total bacterial counts of the soil before inoculation averaged to $9.9 \cdot 10^8$. Since we could not distinguish our strain by color and colony morphology in comparison to the indigenous bacteria on mineral medium amended with 4-CBA or biphenyl, we selected a rifampicin-resistant mutant for tracking our recombinant strain. No indigenous bacteria grew on this medium. Recombinant cells grew in both sterile and non-sterile soil concurrent with 4-CB removal (Figure 18), but did not grow in the non-contaminated, non-sterile control soil. Most significant, however, was the growth of the recombinant strain in the inoculated non-sterile 4-CB contaminated soil and 4-CB removal. Furthermore, analysis of the growth stoichiometry in the inoculated non-sterile soil is consistent with the yield expected from growth on PCBs. The initial growth (0-2 days) may have been partially due to carry over of endogenous reserves of the cells but the growth from day 2 to 10 ($2 \cdot 10^5 \pm 5051$ to $6 \cdot 10^5 \pm 3464$ cells/g), which was 67 % of the 10 day total growth, corresponds to the cell yield of 22.7 μg dry weight of cells/g. Using the molar growth yield, the 22.6-ppm of PCB removed between days 2 and 10 should have produced 25.3 μg of cells on a dry weight basis, similar to the 22.7 μg of dry weight of cells found. Additional growth would have been expected after 10 day from the remaining PCB consumed but this was not observed. This could be due to the restoration of endogenous reserves, oxidation of some intermediates by other populations, or loss of cells to grazing by soil protozoa. The non-inoculated soil showed no removal of PCB providing further evidence that the inoculated strain was responsible for PCB degradation.

Since the 4-CBA degradation pathway is not expected to be under constant selection, information on the stability of degradation in the absence of any selective factor is important for environmental applications. The *fcb* operon appeared stable in both the sterile and non-sterile soil since during 60 days only one of the 700 colonies examined did not yield a *fcb*B amplicon (Figure 18) (Rodrigues et al., 2001). Furthermore, the same isolated colonies grew on solid medium containing 4-CBA as the only carbon source. All colonies also grew on plates containing biphenyl as the C source. The long-term effect of carrying exogenous DNA sequences or expression of new pathways on the fitness of the recombinant strain was not studied (Lenski et al., 1994), however, the period in which aerobic PCB treatment is needed in the field would not normally be much lengthier.

Developing and Testing Molecular Tracking Recombinant Organisms In Situ

The success of any bioremediation process depends upon establishing favorable conditions for microorganisms to degrade the contaminant. Failures in bioremediation more often result from a lack of knowledge about ecological constraints than from the information about the genetic capabilities of the organism. Good data on population dynamics in bioremediation is important for providing insights into better methods of inoculation, maintenance, and management of contaminated sites.

Molecular markers used for detection of microorganisms include antibiotic resistance genes (Burlage et al., 1990), transposons (Recorbe et al., 1992), engineered catabolic genes (Hwan, Farrand, 1997), bacterial luciferase (*luxAB* genes) (Burlage et al., 1990) and green fluorescent protein (GFP) encoding genes (Bloemberg et al., 1997). However, addition or alteration of genetic traits may have deleterious effects on the environmental fitness of microorganisms (Lenski., 1991). Other molecular tracking methods such as amplified ribosomal DNA restriction analysis (ARDRA) (Nüsslein, Tiedje. 1998), single-strand conformation polymorphism (SSCP) (Lee et al., 1996; Schwieger, Tebbe, 1998), denaturing gradient gel electrophoresis (DGGE) (Recorbe et al., 1992; Muyzer et al., 1993), and terminal restriction fragment length polymorphism (T-RFLP) (Muyzer et al., 1998) lack a quantitative component. *In situ* hybridization with fluorescently-labeled oligonucleotide probes (FISH) targeted to the rRNA sequences provides for quantitation and has proven very useful especially in aqueous environments (Delong et al., 1980) but it is labor intensive, does not offer species to strain level specificity and it has been more difficult to use in soil.

Real time PCR is a promising tool for the detection and quantification of microorganisms. This may be especially useful for monitoring bioreactors utilized in the clean up of contaminated soils and sediments. Real time PCR (RTm-PCR) was originally developed for estimating the number of copies of a targeted gene (Dölken et al., 1998; Heid et al., 1996; Higuchi et al., 1993). In this method, a double-labeled probe is used to measure the accumulation of fluorescence of a released reporter dye during PCR. This fluorescence is then correlated to the amount of product formed in real time during amplification when PCR is more quantitative (Higuchi et al., 1993). The method has been used clinically for quantitation of *Listeria monocytogenes* (Bassler et al., 1995), *Yersinia pestis* (Higgins et al., 1998), *Mycobacterium tuberculosis* (Desjardin et al., 1998), and *Borrelia burgdorferi* (Pahl et al., 1999), and for a functional gene of ecological importance, denitrifying nitrite reductase (Grüntzig et al., 2001). We applied this technique to study the population dynamics in soil of a *Rhodococcus* strain genetically engineered to grow on a chlorobiphenyl by quantifying both the 16S rRNA gene and the engineered 4-chlorobenzoate (*fcb*) degradation operon (Rodrigues et al., 2001).

Probe sequences for RTm-PCR were designed using the Primer Express software (Perkin Elmer Applied Biosystems, Foster City, CA). The probe contained FAM (6-carboxy-fluorescein) as a reporter fluorochrome on the 5' end and TAMRA (*N,N,N',N'*- tetramethyl-6-carboxy-rhodamine) as quencher on the 3' end of the nucleotide sequence. Species specific 16S rRNA regions were identified for *Rhodococcus* sp. strain RHA1, using the phylogeny of these strain and close relatives. All candidate primers included more than one species of the genus *Rhodococcus*,

because of the inherent difficulty in designing a 16S rRNA-based strain-specific probe. The RHA1(*fcb*) 16S rRNA gene sequence correspondent to *E. coli* positions 599-619 was chosen for designing the Taqman-16S rDNA probe. Our choice was based on the presence of only two other perfect matches for this primer, *R. marinonascens* and *R. globerulus*. Because of the internal probe design requirements, the S-*- Rco-0599-b-A-21 primer had to be extended to 29 nucleotides. To test the specificity of the newly designed probe, RTm-PCR was performed against genomic DNA isolated from different *Rhodococcus* species and *E. coli*. Fluorescence during PCR amplification was observed for the strain RHA1(*fcb*), *R. globerulus*, *R. marinonascens*, *R. opacus*, and *R. percolatus*. Primers and a suitable probe targeting the *fcbB* gene were similarly designed. 4-CBA degradation operon (*fcb*) isolated from *Arthrobacter globiformis* was introduced into the strain RHA1, allowing the recombinant strain to grow in medium containing 4-CBA. The *fcbB* gene codes for a 4-chlorobenzoate dehalogenase and was chosen due to its importance during PCB mineralization. When the Taqman probe specific for the *fcb*B gene was used against total DNA from the same closely related species, quantitative amplification of the sequence was possible only for the strain RHA1(*fcb*) (Figure 19). The sensitivity of the 16S rDNA and *fcb* probe and primer sets for RTm-PCR was evaluated with 1:10 serial dilutions (from 10^2 to 10^7 copies) of the RHA1(*fcb*) genome and the *fcb*-containing plasmid pRHD34, respectively. Standard curves were linear over six orders of magnitude for both target genes. The same standard curve was obtained for the RHA1(*fcb*)-16S rRNA gene-containing plasmid, pRHA16S, after correction for ribosomal RNA operon copy number. Strain RHA1(*fcb*) was found to have four copies of the 16S rRNA gene per genome. This correction was taken into account for evaluating experimental data.

To test whether the recombinant strain RHA1(*fcb*) could be detected in a background of soil community DNA, soil samples were amended with a known number of cells. Soil samples were obtained from a non-contaminated area near a PCB contaminated site at Picatinny Arsenal, New Jersey. The soil, classified as loamy sand (84.6 % sand, 12.1 % silt, and 3.4 % clay), 3.5 % organic matter, pH 7.6, was sieved through a 4-mm mesh and stored at 4 °C until used. PCBs representing the products of anaerobic Aroclor 1242 dechlorination (mix M 28) were dissolved in acetone (75 ppm) and added to soil. Rifampicin resistant RHA1(*fcb*) cells were grown on 3 mM 4-CBA, washed twice with 50 mM phosphate buffer (pH 7.0), resuspended in K1 medium, and added in different 10-fold dilutions (1 ml) into 5 g of soil. Soil was brought to 30% water content with sterile K1 medium, mixed well, and incubated in triplicate at 30 °C. Total soil DNA was extracted with the Soil DNA Extraction Kit (MoBio Laboratories, Inc., Solana Beach, CA) over a 30-day incubation period. DNA samples were stored at -20 °C until use. For plate counts, cells were recovered from soil microcosms by vortexing 1 g of soil in 9 mL of phosphate buffer for 10 min. Serial dilutions were spread on triplicate LB plates containing rifampicin (50 μg/mL). CFUs were determined after one week of incubation.

RTm-PCR with total DNA extracted from non-inoculated soil did not result in fluorescence increase above the threshold value, indicating that the sensitivity of the probe to the native *Rhodococcus* population was below the detection limit. In soils amended with RHA1(*fcb*) cells, a linear relationship between isolated CFUs per gram of soil and C_t values was observed for both probes, but the sensitivity was decreased by 1.1 and 1.3 orders of magnitude for the *fcb*- and 16S rDNA- probes, respectively, when compared to pure culture standard curve.

TaqMan probes were also tested with PCB contaminated soil inoculated with strain RHA(*fcb*) and incubated for 30 days. The estimated numbers of RHA1(*fcb*) cells per g of soil measured by the two TaqMan probes were similar to the values obtained for culturable rifampicin-resistant RHA1(*fcb*) cells (CFUs g^{-1} soil) throughout the growth cycle (Figure 20). Growth was expected on the particular congeners in the PCB mixture, and the decline may have been due to protozoan grazing although this was not investigated. The method did allow measurement of population dynamics in soil and an assessment of the stability of the engineered gene.

Different factors affecting detection limits in PCR-based methods include: the type and composition of the matrix, the type of target organism, the number and diversity of bacteria in the sample, the *Taq* polymerase used, and the DNA extraction protocol (Löffler et al., 2000). Our experiments suggest that the target sequence can also be an important factor in detection as seen by the different standard curves for *fcb*-targeted gene versus the 16S rRNA-targeted gene. One explanation for different sensitivities might be the size of the strain RHA1 genome (3.0 Mb) (Tonso, 1997) in comparison to the plasmid pRHD34 size (14.4 Kb), limiting the ability of the TaqMan-16S rDNA probe from finding its target. However, chromosomal and plasmid targets of RHA1's 16S rRNA gene gave the same C_t. The lower C_t values observed when the TaqMan-*fcb* probe was used is probably due to the probe efficiency and not the difficulty of genome strand separation when targeting the 16S rRNA genes. Penalty score analysis for the two TaqMan probes indicated differences between the two probes; values of 250 for the TaqMan-16S rDNA and 49 for TaqMan-*fcb* (Perkin Elmer Applied Biosystems. 1998), suggesting that the probe targeting the *fcb* operon met most of the criteria required for RTm-PCR, while the other did not. Use of rDNA phylogenetic probes is hampered by a limited number of specific regions within the hypervariable sequence of the 16S rRNA gene (Stackebrandt, Rainey, 1995). Thus, the TaqMan-16S probe design had to be constructed from a pre-determined position, while the TaqMan-*fcb* probe was chosen from many different possibilities within the entire *fcb* operon.

Conclusively, a real time PCR assay using fluorescently labeled oligonucleotides (TaqMan probes) was developed and tested on model system of the recombinant *Rhodococcus* sp. strain RHA1(*fcb*) in soil. One primer and probe set targeted the hypervariable region of the 16S rRNA gene and the other set targeted the recombinant 4-chlorobenzoate degradation (*fcb*) operon. The 16S rDNA probe detected RHA1(*fcb*) and phylogenetically related species while the *fcb* probe was specific for the recombinant strain. The method had a 6-log dynamic range of detection (10^2 to 10^7) for both probes when cultures were used. Although the sensitivity of the method was less in soil, the estimated number of cells by real time PCR corresponded to the measured number of RHA1(*fcb*) cells determined by colony forming units. The real time PCR is easy to perform, has high throughput, and was reliable for enumerating strain RHA1(*fcb*) in non-sterile soil.

Construction of multiple *ortho*-PCB dechlorinator LB400(*ohb*)

To design organisms capable of growing on the most environmentally important *ortho*-chlorinated PCB species, we have recruited the *P. aeruginosa* strain 142 oxygenolytic *ohb*-operon for *ortho*-dechlorination of mono-, di-, and trichlorobenzoates. Plasmid pRO41 contains ohbABCR genes coding for the ISP_{OHB}, potential ABC transporter and putative transcriptional regulator, respectively, as described in our recent paper (Tsoi et al., 1999). Plasmid pOCC1 in addition contains the *clc* genes for chlorocatechol pathway from D-plasmid pAC27 (Ghosal et al., 1987) under control of *lac*-promoter (Figure 21). The plasmids may conveniently be used for

transformation of laboratory or indigenous biphenyl-degrading organisms, which customarily belong to either Rhodococcus genus or a variety of gram-negative bacteria. Addition of the *clc* genes presumably would allow recombinant PCB degraders to grow on congeners that contain di- or trichlorinated rings (yielding respective di- or trichlorobenzoates that would be *ortho*-dechlorinated by the *ohb* genes to the respective mono- or dichlorocatechols).

Plasmids pRO41 and pOCC1 were introduced in 4 different biphenyl degrading strains, VP44 and LB400 (gram negative), and *Rhodococcus* NY05 and RHA1. Recipient VP44 served as a control since we reported previously expression of the *ohb* genes in this host (Hrywna et al., 1999). Controls were the same recipients transformed with vector pRT1. After 8 (VP44) to 12 (LB400 and NY05) weeks of incubation well grown single colonies (a few per each plating) were seen on 2-CBA in case of plasmids pRO41 and pOCC1. No growth was detected for pRT1. No growth was detected for pOCC1 on diCBAs. No growth was detected so far in case of *Rhodococcus* RHA1. From each original transformant, a few randomly chosen colonies grown on 2-CBA were serially transferred to 2-CBA and Bph and shown to fully grow on 2 mM 2-CBA in 2 to 5 days depending on recipient (fastest – VP44, slowest – NY05). The best growing GEM was LB400(pRO41) which was used in further studies.

In our previous experiments resting cells of LB400 partially oxidized 2-CBA. This correlated with less than expected yield of 2-CBA from oxidation of 2-chlorobiphenyl and 2-2'-CB. However, control strain LB400(pRT1) didn't grow on 2-CBA, thus implicating co-metabolic nature of the 2-CBA oxidation by LB400. Recombinant LB400(pRO41) (Figure 22) efficiently grew on 2-CBA at concentrations of up to at least 3 mM, with stoichiometric release of Cl-.

It appears that many biphenyl degraders can grow on at least 4-CB from oxidation of non-chlorinated ring (pentadiene). Some of them can also grow on low concentrations of 2-CB (Hrywna et al., 1999). Control strain LB400(pRT1) was grown on Bph and inoculated in K1 medium with 1 mM of 2-CB. LB400(pRT1) grew on 1 mM 2-CB, accumulating 2-CBA and Cl^- in culture liquid. The residual 2-CBA and Cl- total to approximately 1.2 mM, as measured, indicating complete consumption of 2-CB. Again, less than stoichiometric production of 2-CBA indicated its partial oxidation. The recombinant LB400(pRO41) was grown on Bph and inoculated in 1 mM 2-CB (Figure 23). The GEM completely utilized both 2CB and 2CBA resulting in production of higher biomass (OD). Estimated 1.2 mM of Cl^- were released confirming complete mineralization of 2-CB.

It should be noted that Haddock et al. (1995) purified biphenyl dioxygenase from LB400 and showed that 97% of 2-CB is dihydroxylated at 2,3 positions of non-chlorinated ring, and only 3% was dihydroxylated/dehalogenated in 2,3-positions of chlorinated ring. Therefore, most of the 0.7 mM of Cl^- released by control strain LB400(pRT1) must have come from dechlorination of 2-CBA. No one apparently tested the bph-dioxygenase (enzyme or genes) for activity on 2-CBA. In conjunction with data by Bedard et al. (1986) on co-oxidation of 2-CBA (without growth), it could be that the BDO from LB400 does have 2,3-dihydroxylation/dehalogenation activity on both 2-CBA and 3-CBA (unless LB400 possesses another chlorobenzoate dehalogenating enzyme). In this case, no catechol would be produced that is in accordance with absence of growth on 2- and 3-CBA. To verify this, we PCR amplified and cloned the BDO genes in *E. coli*. Dechlorination test indicated that the bphA1A2A3A4B genes were responsible for partial oxidation of 2-CBA.

Strain LB400 is one of only few organisms known to efficiently oxidize an environmentally important 2-2'-CB. Haddock et al. (1995; 2000) showed with purified BDO that the initial dihydroxylation of 2-2'-CB occurs in 2,3-positions with release of chlorine. Consequently, the dechlorinated ring is further oxidized to pentadiene, which can be consumed for growth, while second ring converts to 2CBA. The recombinant strain LB400(pRO41) inoculated in medium K1 with estimated mM 2-2'-CB released estimated 2.2 mM of Cl^-, as measured, implicating complete mineralization of 2-2'-CB. Therefore, simultaneous expression of two *ortho*-dehalogenating dioxygenases, BDO and ISP_{OHB} resulted in complete mineralization of 2-2'-CB. In addition, LB400 grew on 3CBA due to the indigenous chlorocatechol pathway. Concequently, LB400(ohb) efficiently grew on 2,3-, and 2,5-dichloribenzoates, and on 2,4-, and 2,6-dCBAs, although the latter two were poor growth substrates.

We showed that efficiency of degradation of 2-CB (top) and 2-2'-CB by recombinant LB400(pRO41) was dependent on preparation of inoculum. While complete mineralization of a simple congener like 2-CB was not affected by whether the inoculum was grown on Bph or 2-CBA, a dramatic difference was found in the case of higher chlorinated 2-2'-CB. In the latter case, complete mineralization was achieved only with inoculum grown on 2-CBA, whereas only partial degradation was observed with Bph-grown inoculum. Indeed, Bph-initiated culture of LB400(pRO41) behaved similar to control strain LB400(pRT1), except that no 2-CBA accumulated in the former indicating that the incomplete mineralization could not be attributed to plasmid instability (loss of the ohb genes). Appears that initial ratio of participating dioxygenases (BDO and ISP_{OHB}) is crucial for difficult substrates when inhibitory effects of reaction products are suspected (such as the case of 2-2'-CB). Consequently, LB400(*ohb*) inocula for bioaugmentation of PCB contaminated soils should be grown prior delivery on CBA.

Growth on defined PCB mixtures

Control strain LB400(pRT1) was grown on Bph and inoculated in K1 medium with 1 mM Mix C. Mix C represents best scenario that would be seen in anaerobically dechlorinated sediments initially contaminated with Aroclor 1242, 1248 and perhaps 1256. There was no previous reports on growth of LB400 on any artificial mixes or naturally occurring dechlorination products. As shown in Figure 24, the control strain grew on the Mix C, and the sum of released Cl-, 2-CBA, 4-CBA and 2,4dCBA totals to approx. 1.4 mM of Cl, compared to theoretically expected 1.5 mM. Out of total 1.5 mM chlorine, 0.65 mM was released as Cl- and 0.7 mM accumulated as 2-CBA in accordance with growth of the LB400(pRT1) on individual 2- and 2-2'-CBs, and with only partial consumption of 2-2'-CB. Contrastingly, recombinant LB400(pRO41) released 1.5 mM of Cl- plus negligible amounts of 4- and 2,4-CBA and chlorocatechol, indicating nearly total mineralization. Further flask experiments were done to characterize growth and degradation of products expected from anaerobic phase of Aroclor remediation. In these experiments, the inoculum of GEM LB400(*ohb*) was prepared on 2,5dCBA, while control strain LB400 was inoculated from Bph-grown culture. Individual flasks in triplicates represented time points. Production of biomass (OD), degradation of PCBs (GC), accumulation of CBAs (HPLC), and release of Cl- were measured. The recombinant LB400(*ohb*) grew efficiently on both lighter Mic C (Figure 24) and heavier Mic M. Importantly, we observed significantly higher biomass production and Cl- release, greatly diminished accumulation of CBAs, and potentially toxic intermediates such as Cl-HOPDAs by the recombinant LB400(ohb), as well as it sustained the

exceptionally high rates of PCB consumption characteristic for the wild type host LB400. These results implicated the GEM superior to its wild type parent for bioremediation purposes.

Validation of PCB remediation strategy in soil (microcosm studies)

In preparation for field test we have conducted series of laboratory experiments both in flasks and in soil microcosms to validate applicability of the proposed two-phase anaerobic-aerobic PCB remediation, and determine which GEMs are most effective. Based on growth in flasks on defined PCB mixtures M and C, on Aroclor toxicity testing and on survival of different GEMs in soil microcosm experiments, we have concluded that using a combination of two GEMs, the longer surviving Gram positive *Rhodococcus* RHA1(*fcb*) and the more active and PCB tolerant Gram negative *Burkholderia* LB400(*ohb*) were the most effective for achieving maximum PCB degradation.

Sediment samples were obtained from the Red Cedar River (Michigan) and contaminated with Arochlor 1242. The sediment was inoculated with anaerobic dechlorinating microorganisms eluted from the River Raisin sediment and submitted to anaerobic conditions for a period of one year. The anaerobic treatment was followed by inoculation with the aerobic GEMs, rifampicin-resistant recombinants RHA1(*fcb*) and LB400(*ohb*). The aerobic inoculums were grown on 3 mM (nominal concentration) medium containing biphenyl and 2,5dCBA, respectively. Cells were added to 1 g of contaminated sediment (50% solids microreactors) to give a density of 10^4 (low density treatment) or 10^6 (high density treatment) cells x g^{-1} of sediment for each recombinant strain. Non-contaminated sediment as well as non-inoculated contaminated sediment submitted to same conditions were used as controls. Flasks were continually shaken at 150 rpm and incubated in duplicates at 30°C for a period of 30 days. Sediment samples were stored at – 20 °C for soil DNA and PCB extractions (Figure 25). Samples were taken periodically, and immediately diluted.

Population dynamics of recombinant RHA1(*fcb*) and LB400(*ohb*) strains were tracked by plating on rifampicin-containing Luria-agar plates. Direct bacterial counts of the sediment before inoculation averaged to 4.82×10^8 cells g^{-1} sediment. No indigenous sediment bacteria could be isolated in rifampicin-containing medium bellow our limit of detection (10^2 cells g^{-1} soil). We were able to distinguish and count the recombinant *Rhodococcus* sp. strain RHA1(*fcb*) from the recombinant *Burkholderia cepacia* strain LB400(*ohb*) because of their distinct morphological characteristics on plates (Figure 26). Bacterial counts increased for both high and low density inoculation treatments (Figure 27).

In the high inoculation treatment, RHA(*fcb*) cell number increased to 7.8×10^6 rifampicin resistan cells g^{1-} sediment while LB400(*ohb*) cell number increased to 1.4×10^7 cells g^{-1} sediment. We found approximately 3.8×10^6 and 6.2×10^6 cells g-1 sediment for RHA1(*fcb*)and LB400(*ohb*), respectively, in the low density treatment at the day 15. No LB400(*ohb*) colonies could be detected on agar media in the non-contaminated treatment, while RHA1(*fcb*) colonies increased from 1.0×10^4 to 2.2×10^5 cells g^{-1} soil. Real time PCR results correlated well with plate count data. The calculated CFUs g^{-1} of sediment for RHA1(*fcb*) using the TaqMan-*fcb* and –16S rDNA probes were 5.3×10^6 and 1.1×10^7 cells g^{-1} of sediment, respectively, at the day 15. Sediment samples taken from non-inoculated control did not yield any increment in fluorescence

above the threshold line. The *fcb* and *ohb* operons in the strains RHA1 and LB400, respectively, appeared to be stable, as verified by PCR amplification with the *ohb* and *fcb* specific primers.

A similar pattern of Aroclor 1242 degradation was observed for high and low inoculation density treatments (Figure 27) by the end or the experiment. After 30 days, PCB removals for high and low inoculation densities were 57% and 54% after aerobic treatment, respectively. Only a small amount of PCBs (4%) were degraded in the non-inoculated treatment after a 30 day period, evidencing that the inoculated recombinant strains were responsible for the degradation of anaerobic products of Aroclor 1242 dechlorination.

It should be noted that degradation of PCBs in these 50% solid microreactors was significantly lower as compared to results of flask growth experiments. To investigate whether these rates were affected by water content, we have also conducted similar test in 20% microslurries. The slurries were aerated at room temperature for 15 days and sacrificed for PCB extraction and analyses. Under these conditions, the Aroclor 1242 dechlorinated products (60 ppm) were degraded to a final concentration of 16 ppm.

Conclusively, using a model system of River Raisin sediment historically contaminated with Aroclor 1242, we validated the proposed two-phase anaerobic-aerobic PCB bioremediation scheme. In these experiments, enhanced anaerobic PCB dechlorination was achieved using Hudson River inoculum and resulted in a shift in the congener profile from highly to lower chlorinated PCB congeners. The anaerobic treatment was followed by inoculation with aerobic PCB-growing GEMs. This resulted in 78% removal of PCBs. While the aerobic incubation caused a slight degree of intrinsic PCB degradation, inoculation with the PCB-growing GEMs was essential to achieve efficient PCB removal. In these experiments, use of surfactant Tergitol was not beneficial.

Developing protocol for inoculum delivery

In order to find out if we could enhance the delivery of inoculum to the soil we compared to methods of inoculation: addition of cells directly to the soil and addition of cells to vermiculite which was then added to the soil. We chose vermiculite over other possible inoculum carriers such as rice hulls, perlite and calcium alginate beads because vermiculite is inexpensive, readily available and graded for size uniformity.

In our trials two cell densities were used, a low density of ~ $10^{\wedge 4}$ cells/gram, and a high density of ~ $10^{\wedge 6}$ cells/gram. Rifampicin resistant cells were grown in liquid K1 mineral salts media with 20mM Biphenyl then transferred to K1 with 2mM of 2-CBA (LB400(*ohb*)) or 4-CBA (RHA1(*fcb*)) to log phase. Cells were then added to fine grade vermiculite. We used vermiculite at a rate of 3% w:w, vermiculite to soil. We diluted our inoculum with nonsterile tap water so that we added a total volume of 1ml dilute inoculum to 1ml of vermiculite. We homogenized the inoculum/vermiculite mixture and then added it to soil. In the nonvermiculite treatment we diluted the inoculum to a volume that would adjust the soil water potential to –0.5 bars. This volume is dependent on the water potential of the soil and must be adjusted for each new case.

Prior to the addition of the inoculum 2-chlorobiphenyl (2CB) or 4-chlorobiphenyl (4CB) were added from a 1M stock to 4ml of acetone which was dribbled into the soil with constant stirring.

The soil was then vigorously mixed in a sealed metal container for 30 minutes and allowed to stand overnight at room temperature for volatilization of acetone from the soil. Two grams of inoculated, chlorobiphenyl contaminated soil was dispensed into each of 24 10 ml serum vials (Fisher) per treatment, and these were then sealed with Teflon coated rubber septum and crimp top. Samples were taken at random, in triplicate over the course of the experiment. Microcosms were incubated at 25°C and sampled on days 0, 2, 5, 10, 15, 20, 30, and 60. Sampling was done by injecting 5ml of 10mM potassium phosphate buffer (pH 7) into each vial. The six vials were vortexed horizontally at one time for 10 minutes at the maximum speed and let stand for 3 minutes to allow sedimentation of soil particles from the water column. Serial dilutions were prepared and spread onto L-agar plates containing rifampicin. Immediately following cell extraction the microcosms were frozen at –20 °C. PCBs were then extracted from these same vials and analyzed using a GC.

Survival of *Rhodococcus* sp. Strain RHA1 *fcb* and *Burkholderia cepacia* strain LB400 *ohb* was greatly improved when vermiculite was used as an inoculum carrier. Growth did occur in the presence of chlorobiphenyl with and without vermiculite. However, the growth was an order of magnitude greater when vermiculite was used as a carrier.

Recommendations for inoculum delivery during pilot test

Recommendations for inoculum delivery during pilot test:

1) Inoculation Density
    a. Organisms will be inoculated at 10^6 cells/gram.
    b. Table 5 Shows the volume of cells grown to an optical density of 0.5 and at 0.8 required to inoculate a volume of soil at the above cell densities
2) Vermiculite
    a. Use 3g vermiculite/100g of soil (for slurry use weight of soil + water) (3% w/w)
    b. Use a medium to fine grade vermiculite for greater surface to volume (mass) ratio
3) Vermiculite Impregnation
    a. Cell Inoculum is diluted to bring the total water content of the vermiculite to a 1ml to 1 gram ratio.
    b. Diluted cells are mixed into vermiculite immediately after they are harvested until homogenous
4) Addition of Impregnated Vermiculite to Soil
    a. Impregnated Vermiculite is added to soil immediately following addition of cells to vermiculite
    b. Homogeneity of Vermiculite and Soil in high solid (land farming), and medium solid treatments must be reached

We are currently in the process of determining the most practical approach for the transportation of the recombinant organisms to WES for the ongoing pilot-test. We are experimenting with room temperature and refrigerated cell concentrates.

Compatibility of anaerobic and aerobic phases in remediation process

In the anaerobic/aerobic bioremediation system to be employed, $FeSO_4$ will be added to the soil to enhance anaerobic reductive dechlorination. In order to find out if residual $FeSO_4$ and or the resulting FeS from this process will have a negative influence on the efficiency of aerobic metabolism we grew both organisms in the presence of these Fe(II) compounds (Figure 28). *Burkholderia cepacia* strain LB400 (*ohb*) rif+ and *Rhodococcus* sp. strain RHA1 (*fcb*) rif+ were grown to log phase in K1 + 2-CBA or 4-CBA respectively or K1 + bph. $10^{\wedge 4}$ cell/ml were added to K1 media with 2-CBA or 4-CBA, or 2mM 2-chlorobiphenyl (2CB) or 2mM 4-chlorobiphenyl (4CB) (CB studies are in progress) with either 20mM $FeSO_4$, 10mM $FeSO_4$ + 10mM FeS, 10mM FeS or an Fe-free control. Cell density was measured with plate counts on Luria agar (L-agar). The pH of each treatment was adjusted to ~ 7. The results of our studies using CBAs as growth substrates indicate that $FeSO_4$ and or FeS do not appear to adversely affect either organism. Given our results the anaerobic stage $FeSO_4$ treatments used to stimulate anaerobic dechlorination should not affect the activities of either aerobic organism in soil.

Microbial-Surfactant Compatibility Experiments

Microbial growth experiments were conducted to determine the effect of surfactants on two biphenyl-degrading strains of bacteria, gram-positive *Rhodococcus erythreus* strain NY05 and gram-negative *Comamonas testosteroni* strain VP44. Three nonionic surfactants were selected based on solubilization potential and susceptibility to microbial degradation. Of the three surfactants, the linear alcohol ethoxylate, Witconol SN-120, completely inhibited microbial growth of both bacterial strains. In contrast, both microorganisms achieved substantial growth in solutions containing the ethoxylated sorbitan monooleate, Tween 80, as the sole carbon source, as well as in solutions containing Tween 80 with either biphenyl or the monochlorinated biphenyl, 4-CBP. The third surfactant, Tergitol NP-15, a nonylphenol ethoxylate, did not inhibit microbial growth when present in solutions containing either biphenyl (BP) or 4-CBP, and Tergitol NP-15 did not support microbial growth when present in solution as the sole carbon source. Examples of representative NYO5 and VP44 growth curves in the presence of Tween 80 and 4-CBP are shown in Figure 29. A summary of the results from the growth experiments for both microbial strains is presented in Table 5.

Plasmid stability studies

During the past six months a matrix of experiment was performed to ascertain whether microorganisms engineered to grow on a particular PCB congener or pathway would lose that ability if allowed to grow on other substrates, including surfactants. Initially, solutions of 4-chlorobenzoic acid (4-CBA) and 2-chlorobenzoic acid (2-CBA) were prepared by dissolving each acid in 1 N KOH. The pH of the solution was brought to below 7.0 by addition of 2 N H_2SO_4. A growth flask was then prepared with approximately 100 ml of K1 nutrient solution, and 2-CBA or 4-CBA was added to reach a concentration of approximately 2 mM. The flasks were inoculated with engineered strains of RHA1, NY05, and VP44 provided by MSU, to which 2 ml of 100 mM CBA solution were added each day. Once the solution in the flask reached an

optical density of 1.0 or greater, 10 ml of the solution were used to inoculate a second flask containing the same K1 and CBA solution and the process was repeated.

After the second flask reached an optical density of 1.0, the solution was centrifuged and the supernatent was discarded. The biomass was resuspended in K1 and centrifuged again. This process was repeated two more times in an attempt to remove all of the CBA from solution. After the final centrifugation, the biomass was resuspended to a concentration of 0.2 g biomass per ml. This solution was equally divided and used to inoculate three growth flasks, the first containing a solution of K1 and biphenyl, the second containing a solution of Tergitol NP-15 and biphenyl, and the third containing a solution of Tween 80. A sequential plating method was used with five plates being produced for each instance with the hope that the fifth plate would produce individual colonies. These plates were labeled, sealed with parafilm and forwarded to MSU for analysis. A summary of the PCR analysis for RHA1+fcb is given in Table 6. Notice that under all of the growth conditions tested, the plasmid gene was detected by PCR analysis.

PCB-surfactant solubilization experiments

Surfactants are commonly known to enhance the apparent solubility of hydrophobic organic compounds, such as chlorinated biphenyls, at concentrations above the surfactant critical micelle concentration (CMC). The monochlorinated biphenyl, 4-CB, has an aqueous solubility of 2 ppm (0.011 mM). The solubilization capacities of two surfactants, Tween 80 and Tergitol NP-15, with respect to 4-CB, were determined from batch solubility experiments conducted in 26-mL glass serum tubes. The lower portions of the tubes were initially coated with 4-CB that was transferred in a solution of highly volatile hexane. Surfactant concentrations were prepared within the range of 50 to >2000 ppm and were added to the serum tubes, which were placed on an orbital shaker at 150 rpm for two to three weeks. The solubility experiments were conducted at 22° C (room temperature) and 30° C (temperature of microbial growth studies).

After two to three weeks, 0.4-mL samples were taken from the serum tubes and mixed with 1.2 mL of isopropanol in chromatography vials, which were analyzed through gas chromatography with an electron capture detector (GC/ECD). Alternatively, for lower concentrations of 4-CB, samples from the serum tubes were transferred as 10 to 15-mL aliquots to new serum tubes to which hexane were added in small volumes of 3 to 5 mL. These serum tubes were centrifuged at 3000 rpm to extract 4-CB from the aqueous solution into the hexane, which was transferred to chromatography vials for GC/ECD analysis.

The solubilization capacity of a surfactant, for a given compound, can be described as molar solubilization ratio (MSR), which expresses the mass of 4-CB solubilized per mass of surfactant, in molar terms. Results from solubility experiments, for both surfactants, are presented in Figure 30.

Mathematical modeling

In order to predict the effect of surfactant additions on the distribution of PCB congeners in a solid-liquid system, it is necessary to account for the potential impact of sorbed-phase and micellar surfactant on PCB bioavailability. Surfactant micelles will act to increase the amount of PCB in solution, however, sorbed-phase surfactant may also increase partitioning of the PCB to the solid phase. This effect will be a function of the surfactant critical micelle concentration

(CMC), the soil sorptive capacity (Sm), and the partitioning of the PCB congener among the aqueous, micellar and solid phases. Conceptually, the overall or apparent solubility of a compound in the presence of surfactant can be represented as the amount of solute associated with surfactant monomers plus the amount associated with surfactant micelles (Equation 1)

Equation 1. The Apparent Solubility of a Compound in the Presence of a Surfactant

$$C_{PCE}^{*}/C_{PCE} = 1 + C_{monomer}K_{monomer} + C_{micelle}K_{micelle}$$

where:
C_{PCE}^{*} is the apparent solubility of solute at the total surfactant concentration (mg/L),
C_{PCE} is the intrinsic solubility of solute in pure water (mg/L),
$C_{monomer}$ is the concentration of surfactant monomers (mg/L),
$C_{micelle}$ is the concentration of micelles (mg/L),
$K_{monomer}$ is the solute distribution coefficient between surfactant monomers and water (L/mg),
$K_{micelle}$ is the solute distribution coefficient between the micelles and water (L/mg).

This approach can be extended to include the effect of sorbed-phase surfactant on the distribution of solute between the solid and aqueous phases as expressed in Equation 2.

Equation 2. The Effect of Sorbed-Phase Surfactant on the Distribution of Solute

$$K_{PCE}^{*} = K_{PCE}(1 + C_{s/om}K_{s/om})/(1 + C_{monomer}K_{monomer} + C_{micelle}K_{micelle})$$

where;
K^{*} is the apparent soil-water distribution coefficient (L/kg),
K is the intrinsic soil-water distribution coefficient (L/kg),
$C_{s/om}$ is the concentration of sorbed surfactant per unit mass of native soil organic matter, $K_{s/om}$ is the solute distribution coefficient between sorbed surfactant and organic matter (K_s/K_{om}), and
Ks is the solute distribution coefficient between the sorbed surfactant and water.

The above equation was incorporated into a macro-based spreadsheet to investigate the effects of system parameters on the overall or apparent distribution coefficient (K*) of DDT as a function of surfactant concentration. Using the measured data reported above, the sensitivity of K* to $K_{s/om}$ was investigated over a surfactant concentration range of 0 to 800 mg/L (Figure 31). The model will be adapted to account for rate-limited sorption and desorption of both the surfactant and the PCB congener. In addition, experimental data will be collected to evaluate the ability of the model to predict the coupled sorption of PCBs and surfactant. An example of the effect of differences is the Ks/om value on the overall partitioning of PCB between the solid and liquid phases is given in Figure 31. Note that once the CMC is exceeded, the effect of surfactant sorption becomes minimal, that is, K* approaches zero. However, at low concentrations partitioning of the PCB into sorbed-phase surfactant can have a substantial impact on the distribution of PCB within the system.

PCB transformation experiments

The microbial growth experiments confirmed the ability of biphenyl-degrading microorganisms to achieve substantial growth in solutions of surfactant that contained monochlorinated biphenyl 4-CB. The disappearance of 4-CB from liquid batch cultures was monitored through additional experiments that were designed to address the fate of both the surfactants and the chlorinated biphenyls. These transformation experiments were conducted in 300-mL glass culture flasks that were sterilized and coated with 4-CB. An aqueous solution of nutrient K1 or solutions of surfactant, either Tergitol NP-15 or Tween 80, was added to the prepared flasks and allowed to mix for ten to thirteen hours at 30° C, 150 rpm. Resting cells of strain NY05, which had been rinsed three times to remove residual biphenyl, were suspended in K1 at 0.2 g/mL and distributed among the flasks to begin the transformation experiments. Samples were transferred from the flasks to 26-mL glass serum tubes to which was added sulfuric acid (2N H_2SO_4) in order to lower the pH to about 3.5 and halt microbial activity. Additional preparation of the samples included hexane extractions, for flasks that did not contain surfactant solutions, and centrifugation to remove biomass after which the samples were transferred to chromatography vials for analysis by GC/ECD. Results from representative degradation experiments are presented in Figure 32 for surfactant and K1 solutions. In order to confirm the disappearance of 4-CB was microbially mediated, controls were included in the transformation experiments in which flasks, both with and without surfactant solutions, were not inoculated with NY05 resting cells. These controls enabled detection of possible abiotic influences, such as vaporization or adsorption to the flasks, which would appear as 4-CB degradation during GC/ECD analysis. Typical results of the abiotic controls, for solutions of both surfactants, are presented in Figure 32 with the respective surfactant solution.

In addition, the transformation of 4-CB by strain NY05 has been shown to result in stoichiometric amounts of the corresponding chlorinated benzoic acid, 4-CBA. The detection of this degradation metabolite provided additional confirmation of the microbially mediated disappearance of 4-CB. Analytical detection of 4-CBA, for a representative degradation experiment conducted in a solution of Tween 80, is presented in Figue 33. Notably, the experiment was initiated well before the system approached equilibrium, and as such, the amount of 4-CBA detected has a greater molar concentration than the initially detected amount of 4-CB in solution.

Current efforts are focused on additional transformation experiments that are allowed to mix a minimum of three weeks prior to inoculation. These extended mixing intervals provide the 4-CB+surfactant systems more time to reach equilibrium before the microbial transformation experiments are initiated. In addition, similar amounts of 4-CB mass are added to each system, both with and without surfactant solution, in order to facilitate comparison of the degradation efficiencies of the different systems. These current transformation experiments will enable better interpretation of the solubility enhancement effects of surfactant solutions on microbial degradation of chlorinated biphenyls when compared with biodegradation of equal amounts of 4-CB mass present as solid phase rather than in solubilized phase.

5 Accomplishments of the Pilot Evaluation

Site Consideration for Field Test

The ideal soil for the demonstration project should contain one of the lesser-chlorinated Aroclors (1242 or 1248) at a concentration of 500 to 800 μg/g and be free of other contaminants such as oils and heavy metals. While more heavily chlorinated PCB mixtures such as Aroclor 1260 could be treated, sequential inoculations would be necessary to obtain extensive enough dechlorination, and this would add to the time required for anaerobic phase. Oils can be expected to slow both the anaerobic and aerobic steps by serving as a phase, to which the PCBs can partition, thus decreasing their availability to the microorganisms. The anaerobic step is relatively immune to heavy metals because under anaerobic conditions they are precipitated as sulfides thus not bioavailable. However, they may be toxic when conditions are made aerobic for the second stage treatment.

Also soil should have a moderate (2 to 3 %) amount of organic carbon to provide substrates for the microorganisms. If the organic carbon level is much higher, sorption of PCBs to the organic matter may begin to limit PCB bioavailability, and growth of non-PCB dechlorinating and degrading microorganisms could be favored (both anaerobic and aerobic stages). A deficiency of organic carbon can easily be made up by addition, preferably of a simple carbon source, such as ethanol or trypticase soy broth, that does not bind PCBs.

Many PCB contaminated sites (Table 7) were evaluated. However, for various reasons these sites were found unsuitable for our field test. Finally GE site, Rome, GA was located and found suitable for performing the field test. Several samples were analyzed for PCB profile and contamination levels (Table 8). The Aroclor 1242 contaminated soil from Rome, GA met all the criteria (as mentioned above) and is currently being used for the ongoing pilot-scale evaluation of the two-phase PCB bioremediation at US Army Engineer Research and Development Center (ERDC), Vicksburg, MS.

The pilot-scale reactor treatment sequence involves an anaerobic phase, designed to dechlorinate the higher-chlorinated congeners, followed by an aerobic phase, to treat the accumulated lower-chlorinated congeners. To enhance the anaerobic treatment process, river sediment inoculum, ferrous sulfate and surfactants are added to the reactors. During the aerobic treatment phase genetically engineered microorganisms will be added using a vermiculite carrier along with a nutrient/surfactant solution (Figure 34).

Traditionally, low solids slurry reactors are used for creating the reduced environment for PCB dechlorination. In these reactors mixing is needed for maintaining the uniform moisture level throughout the system as well as to uniformly distribute the nutrient amendments. Achieving a well-mixed system is dependent on operational capacity of mixing systems, which in turn is dependent on the slurry concentration. Most of the traditional mixing systems can only mix

slurries with consistencies up to 10-20% (solids:water, w/w). Once the treatment goals are achieved, the dewatering costs become prohibitive for the disposal/reuse of the solid material. To overcome these operational limitations, this study is focusing on the economic feasibility and cost benefit analysis of three bioremediation options with different solids loadings.

The main objectives of this pilot-scale evaluation are:

- To validate the usefulness of the two-phase bioremediation scheme that combines enhanced anaerobic dechlorination of Aroclor and genetically engineered aerobic PCB-growing microorganisms (GEMs) for bioremediating PCB contaminated soils.
- To evaluate the effects of different solids loading rates (or moisture contents) on the bioremediation of PCBs and application of GEMs.
- Develop critical design information on the application of GEMs for full-scale bioremediation of PCB contaminated soils.

A pilot-scale demonstration study consisting of side-by-side comparisons of three bioremediation processes is currently underway at the Environmental Laboratory, US Army Engineer Research and Development Center (ERDC), Vicksburg, MS. Three parallel bioremediation processes with different solids loading rates are used to evaluate the effects of solids loading on soil mixing, aeration, addition and uniform distribution of nutrient amendments/GEMs, and final disposal/use of treated material. Each bioremediation process has three separate reactors (treatments) to evaluate the effect of ferrous sulfate and surfactants on PCB dechlorination (Table 9).

Site Description

PCB contaminated soil was collected from GE site in Rome, GA. The PCB concentration in this soil ranges from 500 – 1000 ppm (Table 8). The soil is clayey with little sand (87.4 % fines, 11.6 % sand and 1% gravel). The soil has a liquid limit (LL) of 57 and plastic limit (PL) of 23. Chemical characteristics of Rome, GA soil are summarized in Table 10. Soil was collected from 6 feet below the ground surface. After excavating, the soil was cleaned of any pebbles/debris that was more than 1" in size. The collected soil was sieved through 1" mesh, thoroughly mixed, and homogenized at the site. About 2240 kg homogenized soil was shipped to ERDC in ten 55-gallon drums. The soil was used to load the triplicate treatment reactors for each bioremediation process, and for the initial physical and chemical characterization of the collected soil.

Pilot-Scale Demonstration

The reactor setup consists of three reactors (treatments) for each solid loading under three bioremediation options. The reactors used for three different solids loadings are illustrated in Figures 35 through 37. The low solids reactor (Figure 35) is a stainless steel 430 gallon tank with dimensions of 56" high and 47½" internal diameter. The reactor has a cone shaped bottom for convenient unloading. The reactor is equipped with a variable speed agitator fitted with two propellers for mixing and aerating the slurry. The reactor is similar to a traditional bioslurry reactor commonly used for the ex situ treatment of contaminated soils and sediments. This reactor uses excessive water (90-95 %) to create anaerobic conditions, maximize the distribution

of microbial amendments and to enhance the mass transfer. Inherently the treatment times are considerably short in such types of systems, however, the post-remediation handling of excessive water generated from dewatering the treated material is a major disadvantage of these systems.

The medium solids reactor shown in Figure 36 is a state-of-art shaftless screw reactor capable of mixing soil slurries with solids content up to 70 % w/w. The 140 gallon medium solids reactor has a foot print of 16.5 square feet. The reactor is fitted with three shaftless screws to mix and convey the high solids slurries. The medium solids reactor is a novel idea in bulk material mixing and conveying. The shaftless screws are 76¾" in length and 5¾" in diameter. The screws rotate at variable speed in U-shaped troughs constructed within a rectangular metal housing measuring 76¾" x 31" x 22". The screws simply slide on the wear and chemical resistant lining. Each screw is operated by a 0.75 hp motor equipped with a gear control having a gear ratio of 40. Electrical inverter controllers (Baldor 15H) regulate individual screw speed and rotation. The side troughs are inclined making a 4° angle with the bottom of the reactor. The middle trough is also inclined at the same angle but in the opposite direction of the side troughs. The middle screw rotates twice the speed of side screws in order to transport the material back to the lower end of side screws for continuous mixing.

The shaftless screw reactor significantly reduces the dewatering costs associated with low solids slurries. The shaftless screw reactor enhances the treatment efficiency by reducing the mass transfer limitations in high solids slurries, and increases the soil microbial activity by facilitating the addition of nutrient amendments in high solids slurry, thereby reducing the treatment times and costs.

The high solids reactor shown in Figure 37 simulates the contaminant treatment similar to in situ landfarming. The reactor consists of a galvanized oval shaped iron pan with open top. The top is covered with an airtight plexiglass lid. The total volume of this reactor is 95 gallons with dimensions of 48" x 24" x 24". Contaminated soil at field moisture content is treated in this high solids reactor. The reactor is equipped with leachate collection system at the bottom, consisting of 4" gravel layer covered with geomembrane. Perforated aluminum sheet covers the geomembrane for uniform distribution of soil in the reactor. The process needs minimal maintenance, however the treatment efficiency is low with prolonged treatment times. The major advantage of this bioremediation option is that the treated material does not require any post-remediation dewatering for disposal/reuse. During the aerobic phase soil will be aerated by tilling/mixing the soil with low power rotary cultivator.

Each treatment system will undergo a two phase cycle - anaerobic dechlorination phase followed by an aerobic degradation phase. The anaerobic phase trigger is 6-8 months or a predetermined % dechlorination, which ever comes first. The aerobic phase will follow the anaerobic phase and will last for about 2-4 months.

All the three reactors in each bioremediation process were seeded with anaerobic inoculum obtained from Hudson River sediment. Anaerobic inoculum was applied at 0.8% w/w (dry solid:dry soil). In two of the reactors for each bioremediation process (Table 9) iron (20 mM $FeSO_4$ in pore water) and organic carbon (0.1% w/w, ethanol:slurry) was added along with the anaerobic inoculum. Surfactant (0.5% w/w; Tween 80:pore water) was added in one of the reactors for each bioremediation process. The quantities of soil, water, inoculum, and nutrient amendments in each of the reactors for the three-bioremediation processes is given in Table 11.

After completion of the anaerobic phase, the reactors will be inoculated with GEMs (LB400(*ohb*) and RHAI(*fcb*)). Vermiculite impregnated with GEMs will be applied at the rate of 3 % (w/w vermiculite:dry solids) of contaminated soil in each reactor. Vermiculite will be used because of its beneficial effect on the long-term survival of the GEMs and to improve distribution of the inoculum during mixing.

Sampling Schedule

The sampling analysis schedule for different parameters is given in Table 12. The material in each reactor was analyzed at the beginning for PCB concentration, microbial biomass (PFLA), moisture content, pH, temperature, nutrient levels (NKN, TP, and TOC), and headspace oxygen concentration. Additionally, initial characterization included particle size distribution (PSD), Atterberg properties, metals concentration, particle surface area, and soil leachability. These characteristics will be analyzed again at the end of the experiment. Routine soil/slurry samples are collected every month for PCB concentration, microbial biomass, nutrient analysis and soil moisture and pH. Soil/slurry temperature and headspace oxygen concentration is monitored biweekly to check anaerobic conditions in the reactors. To ensure quality control of analytical results, samples will be analyzed in composite replicates of 7 for high solids, 5 for medium solids and 3 for low solids reactors. Higher replicates are used in high solids reactor because of the level of heterogeneity and variability as compared to well-mixed low solids reactor.

Analytical Methods

Particle size distribution (PSD) was measured by using Coulter LS 100Q particle counter according to instrument specifications. Atterberg Limits test is performed according to Corps of Engineers Laboratory Testing Manual, EM-1110-2-1906, Appendix 3, III: Liquid and Plastic Limits. Bulk Density of the contaminated soil was measured by weighing a known volume of soil at field conditions. Soil pH is determined by a soil-distilled water slurry (1:10, w/w) using a Cole-Parmer® pH meter, and is reported as pH in water (pH_w).

PCB concentration in soil samples is determined by high-resolution gas chromatography with electron capture detection (ECD). Total Kjeldahl nitrogen (TKN) and total phosphorous (TP) analyses are performed by Lachat 8000 Flow Injection Analyzer (FIA). The preparation methods are modified versions of EPA-600/4-79-020 (1983 revision), 365.1 and 351.2, respectively. Total organic carbon (TOC) is determined by Zellweger Analytic TOC analyzer, according to the instrument protocol. Moisture Content of soil samples is analyzed by oven drying at 105 °C for 24 hours. Oxygen concentration is measured in the head space by using a LMSx Multigas Analyzer®.

Surfactant analysis for Tween 80 concentrations in aqueous samples is done by a Hewlett Packard 1100 series. Detection of Tween 80 is achieved using a Diode Array selection HPLC controlled by a HP vectro pentium computer.

Soil leachability is assessed by two alternative leachability methods. The Sequential Batch Leaching Test (SBLT) consists of four repeat extractions of the same sample using deionized

water in at 4:1 (water:solid) ratio. The slurry is mixed for 24 hours, centrifuged, filtered, and the filtrate analyzed for contaminants. The Synthetic Precipitate Leaching Procedure (SPLP) is performed according to SW846, EPA Method 1312. Being a nonvolatile extraction "Bottle Extraction Vessel" like amber jar (1L) with sufficient capacity to hold the sample and the extraction fluid will be used. The method consists of a single extraction using a dilute acid solution. Extraction fluid used to determine the leachability of soil from a site that is in the east of the Mississippi River consists of 60/40 weight percent mixture of sulfuric and nitric acids diluted with reagent water to a pH of 4.20±0.05.

6 Conclusions

Potentially bioremediation results in dechlorination of PCBs and possibly even in mineralization of the contaminant. Energy costs are lower than other forms of treatment. Slurry phase treatment usually requires less time than solid-phase biological treatment due to the increased rates of contaminant mass transfer. Furthermore, it is relatively simple to maintain either aerobic or anaerobic conditions in the reactor and to switch between the two conditions. As noted above, this characteristic is especially beneficial when treating PCBs. Anaerobic conditions can be maintained to allow for reductive dechlorination of the highly-chlorinated congeners. After this stage is complete, aerobic conditions can be established to allow the resulting lightly-chlorinated congeners to be metabolized aerobically.

Principal drawbacks to preexisting bioremediation schemes are slow rates of the anaerobic Aroclor dechlorination in sediments and inability of naturally occuring biphenyl-degrading organisms to dechlorinate and grow on lowere chlorinated PCB congeners resulting from anaerobic phase.

We have addressed these principal barriers and developed processes that resolve the above problems via enhancing rates of anaerobic dechlorination, and allow aerobes to grow on the products from anaerobic Aroclor dechlorination.

Although results of the currently ongoing pilot test are yet to be determined, flask and laboratory scale soil remediation experiments implicated that the designed two-phase enhanced anaerobic dechlorination of Aroclor coupled with GEMs-based enhanced aerobic degradation/ mineralization of lower chlorinated PCBs could be very beneficial in future remediation technologies.

7 Recomendations for Further Transitional Research

The results of our research implicated that the developed bioremediation scheme could be benefitial in future technologies. However, much further research is needed to resolve remaining questions, as well as improve current results by gaining knowledge on how the degradative processes affect and are being affected withing the given metabolic networks in microbial communities. Recent developments in genomic sequencing and DNA microarray technology create opportunities for transferring these approaches to community and populational studies. The latter would allow for rapid advances in our understanding of the complex processes involved in successful biodegradation within a givem microbial population, naturally occurring and/or engineered by introduction of foreign members. Success in this direction will determine practicality of the emerging recombinant technologies for remediation of environmental hazards.

The success of biodegradation may also be hampered by low bioavailability of the contaminant, by mass transfer limitations of the electron acceptor and by low temperature. In addition, most feed soils will require some degree of size reduction, usually to a greater extent than that required with incineration. Particles must be small enough that they can be suspended by the reactor's agitators, and gravel may jam agitator blades. Soils with large amounts of oily or greasy waste have been found to be problematic in slurry treatments. Because these wastes are hydrophobic, dispersants must be used to keep agglomerations of the waste from forming. Even so, the oils are often in large droplets with fairly low surface area with respect to their volume so that microbial attack is hindered. In addition, the oils will adhere to the sides of the reactors and to the agitation equipment, often blocking air nozzles. Further physico-chemical and engineering research is needed to evaluate and improve or design better technological processes.

8 Technology Transfer

We are interacting directly with other members of the flask-to-field bioconsortium working in the PCB area. We will produce a final report. We have produced sevral scintific papers in peer-reviwed journalls describing designing and properties of the PCB-growing organisms. We will produce a technical article that includes WES and GA Tech staff as coparticipants and coauthors. We have made several presentations at national meetings. All information developed in this effort was made available to all consortium members during presentations at the annual Flask-to-Field meeting. Where appropriate, the research investigations developed here will be applied to other 6.2/6.3 research programs and to reimbursable studies for CE and other DoD field offices.

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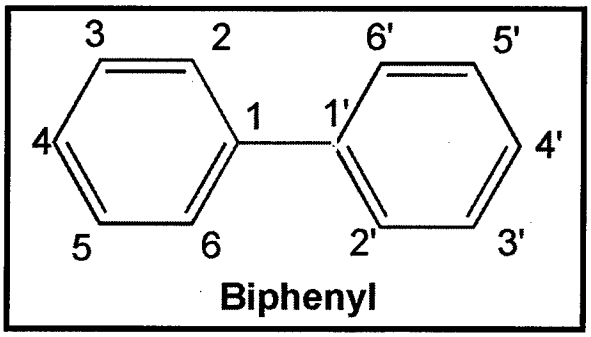


Figure 1. The general structure of polychlorinated biphenyl compounds

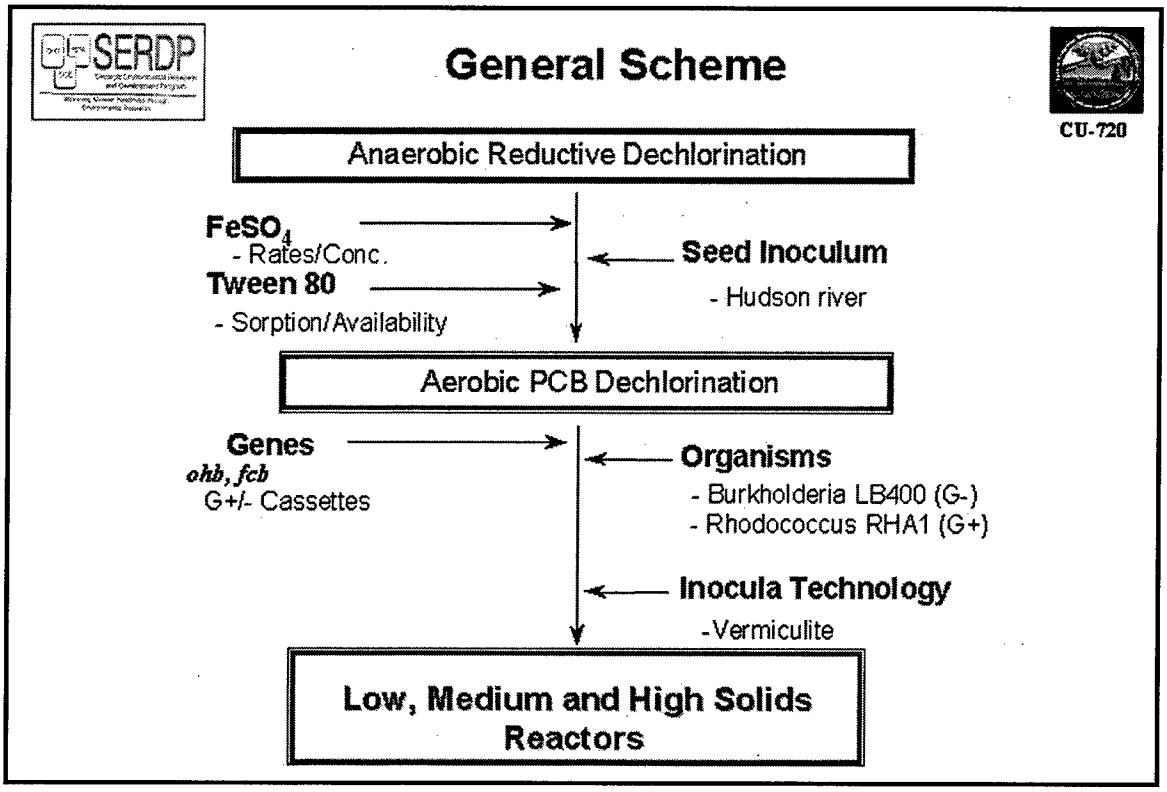


Figure 2. PCB remediation strategy

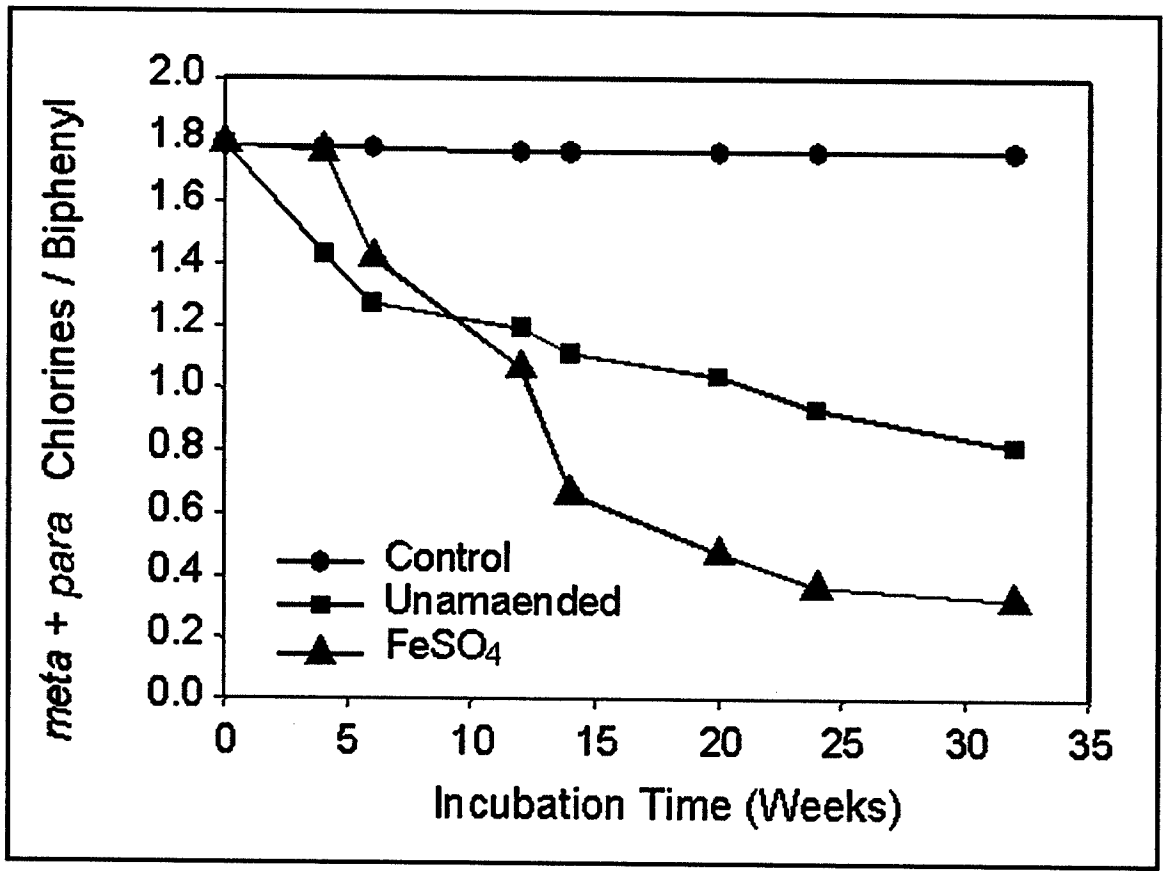


Figure 3. Effect of $FeSO_4$ on dechlorination of Aroclor 1242 from *meta* and *para* positions

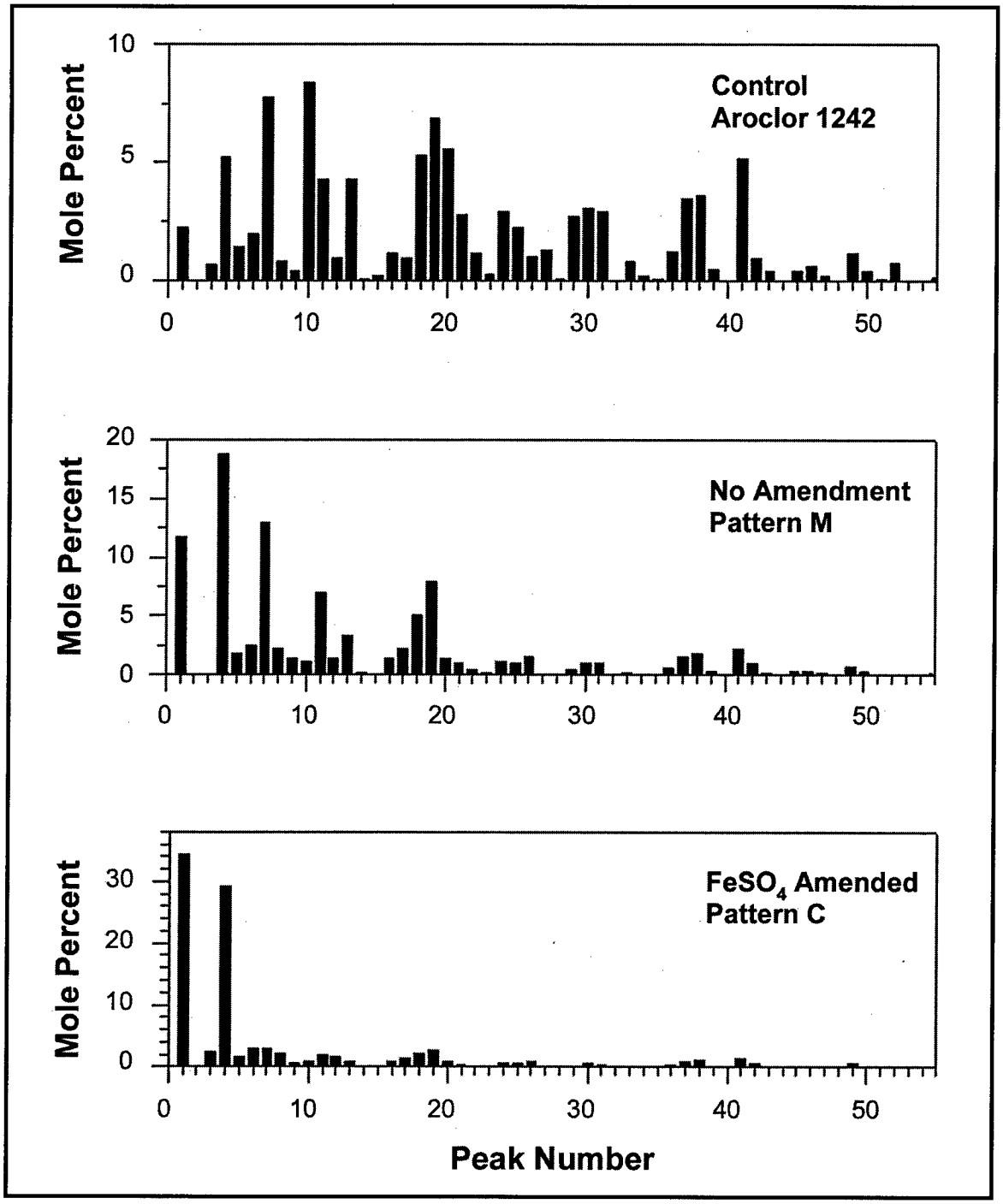


Figure 4. Effect of $FeSO_4$ on dechlorination pattern

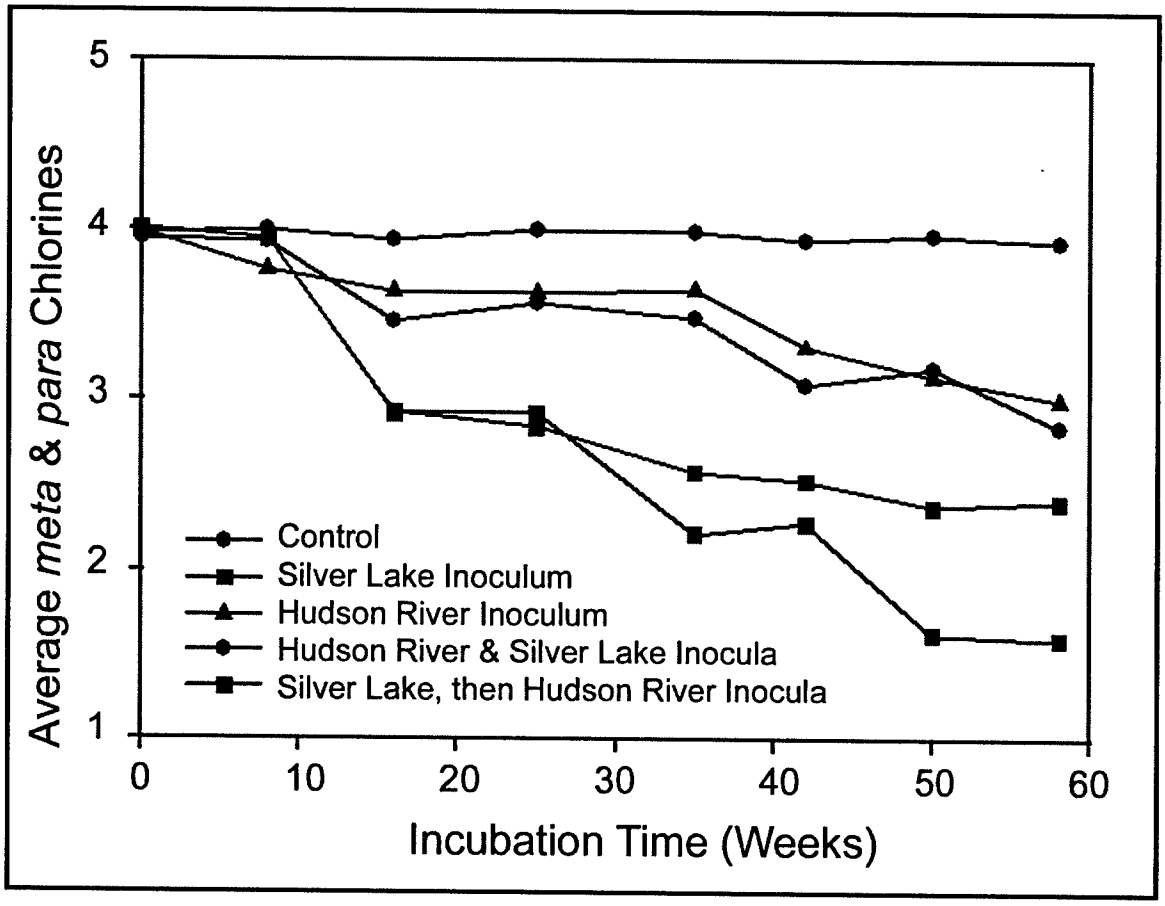


Figure 5. Extent of Aroclor 1260 dechlorination obtained using mixed and sequential inoculations with Hudson River and Silver Lake microorganisms

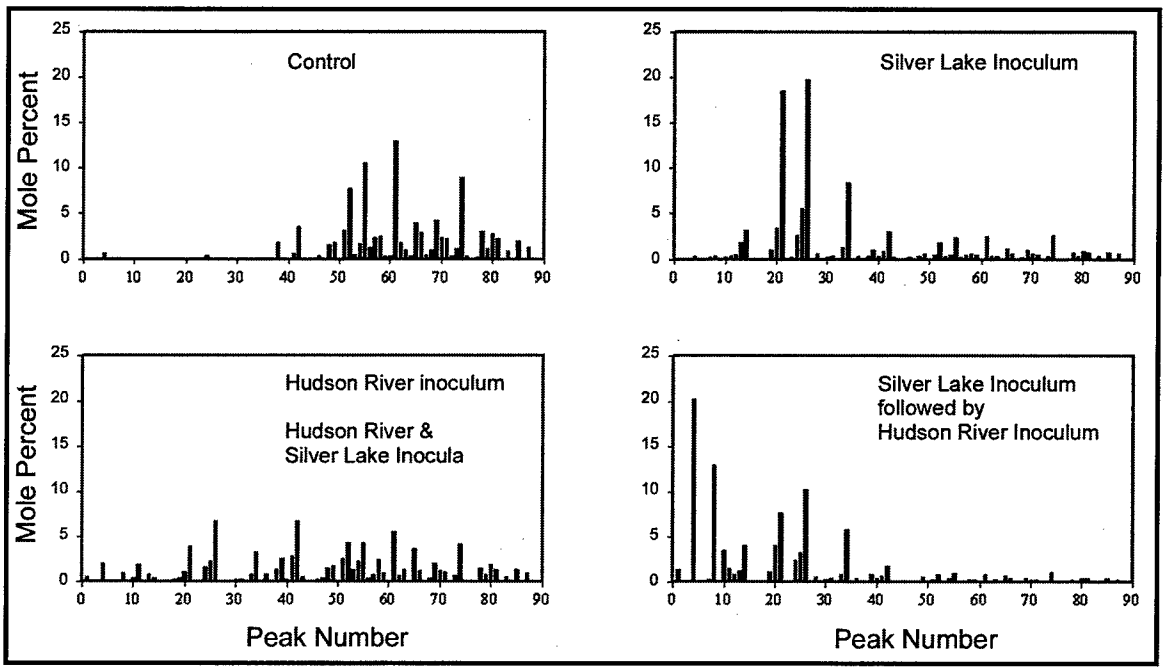


Figure 6. Aroclor 1260 dechlorination patterns obtained using mixed and sequential inoculations with Hudson River and Silver Lake microorganisms

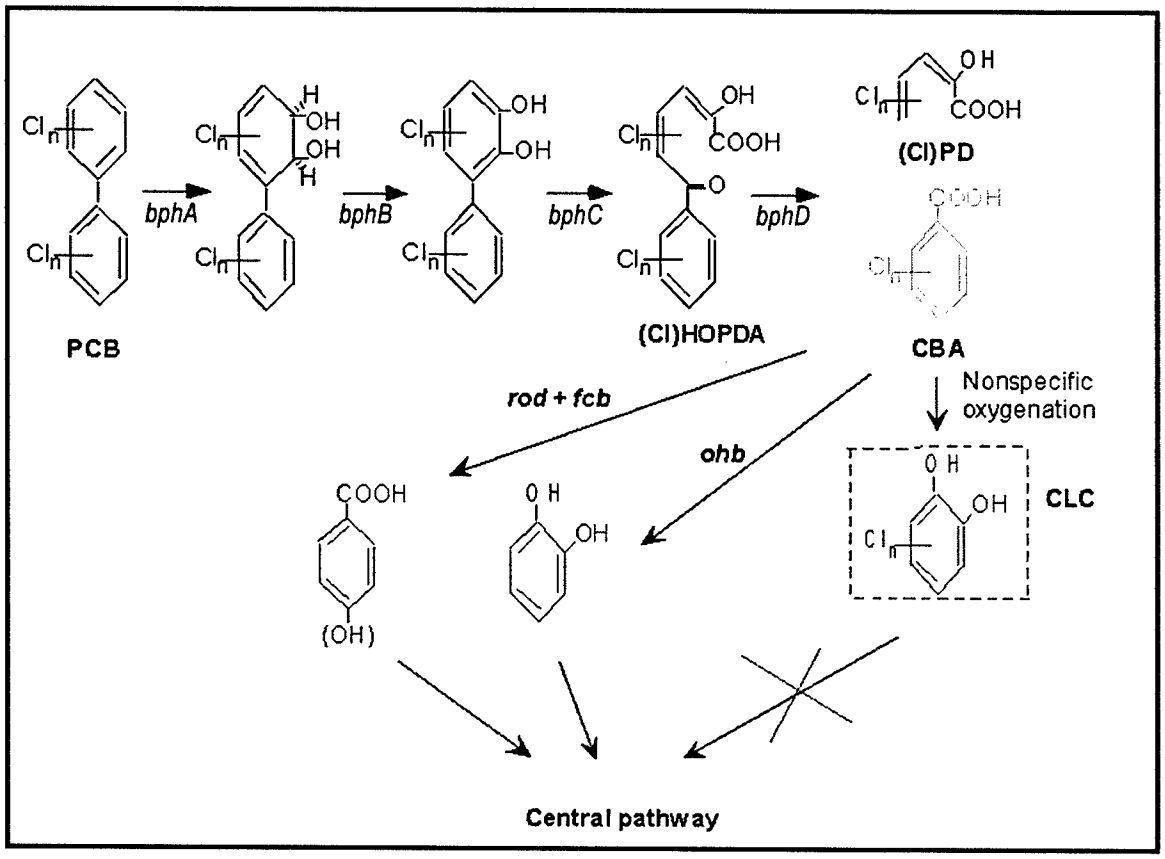


Figure 7. Engineering PCB pathways

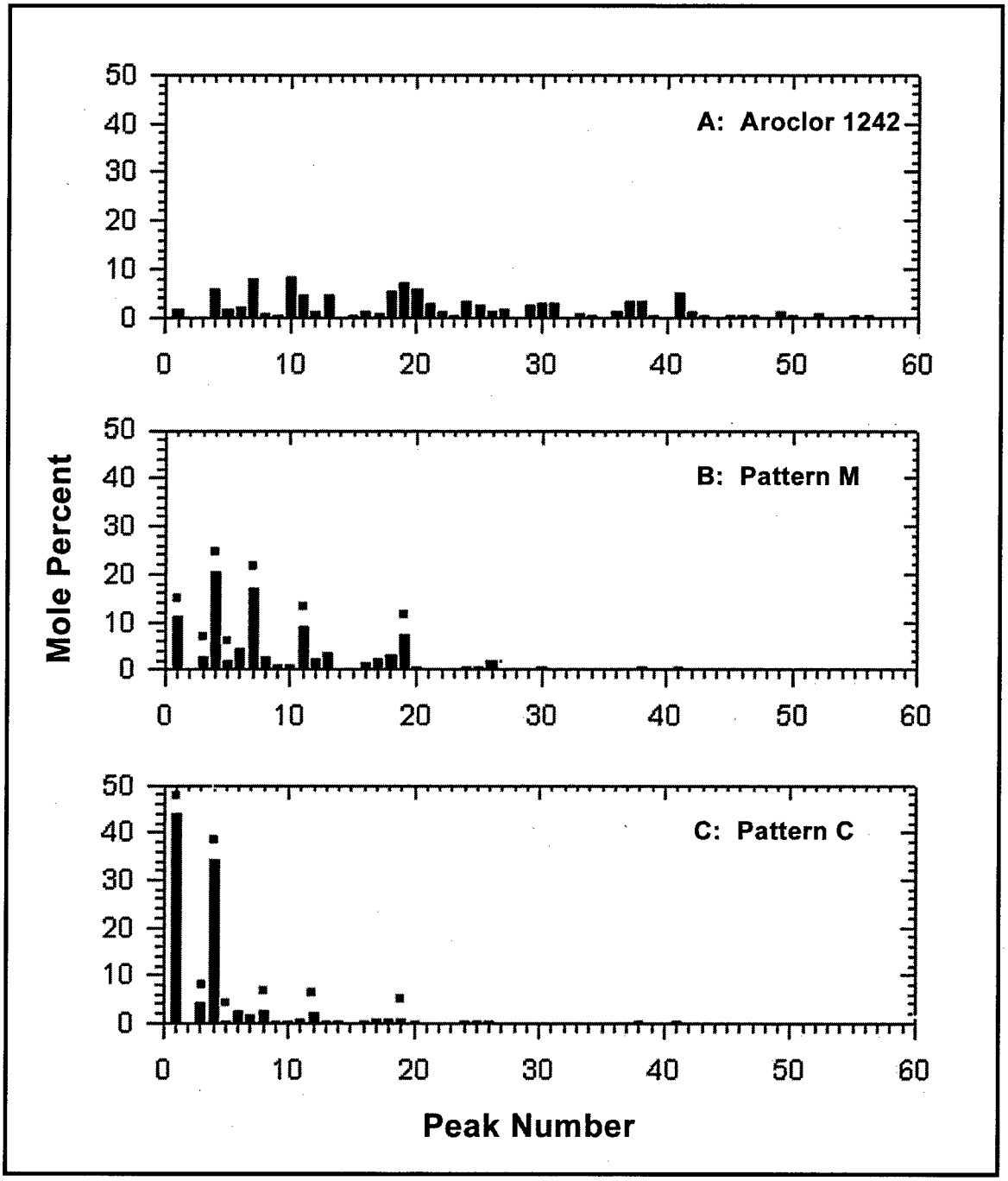


Figure 8. Pattern M and C profiles 1

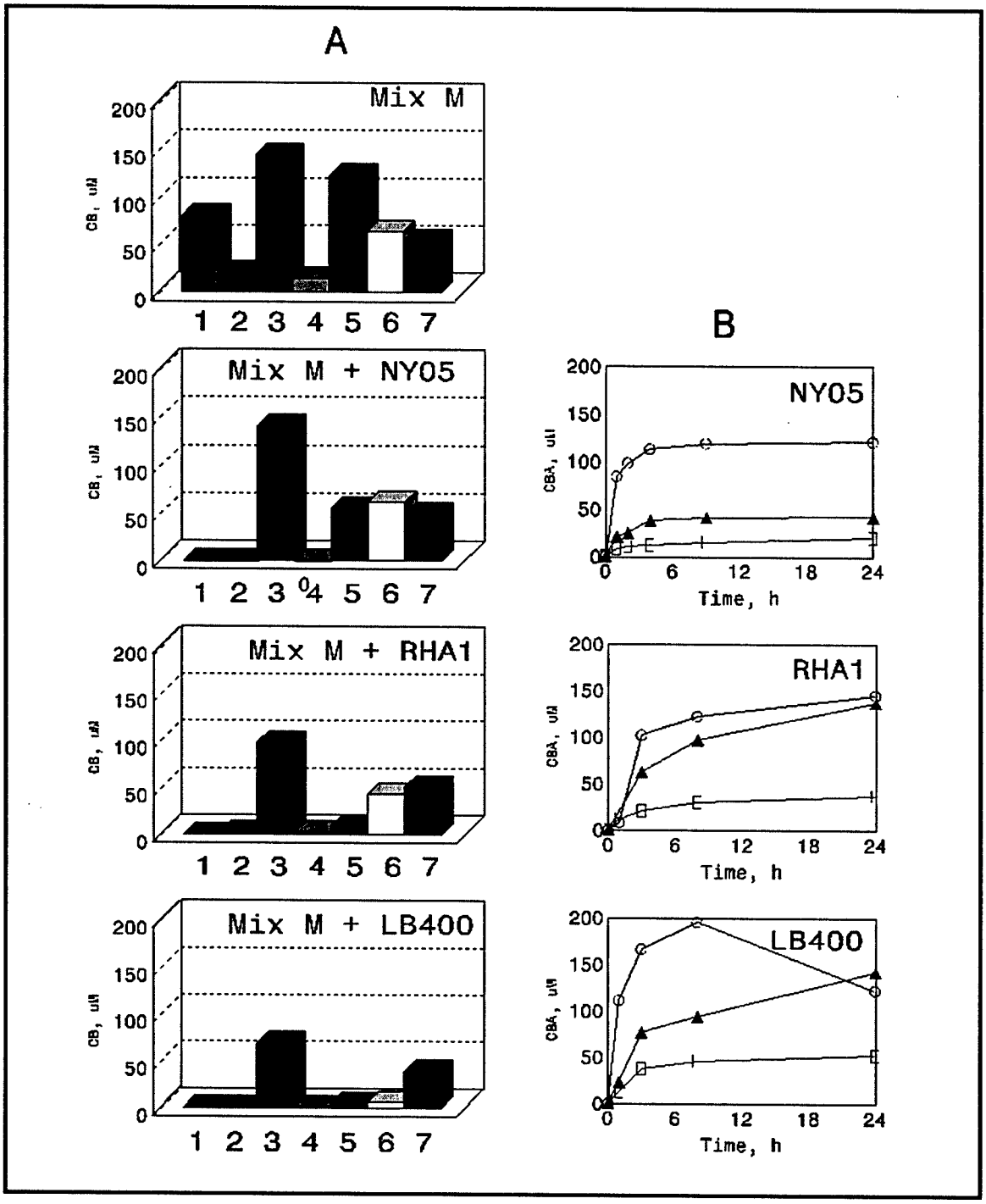


Figure 9. Degradation of Mix M by three Bph-degraders

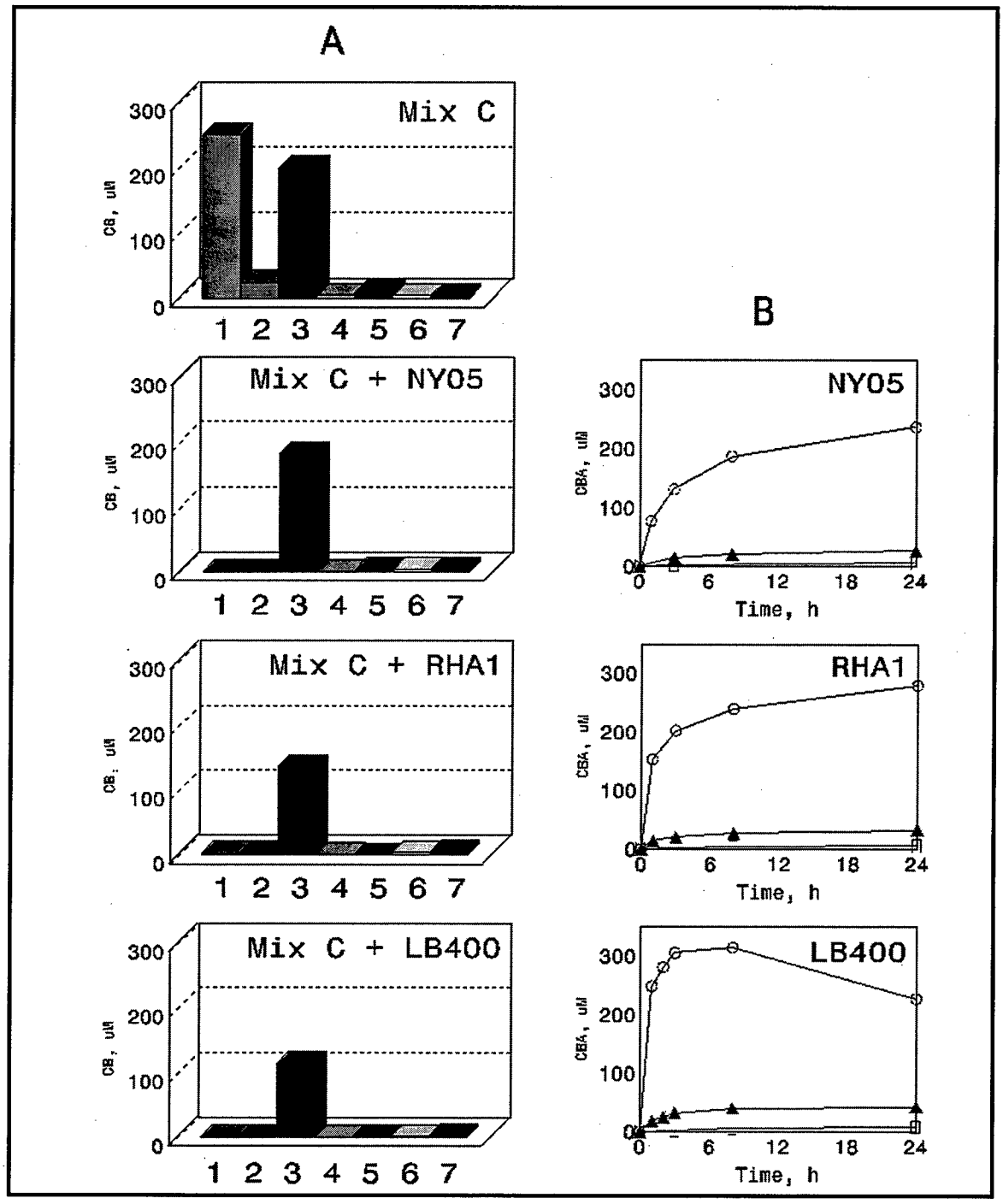


Figure 10. Degradation of Mix C by Bph-degraders

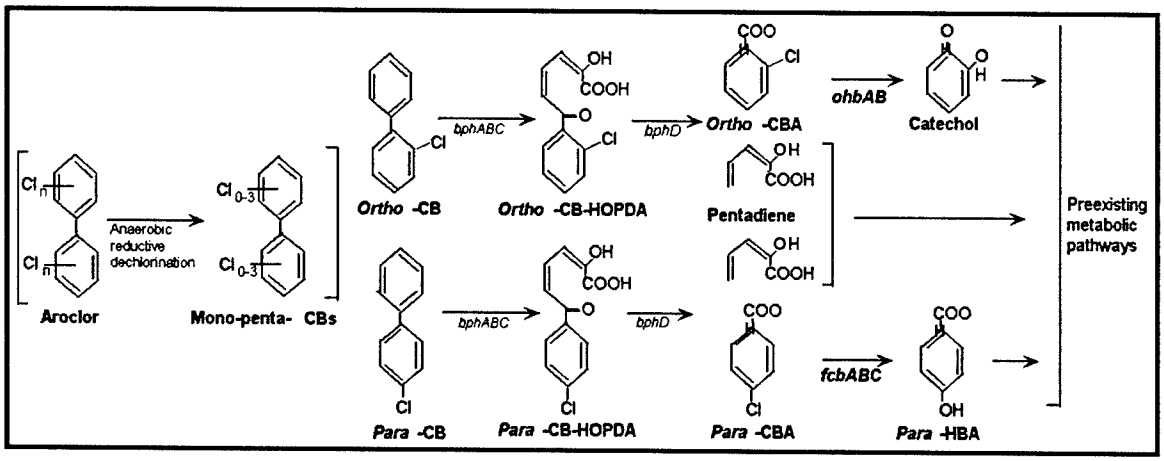


Figure 11. PCB growth pathways in genetically modified VP44

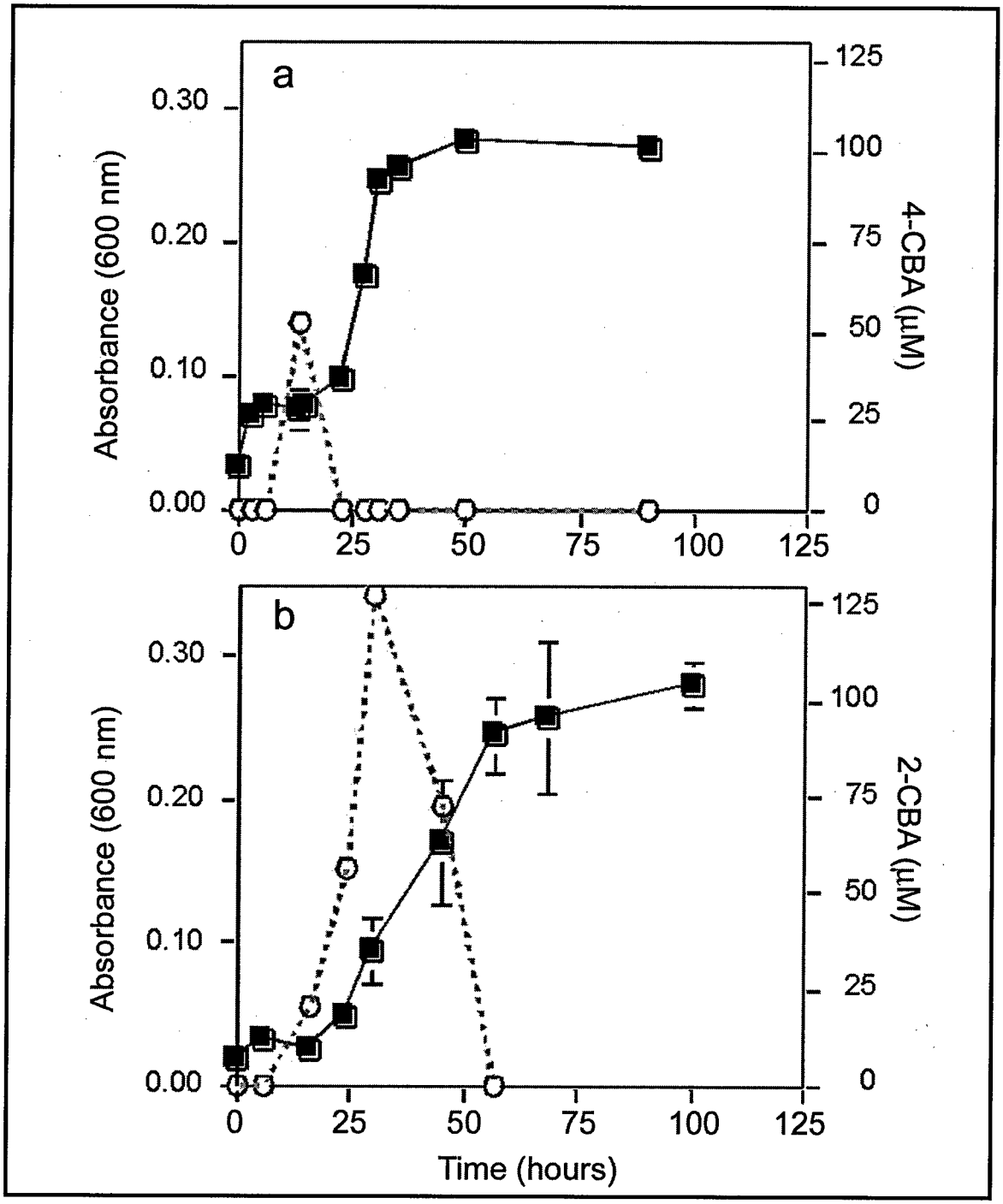


Figure 12. Growth of VP44(pPC3) and VP44(pE43) on 4-CB (a) and 2-CB (b)

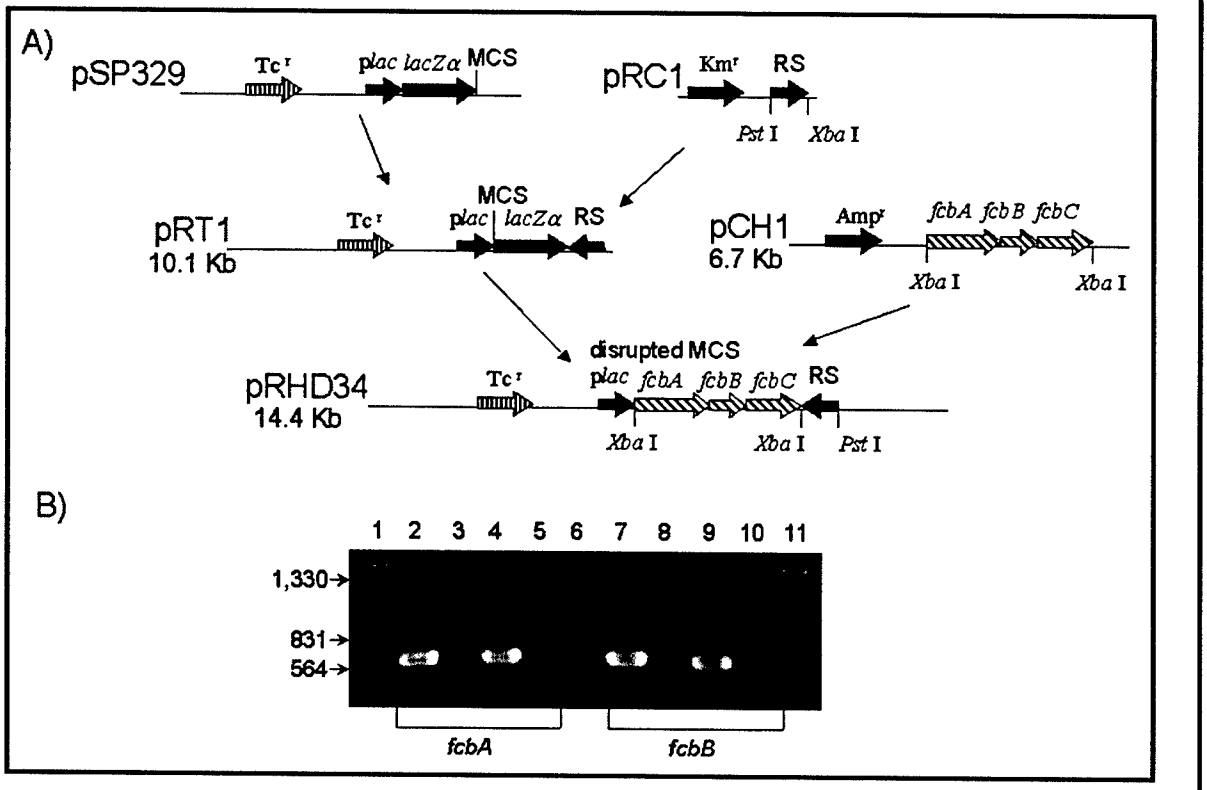


Figure 13. Gene transfer system for G+/G

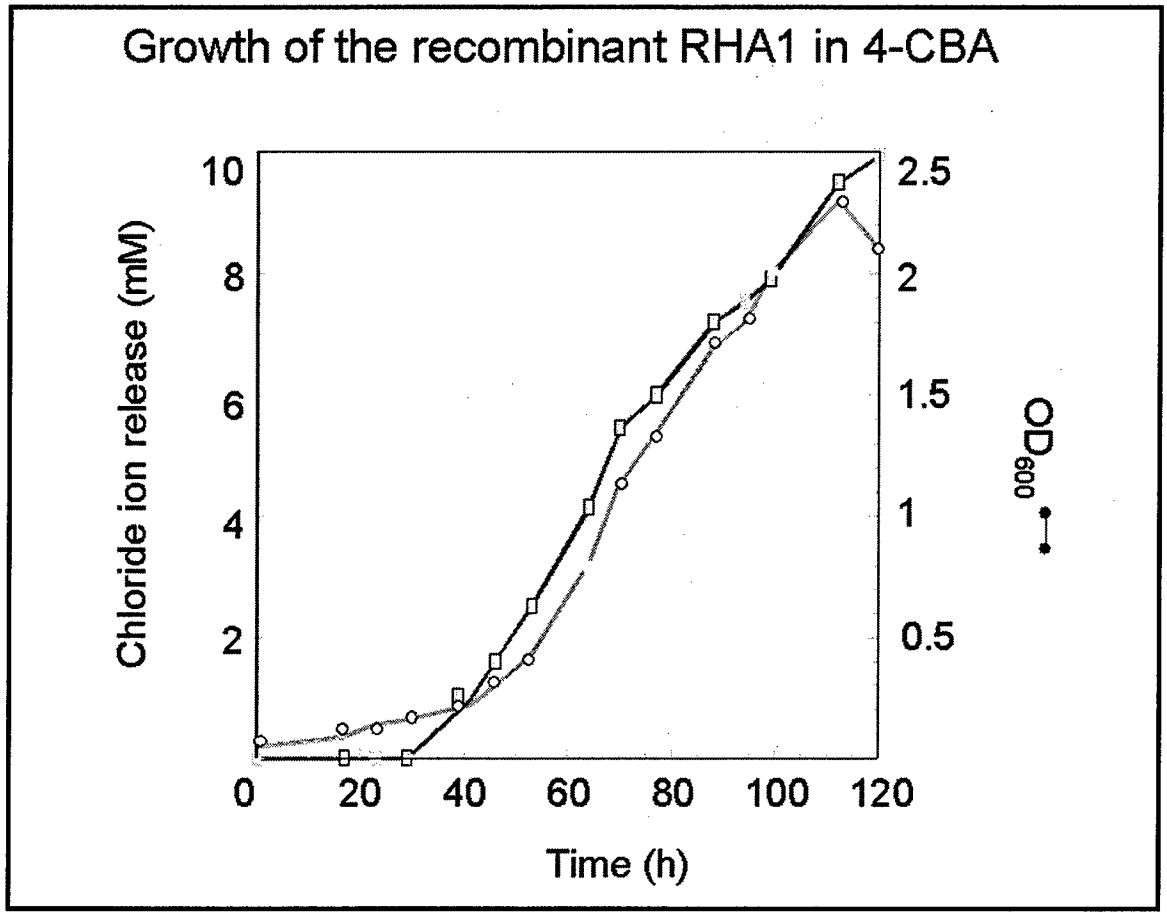


Figure 14. Growth of RHA1(*fcb*) on 4CBA 1

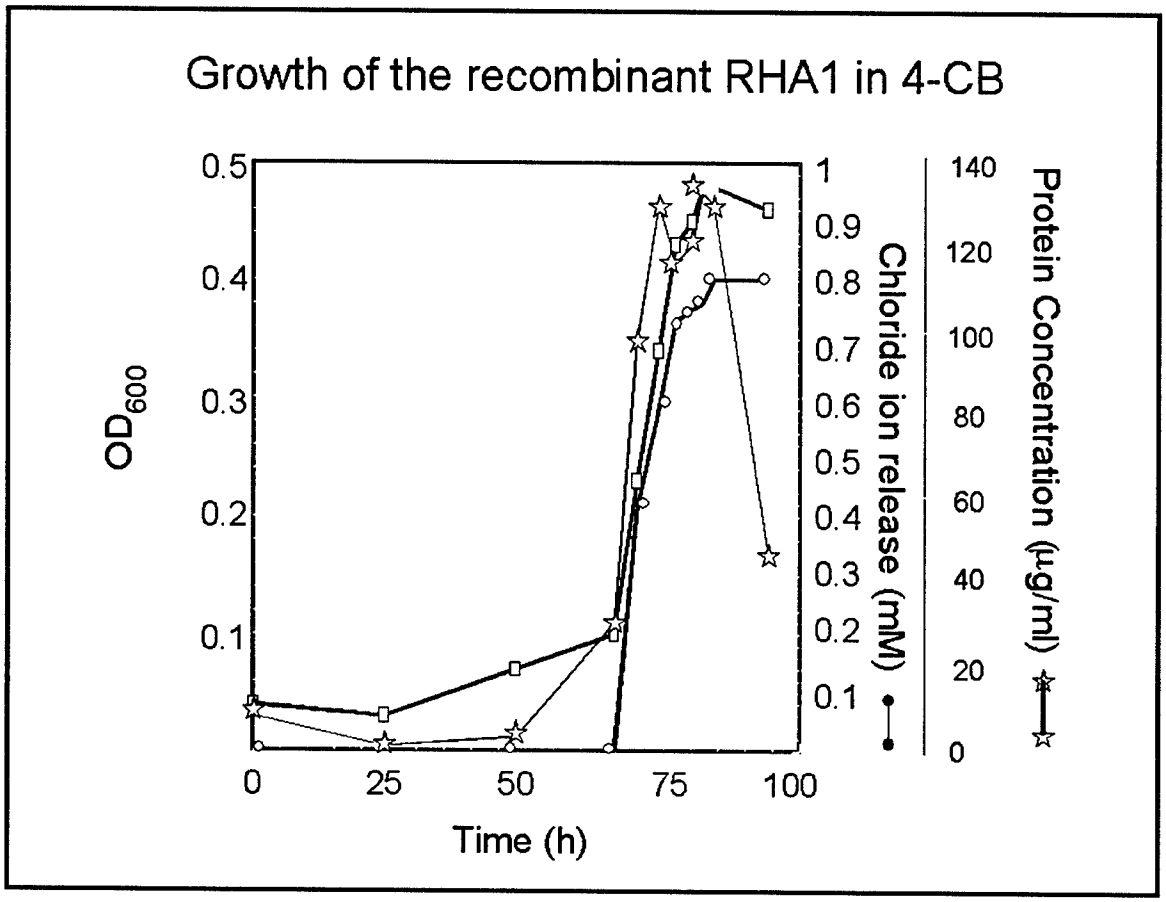


Figure 15. Growth of RHA1(*fcb*) on 4CB

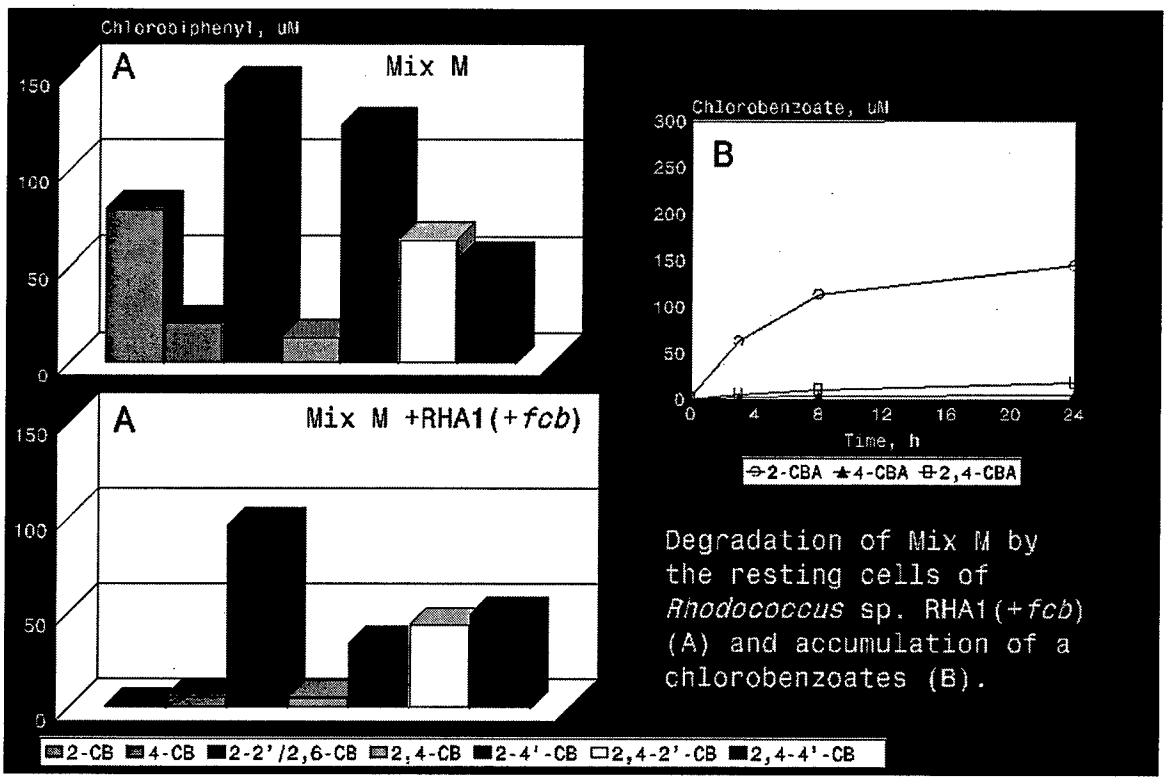


Figure 16. Degradation of Mix M by RHA1(*fcb*)

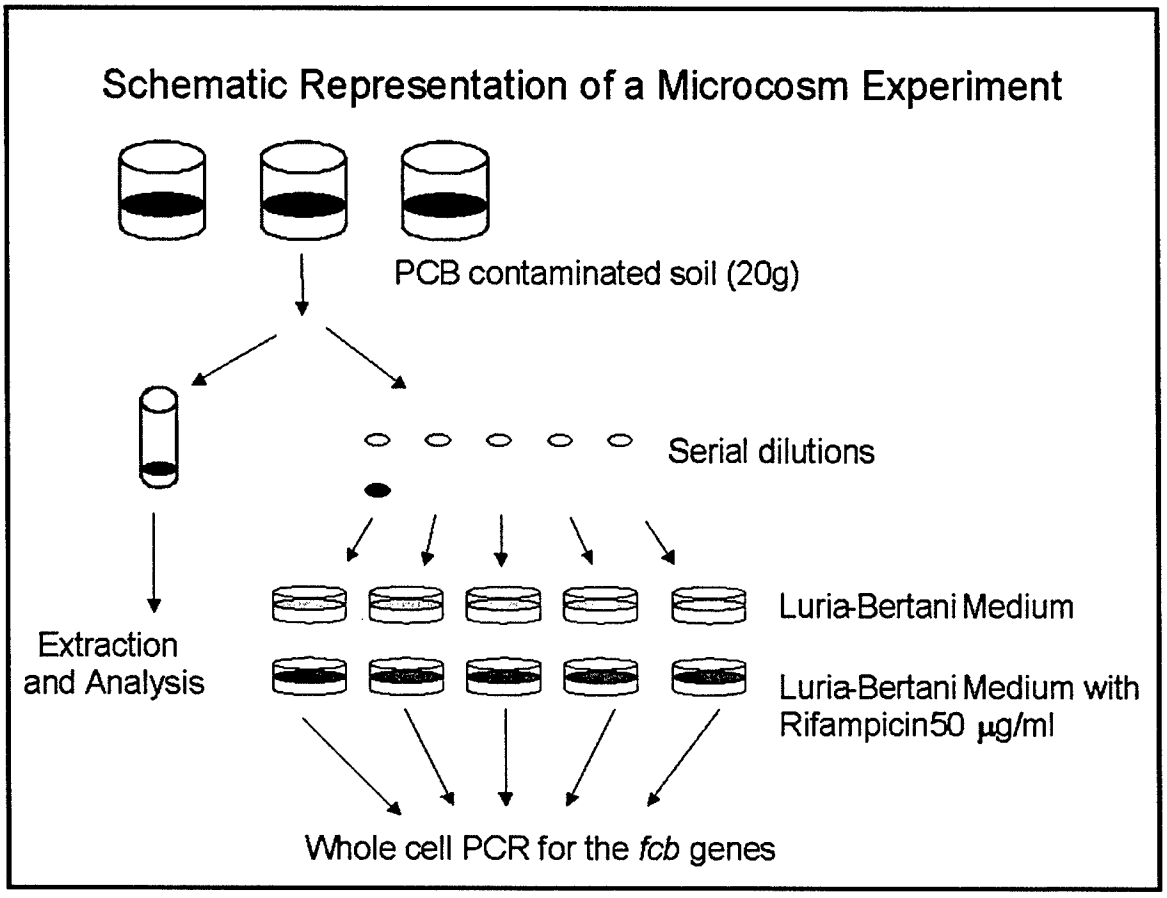


Figure 17. Microcosm experiment

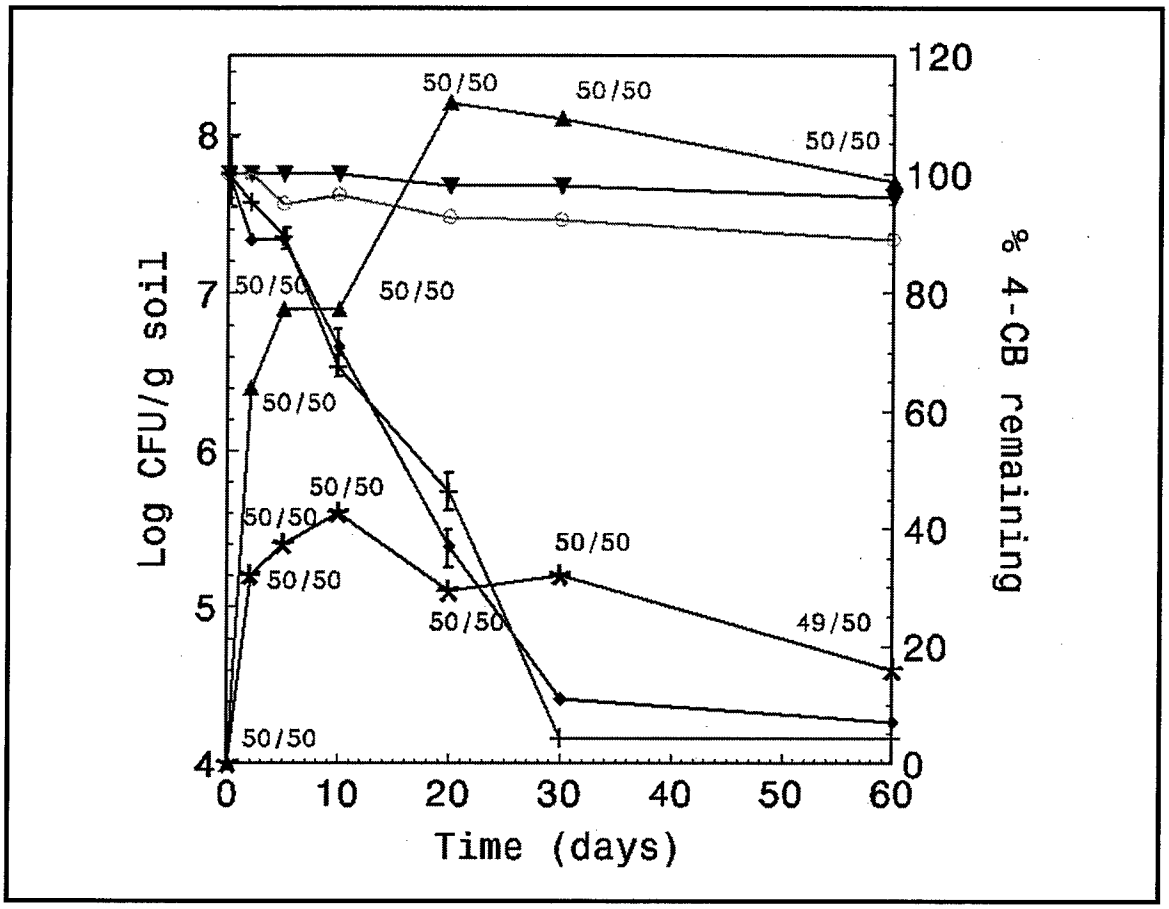


Figure 18. Survival and degradation of 4CB in soil by RHA1(*fcb*)

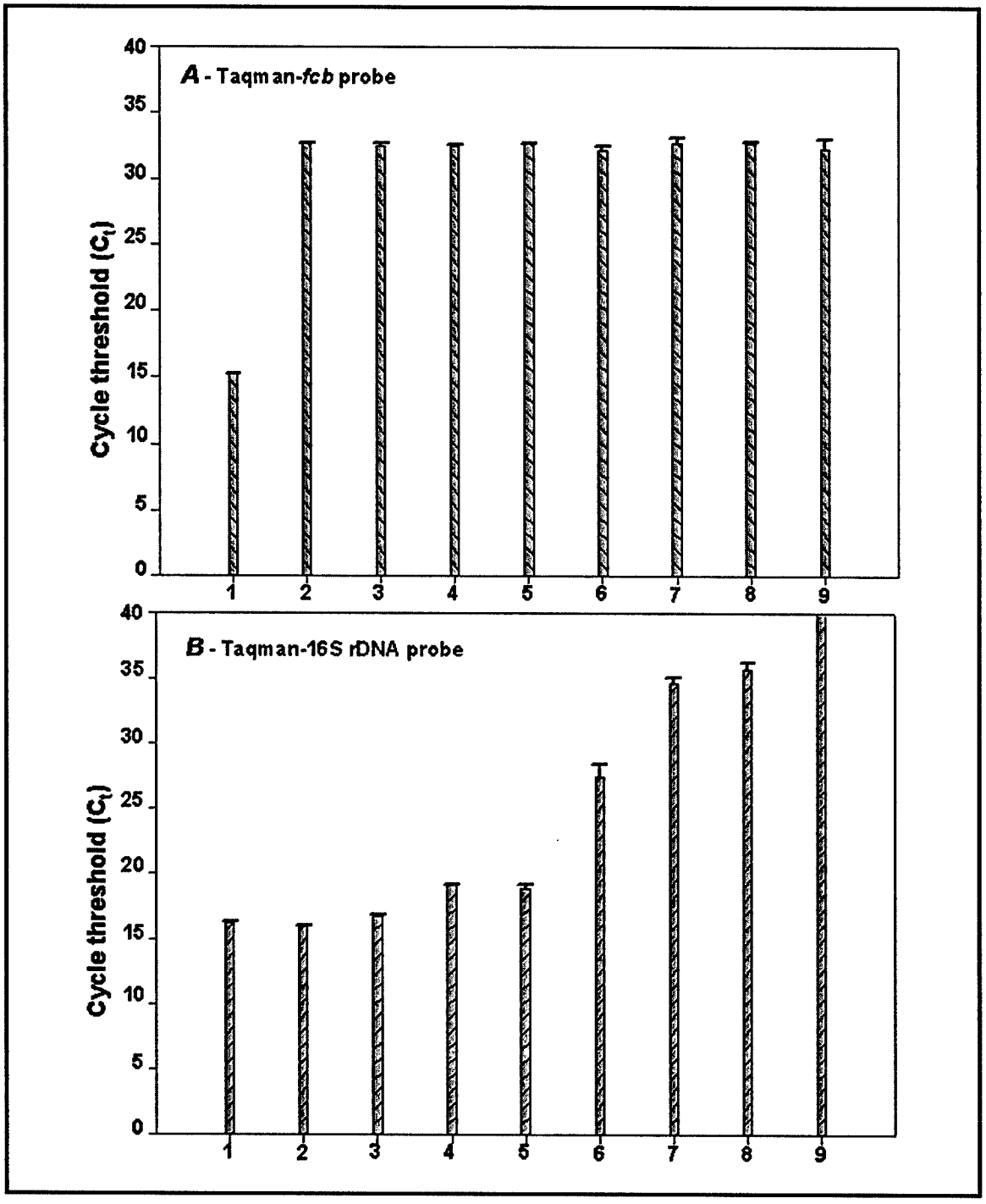


Figure 19. Average cycle threshold values (Ct) for real-time PCR

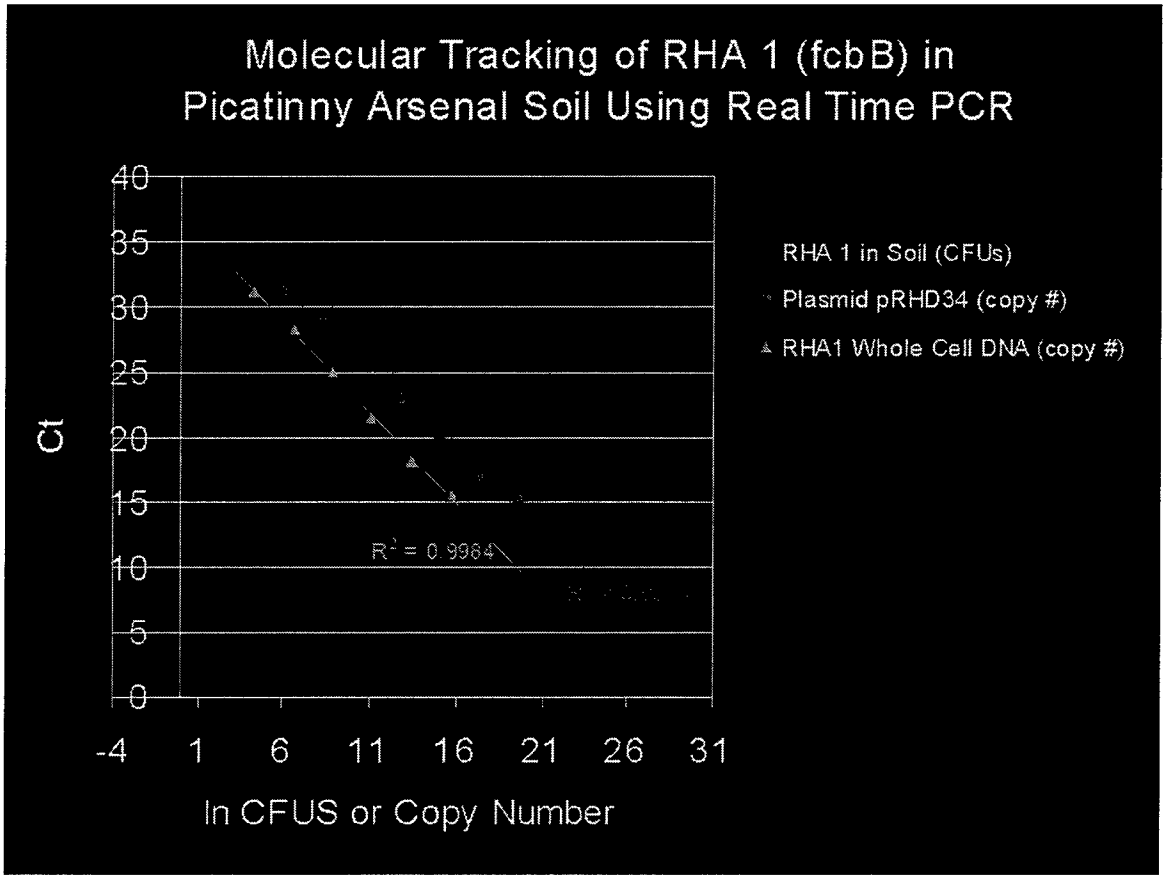


Figure 20. Molecular tracking GEMs in soil

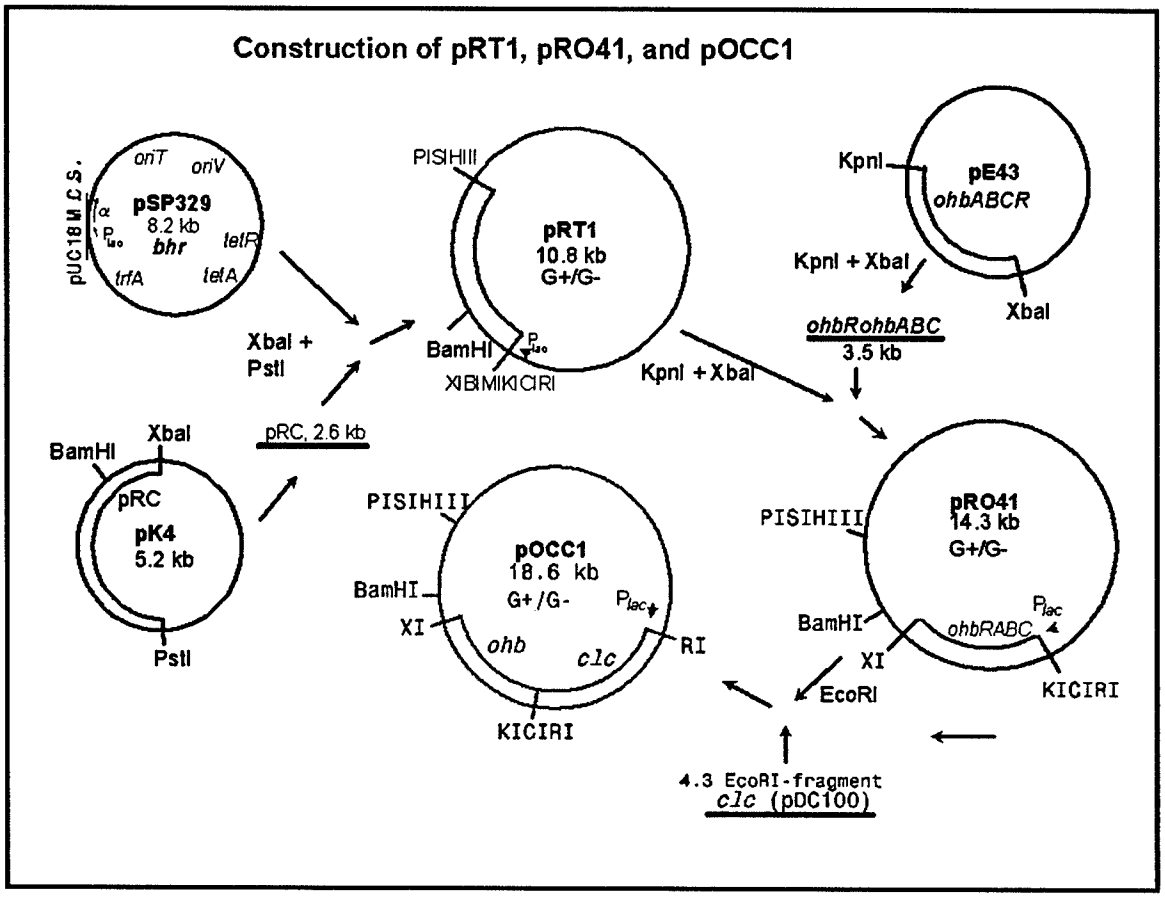


Figure 21. Construction of plasmid pRO41

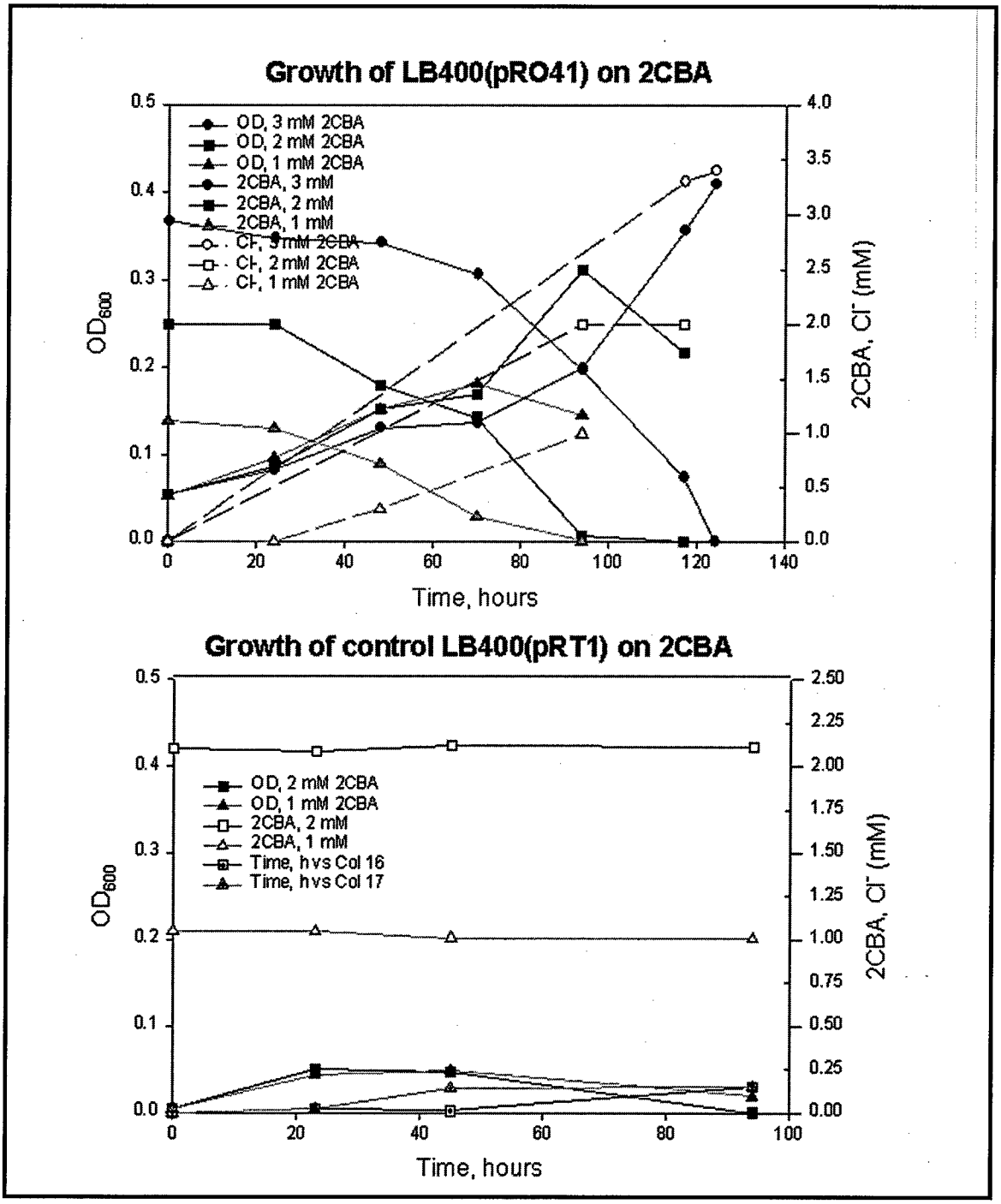
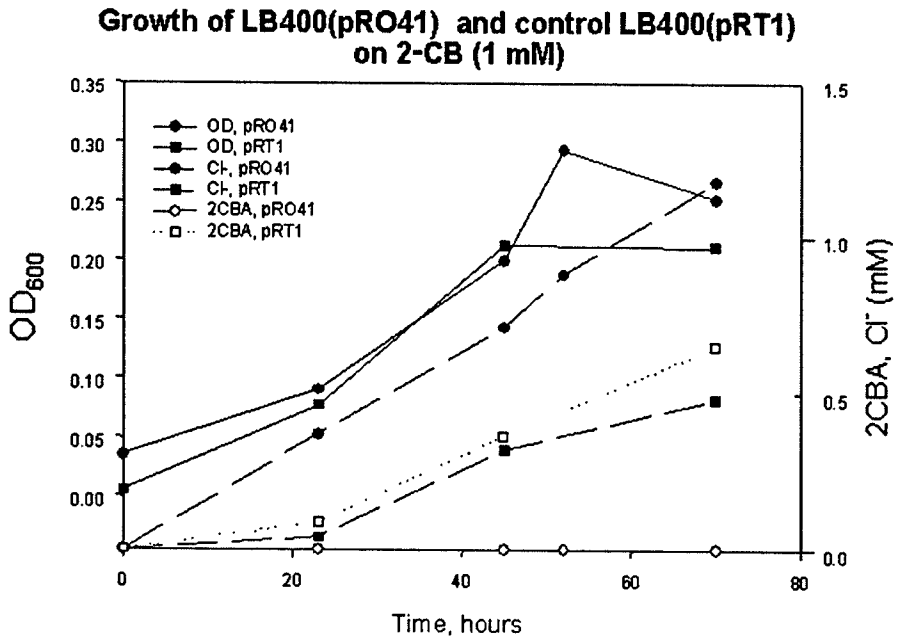


Figure 22. Growth of LB400(*ohb*) on 2CBA



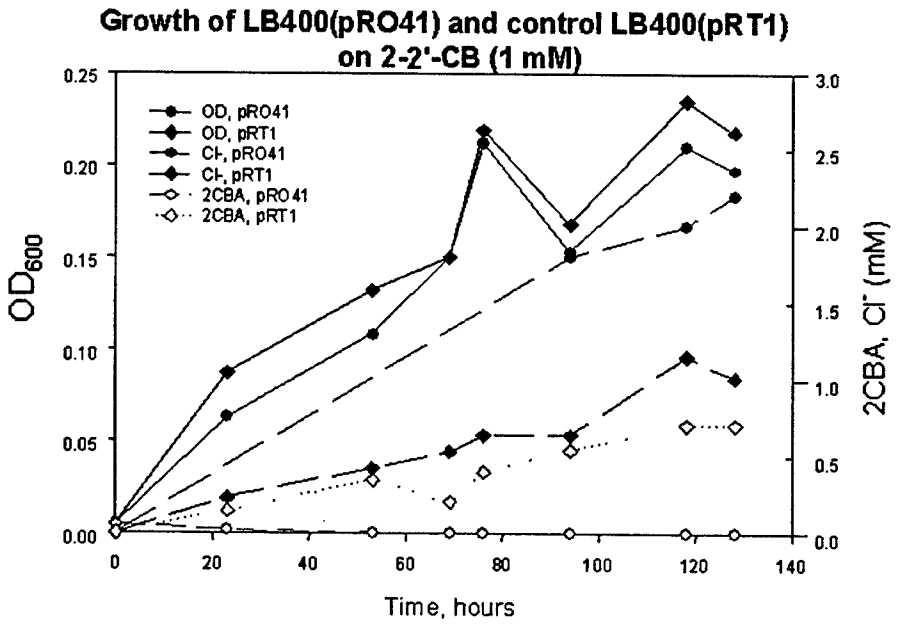


Figure 23. Growth of LB400(*ohb*) on ortho-PCBs

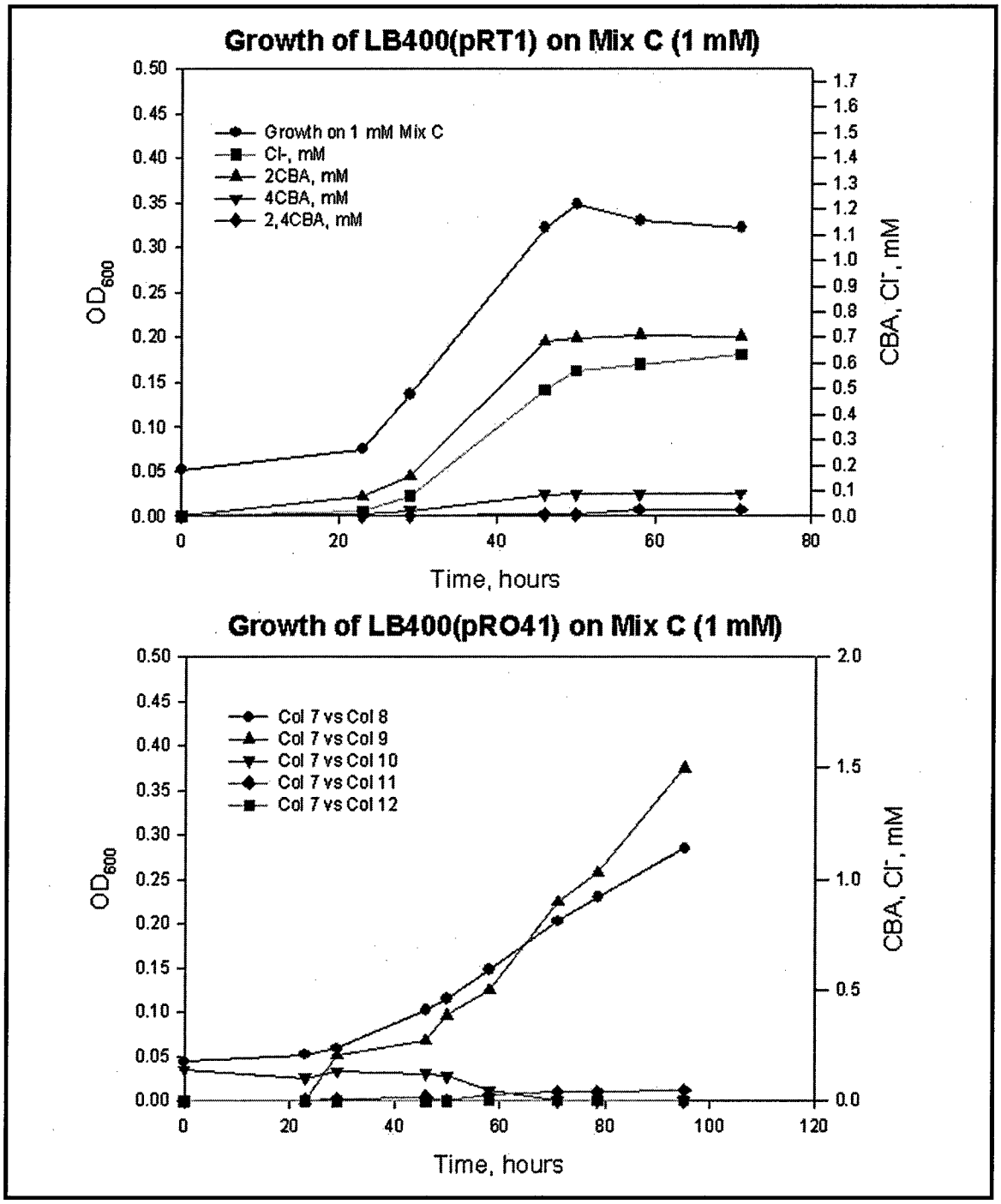


Figure 24. Growth of LB400(*ohb*) on Mix C

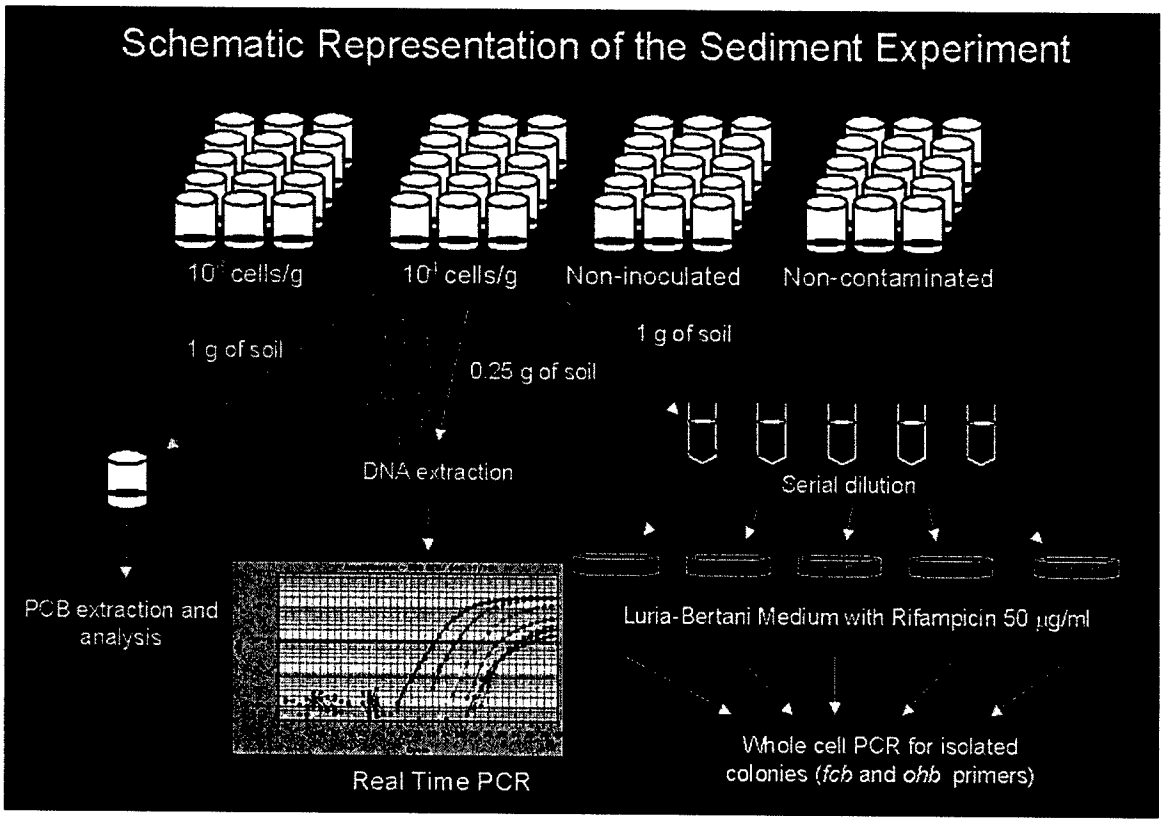


Figure 25. Scheme of microcosm experiment

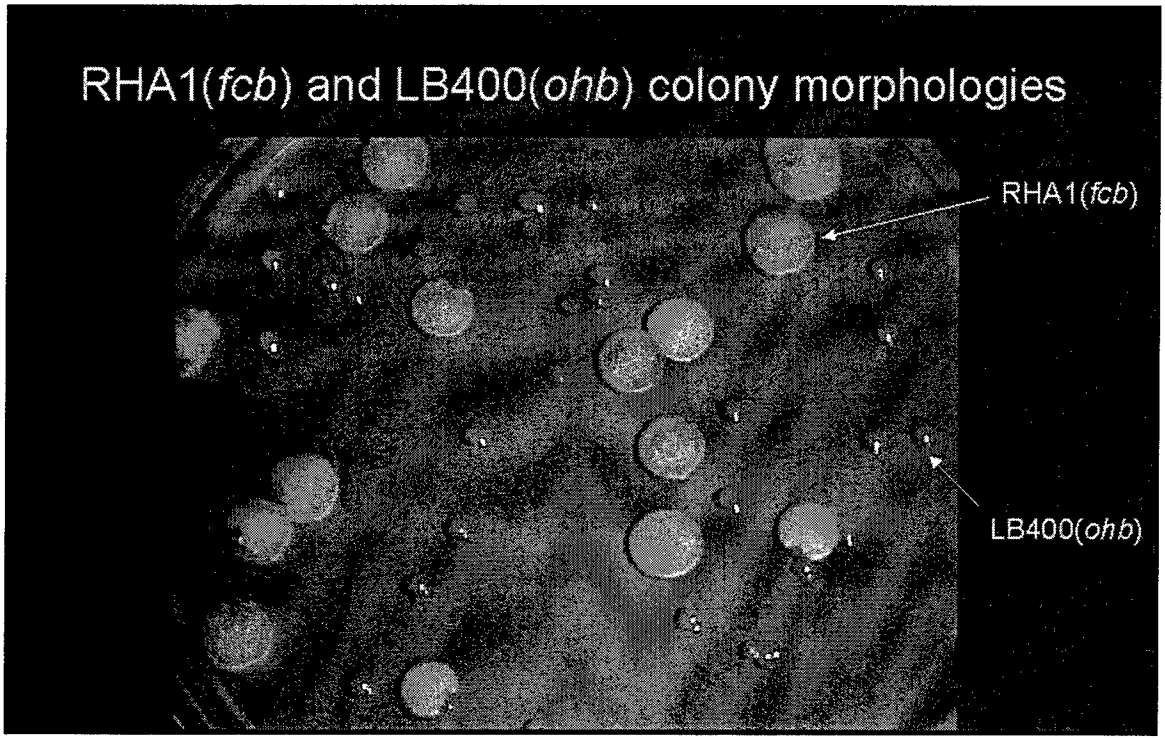


Figure 26. Colony morphology of GEMs 1

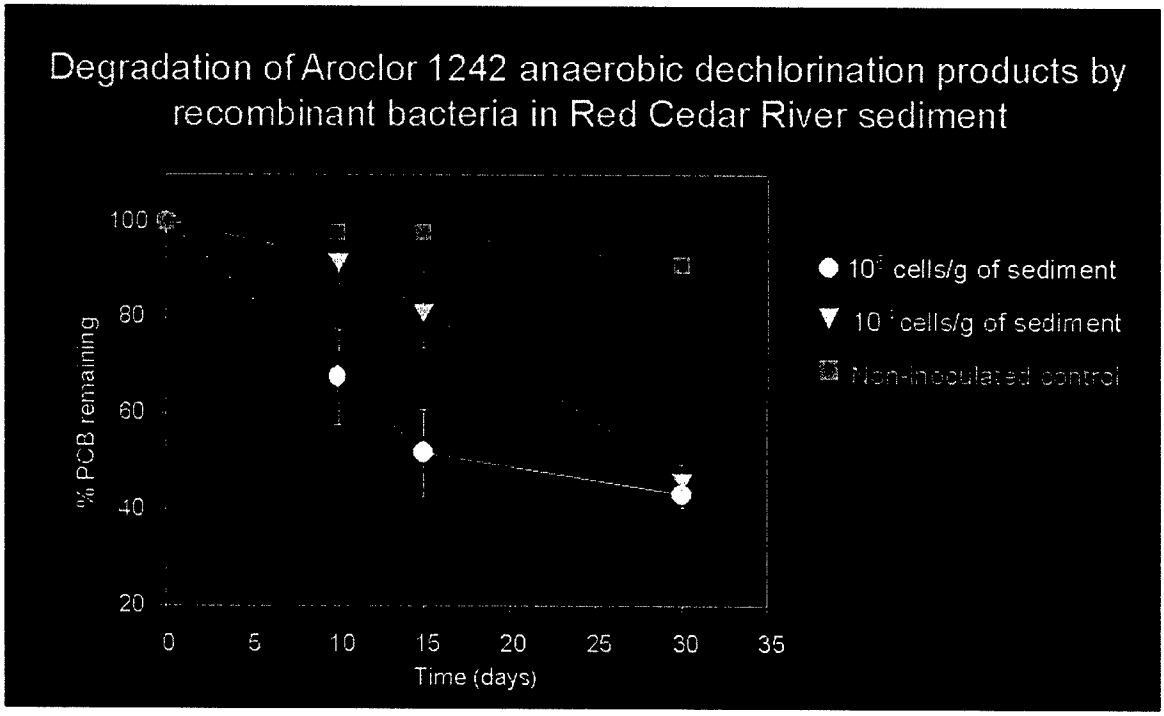


Figure 27. PCB degradation in 50% slurries

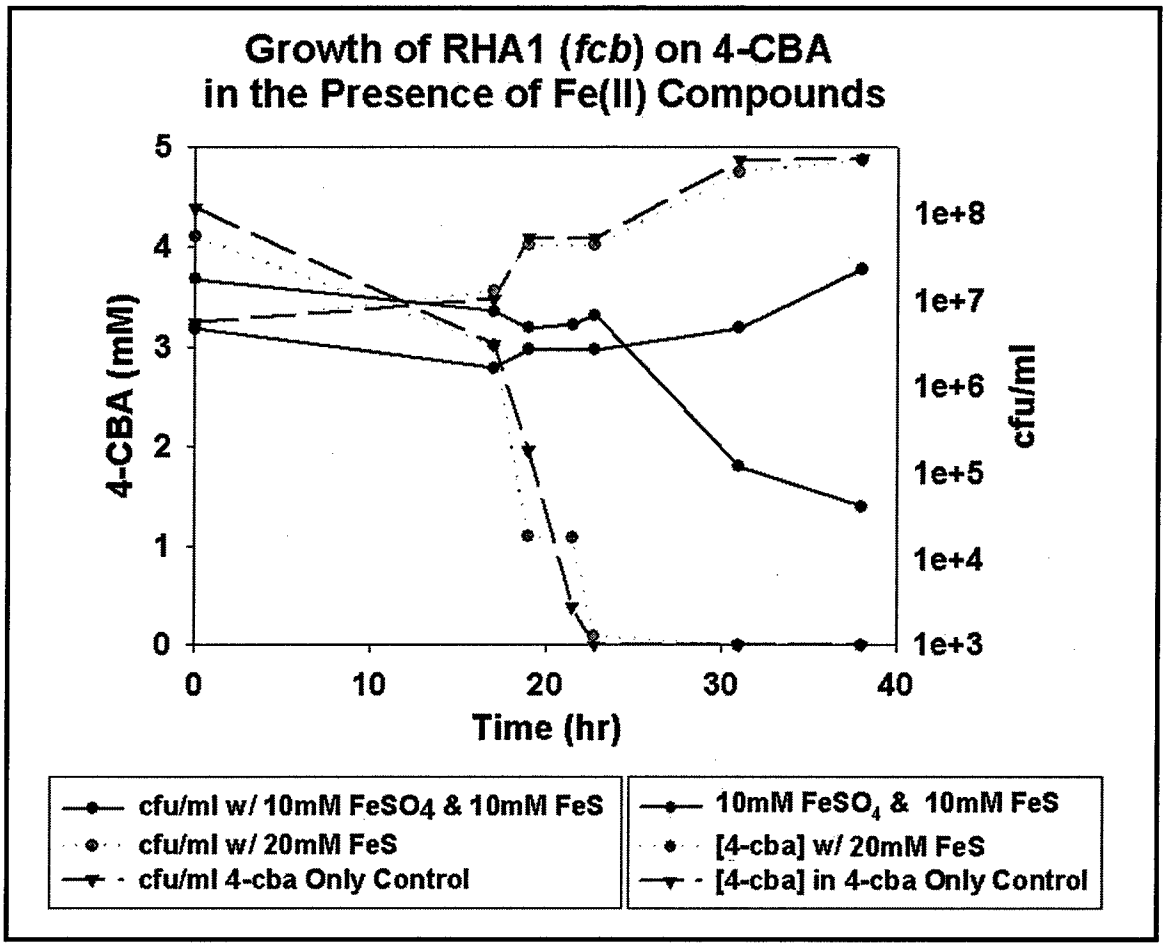


Figure 28. Effect of FeII on growth of RHA1(fcb)

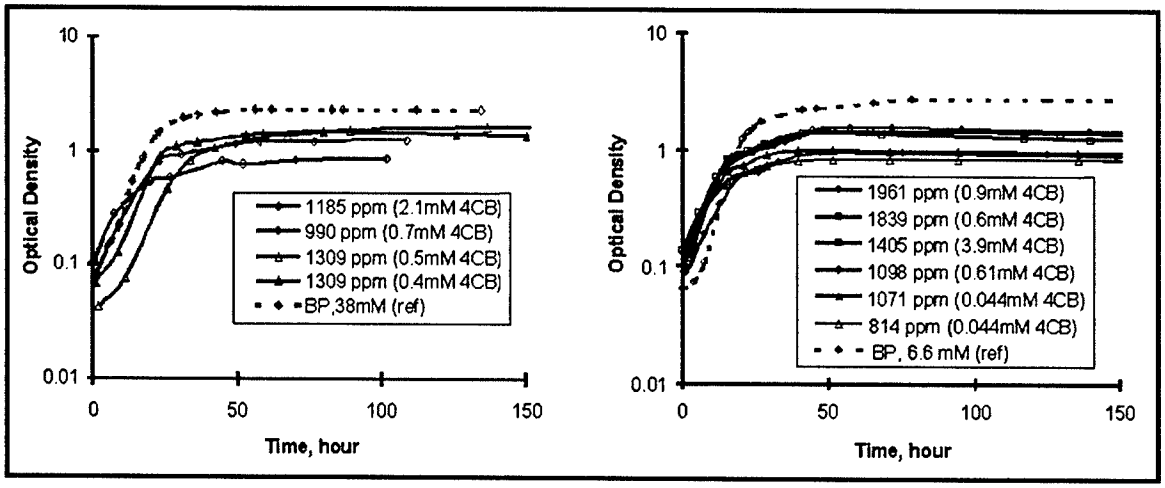


Figure 29. Examples of growth of NY05 and VP44 on Tween80 + 4-CB

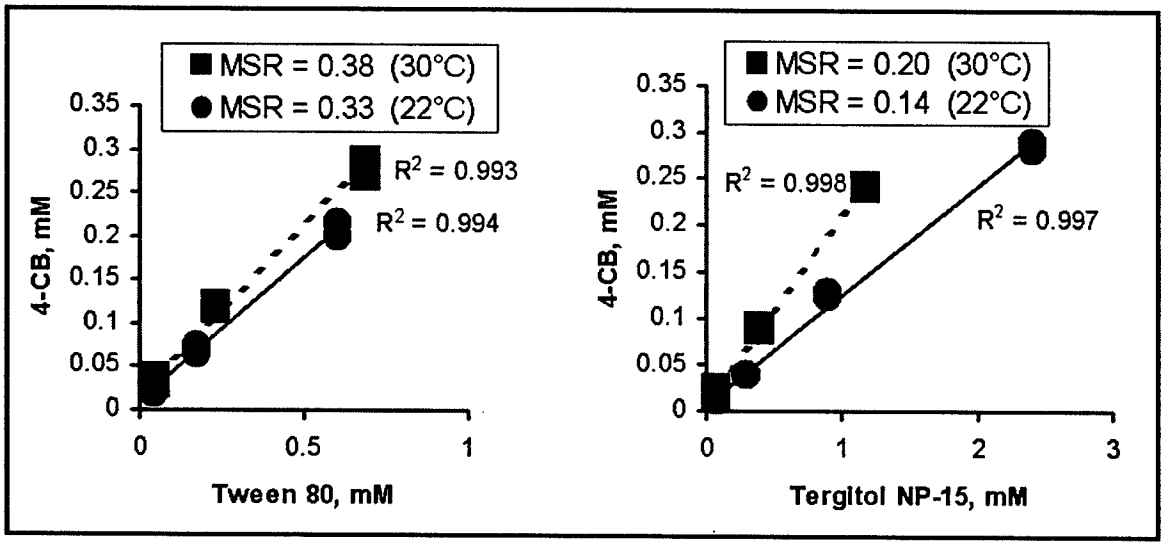


Figure 30. Solubility enhancements of 4-CB in solutions of Tween 80 and Tergitol NP-15 at 22 °C and 30 °C

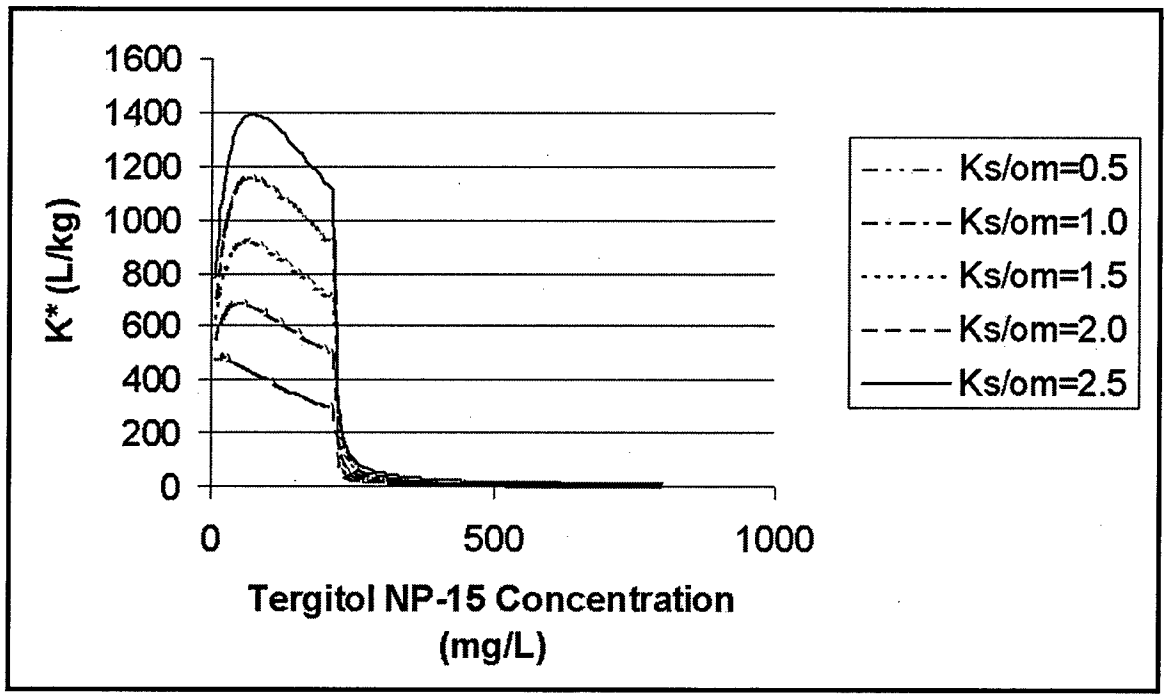


Figure 31. Effect of variations in values of Ks/om on the overall distribution coefficient

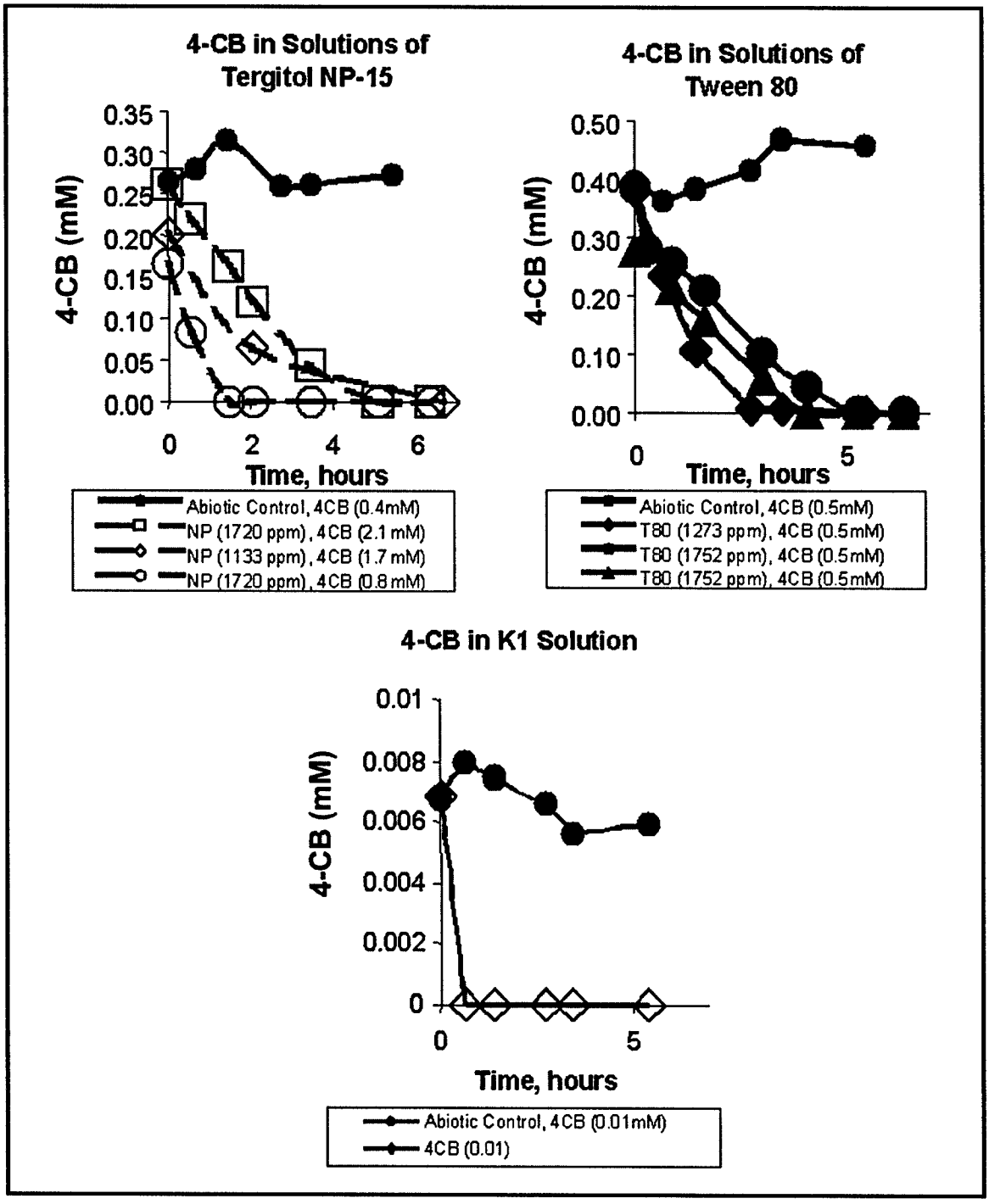


Figure 32. Transformation of 4-CB by NY05 in Solutions of Tergitol NP-15, Tween 80, and medium K1

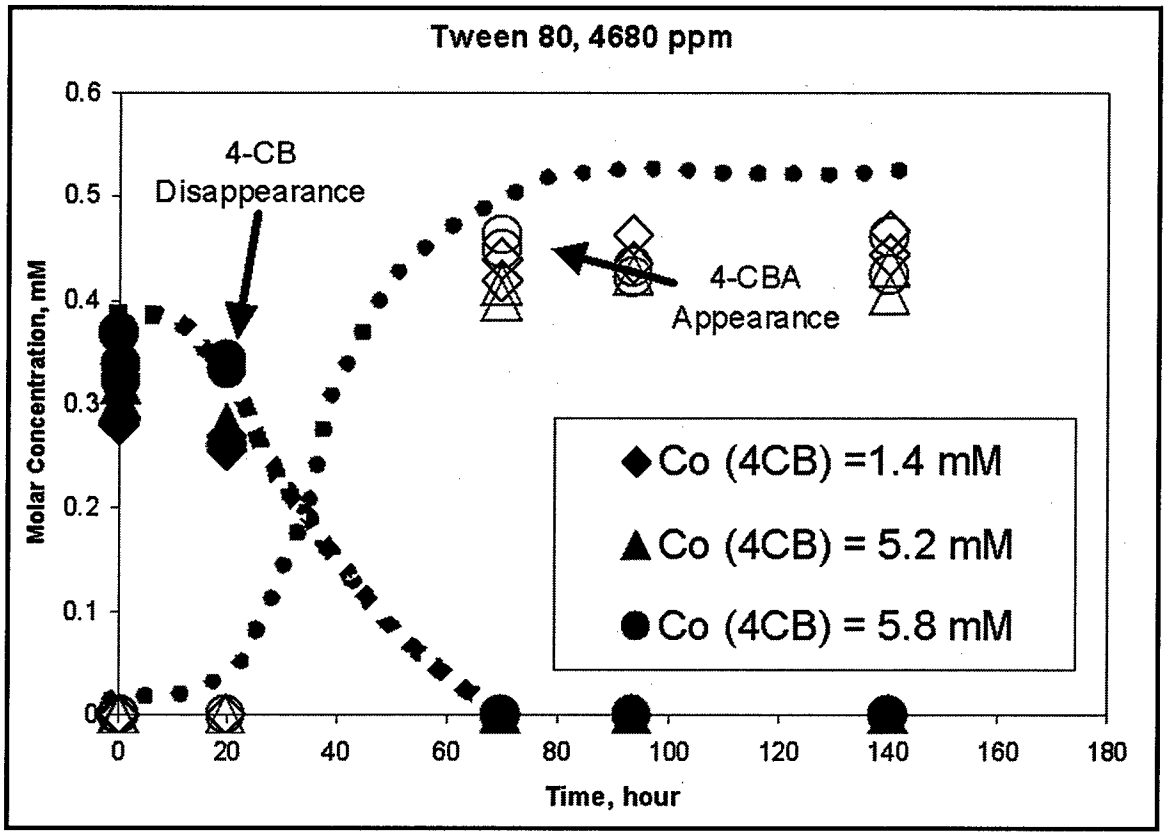


Figure 33. Disappearance of 4-CB and production of 4-CBA by NY05 in solution of Tween 80 (4,680 mg/l)

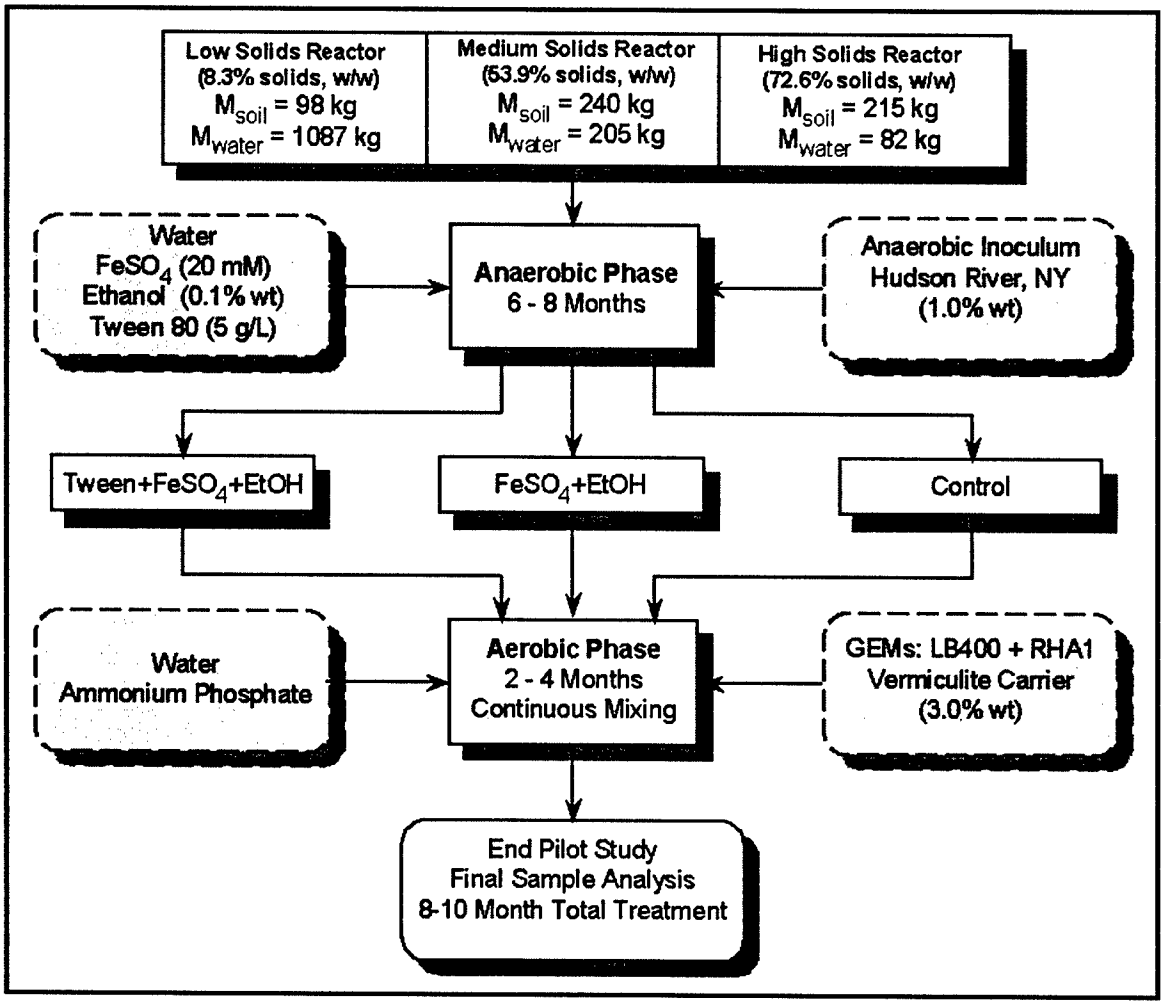


Figure 34. Flow chart illustrating process streams for pilot-scale biotreatment of Rome soil

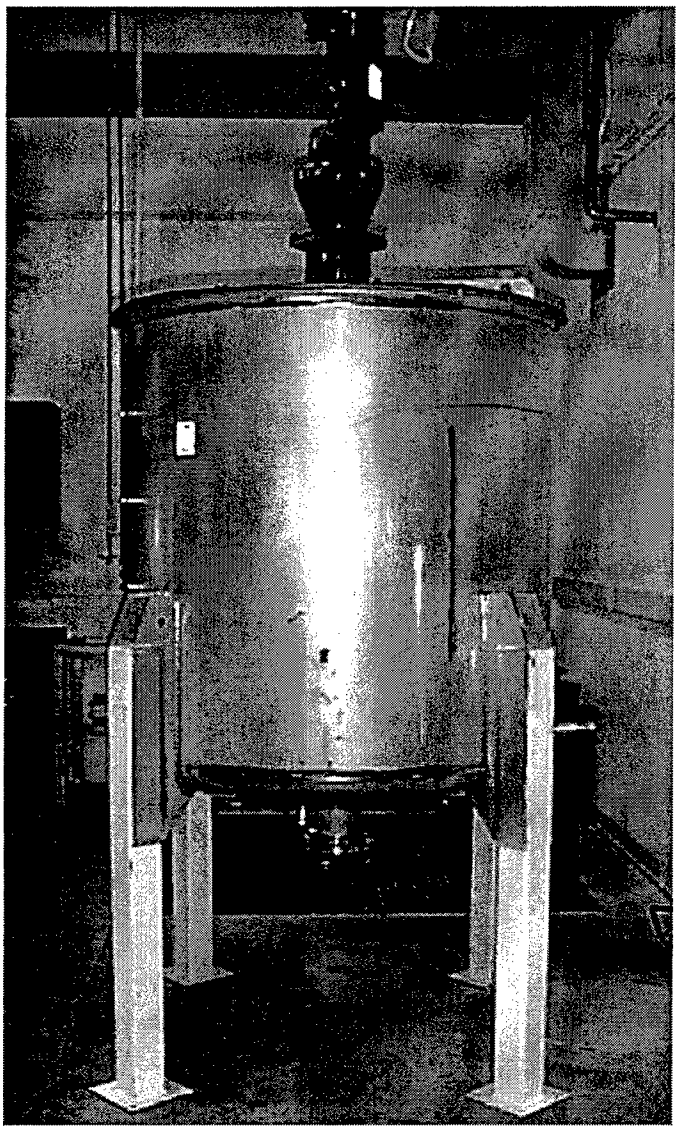


Figure 35. Low solids reactor 1

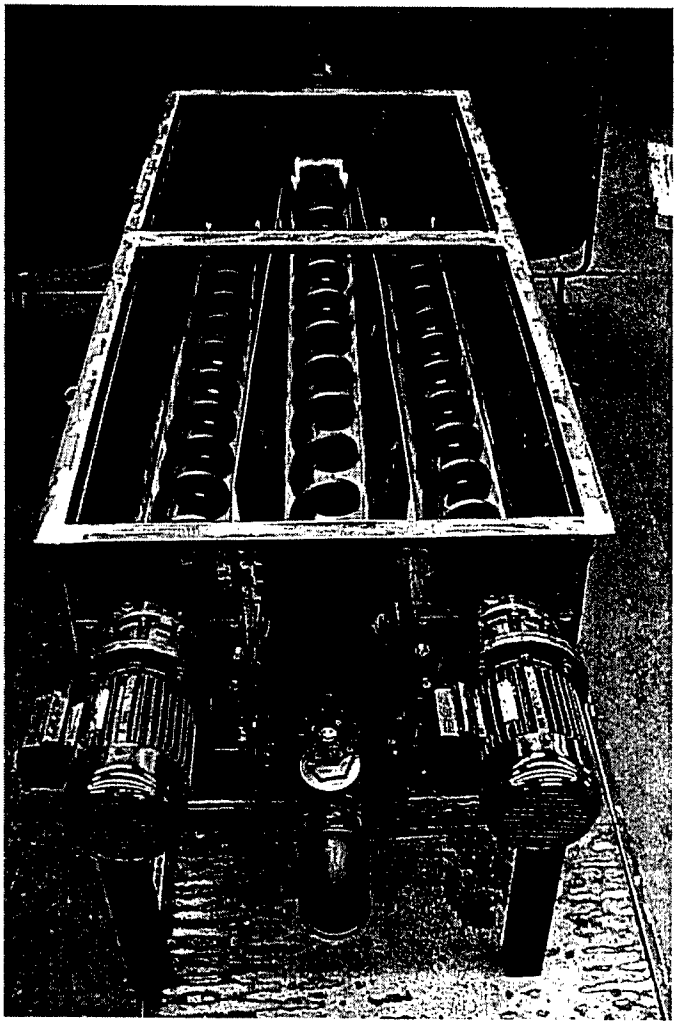


Figure 36. Medium solids reactor 1

Figure 37. High solids reactor

Table 1
Regiospecificites of the Various Described PCB Dechlorination Processes

Dechlorination Activity	Susceptible Chlorines
M	Flanked & unflanked meta
Q	Flanked & unflanked para
H	Flanked para Doubly flanked meta
H'	Flanked para Meta of 2,3- & 2,3,4- groups
P	Flanked para
N	Flanked meta
T	Meta of hepta- & octa-CBs
LP	Unflanked para

Table 2
Transformation of the Individual Congeners Contaning Chlorine on One Biphenyl Ring by Resting Cells of *R. erythreus* NY05, *C. testosteroni* VP44, *Rhodoccus* sp. RHA1 and *Burkholderia* sp. LB400[a]

Congener	Initial conc. (μM)	Disappearance of PCB (%)[b]				Yield of CBA (%)[b]			
		NY05	VP44	RHA1	LB400	NY05	VP44	RHA1	LB400
2-CB	500	90-95	90-100	95-100	95-100	2-CBA 90-95	2-CBA 90-95	2-CBA 55-65	2-CBA 65-75
4-CB	500	95-100	95-100	95-100	95-100	4-CBA 90-95	4-CBA 95-100	4-CBA 90-95	4-CBA 95-100
2,4-CB	200	95-100	95-100	95-100	95-100	2,4-CBA 95-100	2,4-CBA 95-100	2,4-CBA 90-95	2,4-CBA 90-100
2,6-CB	50	0-5	0-5	0-5	5-10	2,6 CBA <1	2,6 CBA <1	2,6 CBA 3-5	2,6 CBA 3-5

[a] Biphenyl grown cells were incubated for 24 h in K_1 medium containing the individual congeners.
[b] Range of six observations.

Table 3
Degradation of PCB Congeners Contaning Chlorine on Both Biphenyl Rings by Resting Cells of *R. erythreus* NY05, *C. testosteroni* VP44, *Rhodoccus* sp. RHA1 and *Burkholderia* sp. LB400[a]

Congener	Initial conc. (μM)	Disappearance of PCB (%)[b]				Yield of CBA (%)[b]				Accumulation of HOPDA[c]			
		Ny05	VP44	RHA1	LB400	NY05	VP44	RHA1	LB400	NY05	VP44	RHA1	LB400
-CB	100	80-90	80-90	95-100	90-100	4-CBA 5-10 2-CBA 2-5	4-CBA 2-5	4-CBA 95-100	4-CBA 80-90	+++++ λ_{max} = 396	+++++ λ_{max} = 396	ND	ND
4'-CB	50	95-100	90-100	75-85	80-90	2,4-CBA 5-10	2,4-CBA 2-5	4-CBA 70-80	4-CBA 50-60 2,4-CBA 2-5	+++++ λ_{max} = 396	+++++ λ_{max} = 396	ND	+ λ_{max} = 437
-CB	100	10-15	25-30	80-90	95-100	2-CBA 5-10	2-CBA 15-25	2-CBA 70-80	2-CBA 55-60	ND	ND	+++ λ_{max} = 394	ND
2'-CB	50	5-10	5-10	95-100	90-95	2,4-CBA 5-10	2,4-CBA 2-5	2,4-CBA 95-100	2,4-CBA 85-90	ND	ND	ND	ND

[a] Biphenyl grown cells were incubated for 24 h in K_1 medium containing the individual congeners.
[b] Range of six observations.
[c] + to +++++ reflect relative intensity of absorption at the indicated wave length; ND—not detected.

Table 4
Protein Yield and Chloride Release by Recombinant Strains VP44(pE43) and VP44(pPC3) Grown on (chloro)benzoates and (chloro)biphenyls

Substrate (mM)	44(pE43)		44(pPC3)	
	Protein [µg/ml] (±SD)	Chloride [mM] (±SD)	Protein [µg/ml] (±SD)	Chloride [mM] (±SD)
Benzoate (5)	181 (39)	0.4 (0.1)	228 (24)	0.7
(0.1)				
2-CBA (5)	169 (15)	5.3 (0.4)	<10	<0.1
2-CBA (10)	373 (16)	10.1 (1.2)	<10	<0.1
4-CBA (5)	<10	<0.1	158 (9)	7.1
(0.2)				
4-CBA (10)	<10	<0.1	257 (16)	10.4
(0.1)				
Biphenyl (2)	154 (10)	0.5 (0.1)	188 (9)	0.1
(0.1)				
2-CB (2)	177 (4)	1.9 (0.2)	69 (7)[b]	0.1
(0.1)				
4-CB (2)	84 (6)[a]	0.7 (0.3)	160 (39)	1.8
(0.2)				

Table 5
Summary of Results Obtained from NY05 and VP44 Growth Studies

	Witconol SN-120	Tween 80	Tergitol NP-15
Surfactant alone	NO growth	Growth	NO growth
Surfactant + biphenyl	NO growth	Growth	Growth
Surfactant + 4-CB	NO growth	Growth	Growth

Table 6
Example of Plasmid Stability Results

Substrate	Plasmid gene	Initial Substrate	PCR Analysis
Biphenyl + K1	RHA1+fcb	4-CBA	5/5
Tween 80 + K1	RHA1+fcb	4-CBA	5/5
TNP15 + K1	RHA1+fcb	4-CBA	5/5

Table 7
PCB Profile and Contamination Levels for Different Sites

Picatinny Arsenal Site, Picatinny, NJ			Rosenberg Superfund Site, Cortland, NY			Richardson Hill Superfund Site, Oneonta, NY		
Sample ID	Conc. (µg/g)	PCB Aroclor	Sample ID	Conc. (µg/g)	PCB Aroclor	Sample ID	Conc. (µg/g)	PCB Aroclor
PA20241	136	1260	RS1-1	0.7	1254	1	4.6	1248
PA20242	122	1260	RS2-1	0.7	1254	2	10.6	1248
PA20243	102	1260	RS3-1	0.4	1254	3	84.4	1248
PA20244	105	1260	RS4-1	0.5	1254	4	72.4	1248
			RS5-1	0.2	1254	5	69.0	1248
			RS6-1	0.3	1254	6	35.6	1248
			RS7-1	0.8	1254	7	29.7	1248
			RS8-1	1.8	1254	8	22.6	1248
			RS9-1	0.5	1254	9	13.9	1248
			RS10-1	0.1	1254	10	29.5	1248
			RS11-1	0.5	1254			
			RS12-1	0.6	1254			

Table 8
PCB Profile and Contamination Level in Rome, GA, Soil

Sample ID	Bore Hole*	Depth (ft)	Sample A Conc. (µg/g)	Sample B Conc. (µg/g)	Sample C Conc. (µg/g)
GT-1	1	1	590.5	2412.9	765.6
GT-2	1	1 – 1.5	940.6	653.1	597.7
GT-3	1	1 – 1.5	254.7	505.3	526.6
GT-4	1	1.5 – 2	798.5	852.2	687.9
GT-5	1	1 – 1.5	577.3	807.6	435.9
GT-6	2	2	294.4	155.2	154.0
GT-7	2	1.5	13.6	1.6	114.0
GT-8	2	1.5	214.4	158.7	208.1
GT-9	1	2	201.8	353.9	100.9

*Bore hole #1 located approximately 0.5 feet from SB9B-5
*Bore hole #2 located approximately 1.5 feet from SB9B-5
The PCB contamination is predominantly Aroclor 1242

Table 9
Experimental Design

Reactor	No. of Treatments	Treatments		
Low solids	3	Surfactant + Iron + Ethanol	Iron + Ethanol	No amendments
Medium solids	3	Surfactant + Iron + Ethanol	Iron + Ethanol	No amendments
High solids	3	Surfactant + Iron + Ethanol	Iron + Ethanol	No amendments

Table 10
Rome, GA, Soil Chemical Characterization

Nutrients	Conc. (mg/g)	Metals	Conc* (µg/g)
TKN	0.51	B	21.27
TP	0.58	As	26.99
TOC	6.27	Cd	6.17
		Cr	90.25
		Co	15.97
		Cu	20.70
		Pb	47.77
		Mn	427.67
		Mo	6.87
		Ni	70.44
		Se	457.47
		Zn	287.37
		K	281.65
		Na	8.16
		Mg	30.82
		Ca	261.84
		Fe	1.79
		Al	1057.46

*Metal concentration is on dry basis

Table 11
Reactor Loading Conditions

	Soil[1] (kg)	Water (kg)	Solids Content (%)	Ferrous Sulfate[2] (kg	Ethanol (kg)	Surfactant[3] (kg)	Inoculum[4] (kg)
Low Solids Reactor # 1	98	1087	8.27	6.05	1.25	5.43	1.78
# 2	98	1087	8.27	6.05	1.25	0.00	1.78
# 3	98	1087	8.27	0.00	0.00	0.00	1.78
Medium Solids Reactor	240	205	53.90	1.15	0.47	1.03	4.33
# 1	240	205	53.90	1.15	0.47	0.00	4.33
# 2	240	205	53.90	0.00	0.00	0.00	4.33
# 3							
High Solids Reactor # 1	215	82	72.58	0.45	0.31	0.41	3.91
# 2	215	82	72.58	0.45	0.31	0.00	3.91
# 3	215	82	72.58	0.00	0.00	0.00	3.91

[1]Soil mass is on dry basis.
[2]Ferrous sulfate was applied as $FeSO_4.7H_2O$
[3]Surfactant was applied as Tween 80
[4]Inoculum was applied as wet Hudson River sediment

Table 12
Sample Analysis Schedule

	t =0	t =1	t =2	t =3	t =4	t =5	t =6	t =7	t =8	t =9	t =10	t=11	t=12	t=13	t =14	t =15	t =16
Day	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240
PSD	X																X
Atterberg Limits	X																X
Bulk Density	X																X
Surface Area	X																X
Leachability	X																X
Metals Concentration	X																X
Temperature	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Oxygen Concentration	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Moisture Content	X		X		X		X		X		X		X		X		X
pH	X		X		X		X		X		X		X		X		X
PCB Concentration	X		X		X		X		X		X		X		X		X
Microbial Biomass	X		X		X		X		X		X		X		X		X
TOC	X		X		X		X		X		X		X		X		X
TKN	X		X		X		X		X		X		X		X		X
TP	X		X		X		X		X		X		X		X		X
Surfactant	X		X		X		X		X		X		X		X		X

t =13 is the tentative start of aerobic phase.

Attachment D-1.
Technology Transfer Documents

Peer Reviewed Journal Articles

Best, E.P.H., M. Zappi and H.L. Fredrickson, (1997) Fate of TNT and RDX in aquatic and wetland-based systems during treatment of contaminated groundwater. *In*: R. Bajpai and M. E. Zappi (eds.), Bioremediation of Surface and Subsurface Contamination, *Annals of the New York Academy of Science*, Vol. 829.

Harvey, S.D., H.L. Fredrickson, M.E. Zappi and D.O. Hill, (1997) Evaluation of bioslurry ecosystems for removal of TNT from contaminated Soil. *In*: R. Bajpai and M. E. Zappi (eds.), Bioremediation of Surface and Subsurface Contamination, *Annals of the New York Academy of Science* , Vol. 829

Hrywna Y., T.V. Tsoi, O.V. Maltseva, J.F. Quensen III, J.M. Tiedje. 1999. Construction and characterization of two recombinant bacteria that grow on ortho- and para-substituted chlorobiphenyls. Appl. Environ. Microbiol. **65**(5):2163-2169.

Howell, D.P., Pennell, K.D. and Tiedje, J.M. Biological compatibility of selected nonionic surfactant with PCB-degrading bacteria. **(Manuscript in preparation).**

Gunnison, D., Fredrickson, H., Kaplan, D. L., Mello, C. M., Walker, J. E., Myrick, G., and Ochman, M. (1997) Application of Continuous Culture Technology for the Development of Explosives-Degrading Microorganisms, *In*: R. Bajpai and M. E. Zappi (eds.), Bioremediation of Surface and Subsurface Contamination, *Annals of the New York Academy of Science* , Vol. 829, pp. 230-241.

Maltseva, O. V., T. V. Tsoi, J. F. Quensen III, M. Fukuda, and J. M. Tiedje. 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation 10:363-371.

Pennell, K.D., A.M. Adinolfi, L.M., Abriola and M.S. Diallo. 1997. Solubilization of dodecane, tetrachloroethylene and 1,2-dichlorobenzene in micellar solutions ofethoxylated nonionic surfactants. Environmental Science and Technology 31:1382-1389.

Rodrigues, J.L.M., O.V. Maltseva, T.V. Tsoi, R.R. Helton, J.F. Quensen III, M.Fukuda, J.M. Tiedje. 2001. Development of a *Rhodococcus* recombinant strain for degradation of products from anaerobic dechlorination of Aroclor 1242. Environ. Sci. Technol. **35**:663-668.

Rodrigues J. L. M., Aiello M. R., Urbance John W., Tsoi T. V., and J. M. Tiedje. Quantification of the PCB degrader *Rhodococcus* sp. strain RHA1(*fcb*) in soil by real time PCR. (Manuscript submitted to Appl. Environ. Microbiol.)

Romanov V., R. Hausinger (1996) NADPH-Dependent Reductive ortho Dehalogenation of 2,4-Dichlorobenzoate in Corynebacterium sepedonicum KZ4 and Coryneform Bacterium Strain NTB-1 via 2,4-Dichlorobenzoyl Coenzyme A. J. Bacteriol., 176(9):2656-2661.

Speakman, R. and K.D. Pennell. An economic analysis of surfactant enhanced aquifer remediation systems. (submitted to Ground Water Monitoring and Remediation)

Taylor, T.P. and K.D. Pennell. Micellar solubilization and recovery of 2,4,6-trinitrotoluene from soil columns flushed with ethoxylated nonionic surfactants (submitted to Environ.Sci. Technol.).

Tiedje J.M., T.V. Tsoi, J.F. Quensen III, J.L.M. Rodrigues, O.V. Maltseva, M.R. Aiello, J.F. Stoddard, C.A. Kachel. Designing and validation of genetically enhanced two-phase anaerobic-aerobic PCB remediation system. (manuscript in preparation).

Tsoi, T. V., E. G. Plotnikova, J. R. Cole, W. F. Guerin, M. Bagdasarian, and J. M. Tiedje. 1999. Cloning, expression and nucleotide sequence of the *Pseudomonas aeruginosa* strain 142 ohb genes coding for oxygenolytic *ortho*-dehalogenation of halobenzoates. Appl. Environ. Microbiol. 65 (5):2151-2162.

Tsoi, T.V., J.R. Cole, E.G. Plotnikova, S. Rech, B. Sun, J. Davis, O.V. Maltseva, J.L.M. Rodrigues, and J.M. Tiedje. Multiple aryl reductive ortho-dehalogenases cloned and found functional in Nocardioides sp. strain KZ4. (Manuscript in preparation).

Yeh, D.H., K.D. Pennell and S.G. Pavlostathis. 1998. Toxicity and biodegradability screening of nonionic surfactants using sediment-derived methanogenic consortia. Wat. Sci. Technol. 38: 55-62.

Yeh, D.H., K.D. Pennell and S.G. Pavlostathis. 1999. Effect of Tween surfactants on methanogenesis and microbial reduction dechlorination of hexachlorobenzene. Environ. Tox. Chem.. Accepted.

Ye, D., J.F. Quensen, J.M. Tiedje, S.A. Boyd. 1999. 2-Bromoethanesulfonate, sulfate, molybdate, and ethanesulfonate inhibit anaerobic dechlorination of polychlorobiphenyls by pasteurized microorganisms. Appl. Environ. Microbiol. **65**:327-329.

Textbooks/Book chapters/Guidance manuals

DESCRIPTION OF GENETICALLY ENGINEERED ORGANISMS (GEMs) TO BE USED IN PCB BIOREACTOR STUDIES. Guidance manual prepared January 2001. (see Attachment D-3)

Patents

Patent on "Mixing Performance of SSR" in draft

Attachment D-2.
PCB Remediation - Technology Comparison

Assessment of PCB Treatment Alternatives

When the public first became aware of the dangers of xenobiotic compounds released into the environment, polychlorinated biphenyls (PCBs) were some of the first contaminants to come under scrutiny. In fact, PCBs are the only substances that were specifically listed by the Toxic Substance Control Act (TSCA) of 1976. Due to their toxicity and carcinogenic effects, the manufacture, transport and use of PCBs were banned after 1979. However, restrictions have since been relaxed to some degree. Exceptions are now often granted for PCB use within contained systems and where there is no unwarranted threat to human health or environment (Erickson, 1993). In addition, EPA has dropped its ban on the importation of PCB-contaminated wastes so that US waste management companies could incinerate or otherwise treat these wastes (Johnson, 1996).

PCBs contain from 1 to 10 chlorine atoms substituted for biphenyl hydrogen atoms, giving rise to 209 possible permutations, or congeners, each of which may have somewhat different chemical and physical properties. PCBs with a large number of chlorines are extremely hydrophobic, have low solubility in water, and readily partition into solid organic phases. Table 1 lists some physical and chemical properties of selected PCB congeners.

Table 1. Properties of selected chlorobiphenyls (CBPs)

Congener	Aqueous Solubility (mg/L)	Vapor pressure (Pa @ 25°C)	Henry's Constant (Pa-m^3/mol)	log K_{ow}
4-CBP	1.20	0.27	42.56	4.40
2, 4'- CBP	1.00	0.147	45.39	5.10
3,3'4- CBP	0.0514	0.014	8.13	5.53
3,3'4,4'- CBP	0.001	5.88E-05	4.37	6.29
2,3,3',4',6- CBP	0.0073	0.00228	17.12	6.27
2,2',3,3',5,6- CBP	0.00091	0.00127	20.67	6.55
2,2',3,4,4',5,5'- CBP	0.00031	0.00013	30.40	7.21
2,2',3,3',4,4',5,5'- CBP	0.000277	2.07E-05	47.52	7.47
2,2',3,3',4,5,5',6,6'- CBP	0.000018	3.08E-05	32.53	8.16
DecaCBP	1.20E-06	1.40E-06	20.84	8.26

PCBs were originally manufactured for their excellent dielectric and insulating properties, allowing heat conduction without conducting electricity. In addition, PCBs

exhibit thermal and chemical stability, low volatility, and are non-flammable (Clark, 1993). As such, PCBs have been used extensively across a wide range of industries, particularly in heat transfer and hydraulic systems, pigments, plasticizers, and as a fire retardant in hydraulic oils (Lang, 1992). Their most common use at present is in dielectric fluids for capacitors and transformers. For most of these uses, PCBs are usually dissolved in mineral oil.

Prior to 1977 most of the PCBs manufactured in the U.S. were produced by Monsanto Corporation and were distributed as mixtures of congeners called Aroclors. Similar mixtures, marketed under different trade names, were produced by other companies around the world (Lang, 1992). Aroclors were assigned a number to describe their degree of chlorination. For example, Aroclor 1242 is a mixture which contained 42% chlorine by weight, with the 12 simply representing the number of carbon atoms in the molecule. Monsanto reduced sales in 1972 to those who manufactured electrical transformers and capacitors, and ceased production altogether in 1979. EPA has estimated that up to and including 1975, between 136,000 and 181,000 metric tons (300 million and 400 million pounds) of PCBs entered the environment. High concentrations are prevalent in industrial areas, in some cases exceeding 4000 mg/L in soil, but PCBs are also found in the air, water, and soils of remote sites as well (Bowlds, 1992). The World Health Organization (WHO) estimates that individuals in industrialized nations consume 5 to 15 μg of PCBs daily (Phibbs, 1996).

When PCBs were first manufactured, there was a lack of recognition of the hazards posed by PCBs. Although data on human health effects are limited, two mass PCB poisonings in East Asia caused symptoms such as liver damage, dermal lesions, neurological system damage and immunodeficiency among others. PCBs have been found to have a carcinogenic effect on people exposed to the mixtures (Lang, 1992). Studies in animals have shown that exposure to PCBs can result in tumors, fetal resorption, birth defects, and high offspring mortality rates. Other responses include respiratory disorders, endocrine effects, and ocular damage (Lang, 1992). Safe (1992) observed that the coplanar polychlorinated congeners (3,3',4 ,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl), as well as their monochlorinated *ortho* derivatives, elicit responses similar those caused by dioxins and can be considered to be the most toxic congeners. Recently, however, data concerning the potency of PCBs as a carcinogen has been reassessed, and the risk of carcinogenicity has subsequently been downgraded by EPA, which may impact future cleanup decisions (Phibbs, 1996). The EPA has established reference doses (RfDs) for two specific mixtures of PCBs. The RfD for Aroclor 1254 is 0.02 μg per kilogram body weight per day, and the RfD for Aroclor 1016 is 0.07 μg per kg per day (EPA, 1996).

Summary of Federal Regulations Concerning PCBs

Since PCBs are specifically listed in TSCA, they are subject to additional federal regulations beyond those of most other hazardous compounds, which are subject to Resource Conservation and Recovery Act (RCRA) of 1976 and the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) of 1980. TSCA is enforced by EPA, and the methods and standards for remediation of PCBs are clearly defined in these federal regulations. Rules have been promulgated for self-implementing cleanups which apply when the contamination does not affect property outside the boundary of the cleanup site or the public health or environment in general. These rules would not apply to the cleanup of surface or groundwater, for example, because the waters do remain within a definable boundary. Likewise, the rules would not apply to marine or freshwater sediments, sewers or sewage treatment systems, drinking water sources or distribution systems, grazing lands, and vegetable gardens (40 CFR 761.61 (a)(1)).

To initiate cleanup of a PCB-contaminated site under TSCA, EPA requires written notification to the EPA Regional Administrator and the state and local environmental protection agencies. The following information must be supplied 30 days before cleanup is to begin:

- The nature of the contamination, including the materials contaminated
- Sampling procedures used, with dates of analysis and dates and locations of sampling
- Location and degree of contamination, including a topographic map
- A detailed cleanup plan, including schedule, approach and disposal
- A contingency plan in case higher than expected concentrations or more widely distributed contamination are found

The extent of cleanup is determined by type of contaminated material and the potential for exposure to PCBs after the cleanup is completed. Therefore, different standards exist for high occupancy and low occupancy areas. A high occupancy area is defined as one where people reside, work or regularly traffic, such as a public park, whereas a low occupancy area is defined as one where there is limited access to the public and which is not regularly trafficked by individuals, for example, a unused section of an industrial campus or a military base. The maximum allowable contamination levels are listed in Table 2. TSCA regulations make allowance for sites that are unable to meet the ideal cleanup levels by allowing higher levels if access is limited by a fence posted with warning signs or if exposure is limited by capping the site with an impermeable layer such as concrete. If a site is decontaminated to the level of a low occupancy level, it must remain low occupancy in perpetuity unless cleaned to the more stringent high occupancy level.

In cases where these contaminant levels are still exceeded after treatment, the wastes must be landfilled in a RCRA facility. Wastes may be transported offsite for treatment provided that they are in containers that meet the Department of Transportation's Hazardous Materials Regulations. EPA may impose more stringent standards in some cases, depending on the proximity of residential areas, schools, fisheries, environmentally sensitive areas, and national parks. EPA approval is not required for on- site remediation or soil washing provided certain conditions are met. Namely, if solvent washing is implemented, a non-chlorinated solvent must be used and disposed of properly. Furthermore, a secondary containment mechanism must be in place to prevent solvent releases from the treatment site. In addition, the process must occur at ambient temperature with no external heat applied and no exothermic heat production.

Table 2. Cleanup level requirements

Type of material	Exposure level	Clean up concentration (total PCBs)
Bulk (e.g. soils and sediments)	High occupancy - unsecured	≤1 mg/kg
	High occupancy - capped	>1 and ≤10 mg/kg
	Low occupancy -unsecured	≤25 mg/kg
	Low occupancy - fenced	>25 and ≤50 mg/kg
	Low occupancy - capped	>25 and ≤100 mg/kg
Non-porous surfaces	High occupancy	≤10 μg/100 cm^2 surface area
	Low occupancy	≤100 μg/100 cm^2 surface area
Porous surfaces	High occupancy	≤1 mg/kg
	High occupancy - capped	>1 and ≤10 mg/kg
	Low occupancy -unsecured	≤25 mg/kg
	Low occupancy - secured	>25 and ≤50 mg/kg
	Low occupancy - capped	>25 and ≤100 mg/kg
Liquids	Closed systems	<200 ≤g/L
	Discharged to treatment facility or waterway	<3 ≤g/L
	Unrestricted use	≤0.5 μg/L

PCB Treatment Alternatives

When CERCLA was originally passed in 1980, a great deal of emphasis was placed on cost balancing, with the cost of clean-up playing a major role in the decision of the treatment technique. The result was that, more often than not, PCB-contaminated and other contaminated soils were either landfilled or the sites were capped to prevent water entry, which were typically the least expensive available alternatives. Unfortunately, since these techniques did nothing to destroy the compounds, landfilling had the effect of creating additional Superfund sites (Hesketh *et al.*, 1990). When Congress passed the Superfund Amendments and Reauthorization Act (SARA) in 1986, the cost emphasis was removed, and Congress specifically stated that landfilling and capping were undesirable without first permanently reducing the toxicity, volume or mobility of the contaminant (Hesketh *et al.*, 1990).

To achieve contaminant reductions, EPA permits only a few conventional treatment technologies for PCBs, but allows and encourages research into more innovative

methods. The most commonly used method for treatment of PCB-contaminated soil to date has been incineration, which is discussed later in this report. A properly functioning incinerator can achieve 6-Log (99.9999%) or greater removal of PCBs. Another treatment technology may be used if it can be shown to achieve comparable levels of performance.

The following sections describe many of the technologies that have been considered and tested for treatment of PCB-contaminated soils and sediments. The principal advantages and disadvantages of each are presented, and where possible, appropriate case studies are described. Some of the technologies result in PCB destruction or detoxification, whereas others are implemented to reduce either the volume of contaminated waste or its mobility in the environment. Only a few of these alternatives are intended for use as the sole means of remediation. The processes are usually combined with the aim of finding the most economical solution. Some of the advantages and disadvantages of the technologies are summarized in Table 7.

Incineration

Incinerators are usually capable of eliminating more than 99.9999% of PCB contamination from hazardous wastes. Therefore, incineration remains the most common means of PCB treatment despite its high costs, $1.65 to $6.60 per kg for soils contaminated with PCBs. Other treatment processes can be used only if it is demonstrated that they can achieve the same Destruction and Removal Efficiency (DRE) as incineration. At smaller sites, it is generally more economical to transport contaminated soils to an offsite incinerator. However, various types of mobile or transportable incinerators are commercially available for on-site incineration at large sites. The most common types of incinerators, whether off-site or on-site, are rotary kilns, infrared conveyor furnaces, and fluidized bed incinerators. Schematic diagrams of these incinerators are given in Figures 6 through 8.

- *Rotary kilns* are the most conventional type of incinerators and have proven to be adequate for small- and medium-sized sites, i.e. those with up to 30,000 tons of contaminated waste (Hesketh *et al.,* 1990). Rotary kilns can be used with most types of contaminated wastes, including soils, non-aqueous phase liquids (NAPLs), or drummed waste.
- *Infrared conveyor furnaces (IRCFs)* can also be used at small- to medium-sized sites as well. However, they are not well-suited for sites where drummed wastes or NAPLs are present. They are heated electrically and are strongly affected by both the moisture content and heat content of the soil, which are discussed in further detail below. Soil moves into the IRCF chamber on a woven metal belt. Because there is little mixing of the waste, this type of incinerator requires the particle sizes to be less than 3/4 to 1 inch in diameter. Chlorinated organic compounds may also cause the belt to corrode (Hesketh *et al.,* 1990).

Table 7: PCB Treatment Technologies

Technology	Advantages	Disadvantages	Estimated cost per kg
Incineration	Destroys PCBs	Expensive	$1.65 - $6.60
	99.9999% DRE	Possible dioxin formation	
	Quick; well-documented	Public opposition	
Thermal desorption	Insensitive to co-contaminants	PCBs not destroyed	*In situ:* $0.02 - $0.08
	Effective at high PCB concentrations	Soil sterilized	*Ex situ:* $0.045 - $0.33
		High clay and moisture content decrease efficiency	
Chemical dehalogenation	Destroys PCBs	High PCB concentrations require more chemicals	BCD: $0.11 APEG: $0.22 - $0.55
	Stand-alone technology	Size reduction required	
		High clay and moisture content decrease efficiency	
Solidification/ stabilization	Can be implemented *in situ*	PCBs not destroyed	$0.011
	Effective on inorganic co-contaminants	Contaminant leaching possible	
		Long-term monitoring required	
Vitrification	Destroys PCBs	Sensitive to soil moisture content	$0.16 – $0.47
	Effective on co-contaminants	Off-gases must be treated	
	Effective on wide range of soils		
Soil washing	Effective on co-contaminants	PCBs not destroyed	$0.02 - $0.19
	No off-gases	Sensitive to ambient temperature	
Chemical extraction	Effective at high PCB concentrations	PCBs not destroyed	Solvent extraction: $0.11 -$0.44
	Insensitive to co-contaminants	High capital costs	SFE: $0.12 - $0.19
		High clay and moisture content decrease efficiency	
Slurry-phase bioremediation	Destroys PCBs	Many parameters to contend with	$0.10 - $0.12
	Low energy costs	Desorption kinetics may limit degradation rates	
Landfarming	Destroys PCBs	Large area of land needed	$0.06
	Low maintenance	Long period of time required	
	Low operations/capital costs	May require extensive environmental controls	
Composting and biopiles	Destroys PCBs	Large area of land needed	Windrow: $0.15 Biopile: $0.18
	Low capital/maintenance costs	Many parameters to contend with	In-vessel: $0.23

- *Fluidized bed incinerators* include both bubbling beds and circulating beds. Both types of systems are very efficient for combustion but require feed size reduction in order for the beds to remain fluid. To maintain fluidity, metals should not be present in the waste feed due to their relatively low melting points, nor should there be high concentrations of sodium or potassium salts, which may react to form compounds with low melting points (Hesketh *et al.*, 1990). In their liquid state, these melted

substances would flocculate to form particles too heavy to remain suspended in the air stream. Fluidized beds are generally effective for medium-size sites (5 million to 30 million kg of waste) and for most contamination scenarios except drummed wastes, which may require substantial preparation.

- The US EPA has clearly dictated the procedures to be followed for PCB incineration. TSCA requires an incinerator destruction removal efficiency (DRE) of 99.9999%. Liquid wastes must have a minimum residence time of 2 seconds at 1200°C with 3% excess oxygen in the stack gas or 1-1/2 seconds at 1600°C with 2% excess oxygen in the stack gas. Solid wastes must also have PCB air emissions no higher than 1 *Fluidized bed incinerators* include both bubbling beds and circulating beds. Both types of systems are very efficient for combustion but require feed size reduction in order for the beds to remain fluid. To maintain fluidity, metals should not be present in the waste feed due to their relatively low melting points, nor should there be high concentrations of sodium or potassium salts, which may react to form compounds with low melting points (Hesketh *et al.,* 1990). In their liquid state, these melted substances would flocculate to form particles too heavy to remain suspended in the air stream. Fluidized beds are generally effective for medium-size sites (5 million to 30 million kg of waste) and for most contamination scenarios except drummed wastes, which may require substantial preparation.

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The rate and quantity of PCB-contaminated wastes fed into the combustion chamber must be measured at intervals of 15 minutes or less, requiring an automated recording system in most cases (Brunner, 1991). Stack emissions must also be monitored continuously for oxygen and carbon monoxide, and periodically for carbon dioxide. In addition, when an incinerator is first started up or after any modification are made, emissions must also be monitored for nitrogen oxides, hydrochloric acid, total chlorinated organics, PCBs and total particulate matter. If any of the monitoring systems fail or if the temperature or excess oxygen levels fall below specifications, the feed to the incinerator must shut down automatically. Water scrubbers, or an approved equivalent technology, must be used to remove HCl from the stack emissions.

Incineration residuals (i.e. the bottom ash and fly ash) are still considered to be hazardous substances and may require transportation and disposal in a RCRA approved landfill (Hesketh et al, 1990). A substantial cost may therefore be involved after incineration.

This additional cost can often be avoided by applying to have the incinerator residue delisted. Delisting is possible if it can be demonstrated that the residue no longer meets the criteria which caused it to be considered hazardous beforehand and meets the criteria listed in Table 2. Furthermore, the residue must contain no additional hazardous constituents, such as conbustion byproducts.

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The presence of contaminants other than PCBs may affect the decision to incinerate, particularly if the co-contaminants, for example, heavy metals, are not readily removed by incineration. If toxic constituents are still present after incineration, the residual ashes cannot be delisted and will require transport to a suitable landfill. The presence of co-contaminants is common at sites with PCB contamination, since quite often they are sites of transformer or generator oil spills in which PCBs were dissolved.

One important parameter affecting incinerator performance is the soil moisture content. An incinerator that is capable of processing five tons of waste per hour at 15% moisture may only handle about 3.2 tons of waste per hour at a 30% moisture content (Hesketh *et al.*, 1990). Another parameter is the heating value of the soil which is a measure of the energy that will be released when it is combusted and is expressed as energy per mass (e.g. Btu/lb). Substances with a higher heating value will produce additional heat when combusted, thus reducing the amount of additional energy that needs to be supplied to the kiln. While soils overall have a low heating value, those that are high in organic carbon may contribute to combustion (Hesketh *et al.,* 1990). The heating value of the contaminant also plays a role. PCBs dissolved in oils will contribute a great deal more energy than "dry" (i.e., no oil present) PCB contaminated soils.

The size distribution of soil particles will also affect incinerator performance. Unless the soil or sediment consists entirely of sand or smaller particles, most incinerators require some degree of size reduction of the feed in order to operate efficiently. This reduction may be carried out by soil washing, but more commonly, mechanical methods are used. Grinding or pulverizing is a common option, although screening may be more efficient at sites with large cobbles and boulders, despite the fact that these large pieces would be need to be washed or decontaminated by some other method.

The following section summarizes the advantages and disadvantages of incineration, and provides examples of several case studies.

Advantages: Incineration is a proven technology. Incineration methods and requirements are clearly described in EPA regulations. In cases where time is an important consideration, incineration also provides the quickest means of treating PCB-contaminated soils

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Disadvantages: Incineration is an expensive treatment option, and costs will vary according to fuel prices. Soils have a low heating value. Therefore, it may be necessary to mix in additional fuel, for example, fuel oil, propane or natural gas, to maintain proper temperatures. Auxiliary fuels can be one of the largest cost factors in soil incineration (Hesketh *et al.*, 1990). In addition, incomplete combustion may lead to formation of chlorinated dibenzo-*p*-dioxins, substances which are more toxic than the original PCB (Figure 9). Perhaps most importantly, however, public opinion often opposes the incineration of toxic substances due to the possibility of a release of PCBs or dioxin to the atmosphere and to the potential threats to human health and the environment that might result. The time and cost of litigation or a public relations effort may justify the examination of alternative treatment processes.

Case studies. At the Bridgeport Refinery and Oil Services Superfund Site in Logan Township, New Jersey, on-site incineration was used to treat 156,000,000 kg of soil contaminated with PCBs at concentrations greater than 500 mg/kg along with VOCs and metals. A direct-fired rotary kiln was used in conjunction with a Secondary Combustion Chamber (SCC). Total costs, including excavation, size reduction and disposal of residue, were $187,000,000 or $1.20 per kilogram of soil (FRTR, 1999). A similar method was used to treat 8,800,000 kg of soil at Coal Creek Superfund Site in Chehalis, Washington. The soil was contaminated with PCBs, lead and other metals. The required DRE was met and total costs, including excavation, size reduction and disposal of residue, were $8,100,000 or $0.92 per kilogram (FRTR, 1999).

At the Rose Township Dump Superfund Site in Holly, Michigan, a on-site infrared destruction unit was used along with a SCC to treat 30,800 kg of soil contaminated with PCBs, metals, VOCs and SVOCs. Total costs, including excavation, size reduction and disposal of residue, were approximately $12,000,000 or $0.39 per kilogram. Other cases are summarized below. Information on these cases was supplied by the Federal Remediation Technologies Roundtable.

Site:	Ogden's Research Facility, San Diego, CA
Technology:	Circulating bed incinerator (>1600°C)
Contaminant:	PCBs, dioxins, other organic compounds
Media:	Soil, sludges, liquids, slurry, solids
Results:	99.99% DRE
Cost per kg:	Unknown

Site:	Peak Oil Site, Tampa, FL
Technology:	Infrared incineration unit (>1200°C)
Contaminant:	PCBs, lead
Media:	Soil, sludge
Results:	Unknown
Cost per kg:	$0.18 to $0.80

Site:	Lauder Salvage Yard, Beardstown, IL
Technology:	Unspecified transportable incineration unit (>1600°C)
Contaminant:	PCBs: 12,000 ppm
Media:	Soil
Results:	PCBs < 1 ppm
Cost per kg:	$0.20

Site:	Times Beach Superfund Site, Times Beach, MO
Technology:	Rotary kiln (>1600°C)
Contaminant:	Dioxin: up to 1800 □g/kg
Media:	Soil and debris – 265,000 tons
Results:	99.9999% DRE
Cost per kg:	$0.46

Thermal Desorption

The principle behind thermal treatment processes is to enhance desorption of contaminants by supplying heat to the soil matrix. As the temperature increases, contaminants will be transferred from the solid phase into the aqueous or gaseous phases. Various methods of supplying heat to contaminated soil have been employed. Thermal treatment processes can be utilized for both *in situ* or *ex situ* applications. *In situ* thermal desorption methods can be implemented in conjunction with conventional soil vapor extraction (SVE) wells to capture off-gases. Injection wells are installed around a contaminated area with an extraction well placed in the center of the area. Hot air or steam is then injected into the ground. The extraction wells create a pressure differential causing the heated air or vapor to flow through the contaminated zone toward the extraction wells, where the volatilized contaminant is captured for treatment. Other methods include resistance heating, in which an electrical current is applied to the soil,

and radio frequency heating, in which electromagnetic radiation is applied. Cost estimates for *in situ* thermal desorption range from $0.02 to $0.08 per kilogram, assuming a soil bulk density of 1.68 g/cm^3 (FRTR, 1999).

Ex situ thermal desorption is a more common option for treating PCB-contaminated soils. The typical process flow diagram is illustrated in Figure 10. The contaminated soil is excavated and placed in a kiln and heated to 320 to 560°C (600 to 1000°F) to achieve high temperature thermal desorption (HTTD), or 90 to 320°C (200 to 600°F) to achieve low temperature thermal desorption (LTTD). A thermal desorber typically consists of two separate chambers. The first chamber is operated at temperatures high enough to volatilize organic contaminants, but not high enough to oxidize them, thus saving on fuel consumption. The second chamber can either be a combustion chamber or a chemical or physical flue gas treatment system. In the former case, the thermal desorber can actually be considered a form of incinerator. In the latter case, however, the contaminant is not destroyed. The system condenses the organic material by cooling, recovering the condensed contaminant for final disposal.

Estimates for *ex situ* thermal desorption range widely from $0.045 to $0.33 per kilogram. Means (1999) estimates *ex situ* costs at $0.15 per kilogram. However, these cost estimates are for petroleum hydrocarbons and are likely to be higher for PCBs. For *ex situ* treatments, excavation and replacement with clean soil ranges from $5 to $10 per metric ton ($0.005 to $0.01 per kilogram). Additional costs will be incurred for off-gas treatment and contaminated water treatment, and if necessary, for dewatering the soil prior to treatment.

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Advantages. Thermal treatments are able to reduce the volume of contaminated material by extracting and concentrating the contaminant. The processes are also insensitive to the presence of co-contaminants. Most of the equipment required is readily available from commercial vendors. *In situ* thermal treatments, like other *in situ* technologies, limit the exposure of workers and the environment to the contaminant by eliminating the need for excavation and transport of the contaminated media.

Disadvantages and limitations. Thermal treatments are generally ineffective against inorganic co-contaminants. Therefore, the residue may require stabilization and appropriate disposal, although in the case of HTTD, some metals may be volatilized. Other disadvantages include the fact that thermal desorption does not destroy PCBs, requiring treatment of all off gases and water removed, unless the secondary chamber is an incinerator. In addition, thermal treatments are likely to sterilize the soil, thereby limiting subsequent biological activity.

Thermal treatments are also affected by soil characteristics. Hydrophobic contaminants such as PCBs tend to sorb strongly to those soils containing high clay or organic carbon contents. Therefore, such soils will require more time or greater temperatures for the contaminants to desorb and will tend to have a much lower extraction efficiency. The soil may also need to be dewatered prior to treatment. The disadvantages of *in situ* thermal treatment processes in particular are those inherent to all SVE systems. Soils with low permeability or high moisture content, as well as those with a high organic or clay content, greatly reduce the extraction efficiency, while highly heterogeneous soils may be subject to preferential air flow. In addition, the depth of treatment may be limited to approximately the upper 12 inches (30 cm) of soil.

Case studies. Researchers at General Electric Corporate Research and Development performed a pilot-scale study of an *in situ* method using a thermal blanket to remediate a

site contaminated with PCBs, principally Aroclor 1242 (Iben *et al.*, 1996). Heat flowed downward from the blanket into the soil via thermal conduction and was able to remove PCBs from concentrations as high as 2000 mg/kg soil to below 2 mg/kg soil. The success of this experiment was due in large part to the shallow depth at which the contaminants were found, which was no more than 15 cm (6 in.). The method would only be suitable for surficial contamination. For the thermal blanket, Iben *et al.* (1996) estimate costs to be between $45 and $60 per square meter ($0.15 to $0.20 per kg) of soil. Cases where *ex situ* thermal treatment was used are summarized below.

Site:	Outboard Marine Corporation, Waukegan, IL (1/92 - 6/92)
Technology:	LTTD (250-275°C)
Contaminant:	2400 to 23,000 mg/kg PCB
Media:	Soil/sediment, 12,755 tons, 12.9% moisture content
Cleanup goal:	Soil PCB - 97% removal by mass; Air: DRE: 99.9999%; dioxins/furans: 30 ng/dscm
Results:	Average mass removal: 99.98%. Treated soil concentrations: 0.4 to 8.9 mg/kg. Stack gas requirements met.
Cost:	$2,474,000 plus $900,000 for mobilization, preparatory work, monitoring, sampling, testing and analysis
Cost per kg:	$0.29

Site:	Re-Solve, Inc., North Dartmouth, MA (6/93 - 12/94)
Technology:	HTTD (450-500°C)
Contaminant:	PCBs and VOCs, concentrations not known
Media:	Soil: 44,000 tons
Cleanup goal:	Soil PCB conc: 25 mg/kg; Air emissions: Max. 0.38 lb/hr
Results:	Soil PCB conc: 0.59 - 21 mg/kg Stack gas requirements met.
Cost:	$6,800,000
Cost per kg:	$0.17

Site:	Wide Beach Development, Brant, NY (10/90 - 9/91)
Technology:	HTTD (450-500°C)
Contaminant:	PCBs: 10 to 5000 mg/kg (feed conc.: 11 to 68 mg/kg
Media:	Soil: 42,000 tons, MC: 18.3%; 12.8% clay
Cleanup goal:	Soil PCB conc: 2 mg/kg; Air emissions: 3.33E-5 lbs/hr
Results:	Soil PCB conc: <2 mg/kg Stack gas requirements met.
Cost:	$11,600,000 plus $908,000 for mobilization/prep.work and $3,400,000 for disposal.
Cost per kg:	$0.42 (0.30 for direct costs)
Notes	Technology combined with APEG dehalogenation

Site:	Naval Air Station Cecil Field, Site 17, OU 2, Jacksonville, FL
Technology:	HTTD (>450°C)
Contaminant:	BTEX, dichlorobenzene <18 mg/kg, naphthalene <19 mg/kg, methylnaphthalene < 47 mg/kg
Media:	Soil: 11,768 tons
Cleanup goal:	Petroleum or volatile hydrocarbons <50 mg/kg; PAH < 1 mg/kg
Results:	94% of total reached goal after 1 pass. All goals met.
Cost:	$1,946,122

Chemical dehalogenation

Chemical dehalogenation processes are used to convert PCBs into less hazardous forms by direct removal of chlorine atoms. In this process contaminated soil is excavated and mixed with chemical reagents that react with the PCBs, either replacing the chlorine

molecules or causing the biphenyl ring to cleave. EPA's Risk Reduction Engineering Laboratory developed a Base-Catalyzed Decomposition (BCD) process which especially targets PCBs, dioxins and furans. Contaminated soil is mixed with solid-phase sodium bicarbonate ($NaHCO_3$). The weight of sodium bicarbonate added may be from 5 to 10% of the dry weight of the soil. The mixture is then heated to above 330°C (630°F), which causes the contaminants to partially decompose and volatilize. After being separated from dust particles with a cyclone and baghouse, the volatilized PCBs then are captured by air scrubbers and cooled to allow them to condense. These contaminants can be then burned or treated with an alternative technology. The volume of the condensed contaminants is a fraction of that of the original contaminated material.

The reaction mechanisms involved in chemical dehalogenation are not well understood but may include nucleophilic substitution of hydrogen for the chlorine atoms (Chen *et al.*, 1997). Bench-scale or pilot-scale testing may be necessary to determine the optimal concentration of sodium bicarbonate ($NaHCO_3$) reagent to apply. Based on bench-scale tests, Chen *et al.* (1997) estimated a unit treatment cost of $0.11/ kg for contaminated sediments based on treating 144,000,000 kg per year for 10 years.

A second chemical dehalogenation process utilizes an alkaline (usually potassium) polyethylene glycol (APEG) reagent that is mixed with contaminated soil and heated. The reagent is formed by mixing the PEG with potassium hydroxide (KOH) or other alkaloid. In this process, the halogen molecules are replaced by the polyethylene glycol, forming a glycol ether or hydroxylated compound and an alkali-metal salt (KCl), both of which are water-soluble and either less toxic than PCBs or nonhazardous (Figure 11) (De Filippis *et al.*, 1997). The wastewater generated by this reaction must be collected and treated separately by filtration, incineration or some other means to remove the glycolated biphenyl byproducts.

Chemical dehalogenation processes are used to convert PCBs into less hazardous forms by direct removal of chlorine atoms. In this process contaminated soil is excavated and mixed with chemical reagents that react with the PCBs, either replacing the chlorine molecules or causing the biphenyl ring to cleave. EPA's Risk Reduction Engineering Laboratory developed a Base-Catalyzed Decomposition (BCD) process which especially targets PCBs, dioxins and furans. Contaminated soil is mixed with solid-phase sodium bicarbonate ($NaHCO_3$). The weight of sodium bicarbonate added may be from 5 to 10% of the dry weight of the soil. The mixture is then heated to above 330°C (630°F), which causes the contaminants to partially decompose and volatilize. After being separated from dust particles with a cyclone and baghouse, the volatilized PCBs then are captured by air scrubbers and cooled to allow them to condense. These contaminants can be then burned or treated with an alternative technology. The volume of the condensed contaminants is a fraction of that of the original contaminated material.

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Other dehalogenation technologies have been tested at the bench scale but have not yet yielded encouraging results. EPA's National Risk Management Research Lab (NRMRL) investigated treatment of soils contaminated with PCBs and with polychlorinated dibenzo-*p*-dioxins (PCDDs) with either artificial and natural ultraviolet light. Other PCB contaminated soils in the experiment were treated with chemical oxidation using Fenton's reagent (USEPA, 1995). The process of oxidation with Fenton's reagent is implemented by adding iron sulfate ($FeSO_4$) to the substance to be oxidized. Hydrogen peroxide (H_2O_2) is then added. The $FeSO_4$ acts as a catalyst to break the H_2O_2 into hydroxyl radicals which are strong oxidizing compounds.

The purpose of these tests, however, was not to remove all of the contaminant, but rather to determine the feasibility of improving the rate of biological treatment by first chemically removing chlorines from the more recalcitrant, highly-chlorinated species. In none of the cases, however, was the subsequent biodegradation rate of PCBs increased. It may be that pH of the treated soil inhibited microbial growth. However, it is also likely that PCB degradation was halted by accumulation of the inhibitory acylchloride products discussed above.

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Advantages: The principle advantages of chemical dehalogenation are that the process reduces the toxicity of PCB congeners by removing chlorine atoms and that it can be used as a stand-alone technology. Both the BCD and APEG chemical dehalogenation processes described above have been approved by EPA's Office of Toxic Substances. Relative to other PCB remediation technologies, the treatment time is short, being on the order of weeks, depending on the amount of soil to be treated, as opposed to possible years for bioremediation. Energy requirements are moderate in comparison to such technologies as incineration. Operation and maintenance costs are relatively low due to the short operation time. In addition, the technology can implemented on site, minimizing transportation of the hazardous waste.

Disadvantages and limitations: Costs increase as the amount of chemical reagent increases, so these processes tend to be more amenable to sites with low concentrations of PCBs and smaller sites. In addition, treatment costs rise as the clay content and moisture content of the solid phase increase. Usually, some degree of size reduction is required and is usually performed by screening and crushing the contaminated feed material. In some cases, capture and treatment of residuals of the BCD process may be difficult.

Case studies: The BCD dehalogenation process has been tested at the pilot-scale but has not been applied at a full-scale level. Pilot tests of the process were conducted at the Koppers Superfund site in North Carolina with inconclusive results. However, at the Public Works Center in Guam, approximately 15,000,000 kg (33,000,000 lb) of contaminated soil were successfully treated by the Navy as part of a pilot-scale test. In this study, PCB solid-phase concentrations of 2500 mg/kg were reduced to less than 10 mg/kg. The concentrations of sodium bicarbonate used were not reported.

The APEG dehalogenation process, on the other hand, has been used for full-scale treatment. The process was selected for use at three Superfund sites: Wide Beach Development (New York), Re-Solve (Massachusetts), and Sol Lynn (Texas). At Wide Beach, approximately 51 million kg of soil was treated and disposed of on site. Initial PCB soil concentrations as high as 120 mg/kg were reduced to less than 2 mg/kg. At the two other sites, concentrations as high as 45,000 mg/kg where oils were present were reported to be reduced to less than 2 mg/kg per individual congener. EPA estimates costs in the range of $0.22 to $0.55 per kilogram of contaminated soil, excluding excavation, refilling, residual treatment and analytical costs (FRTR, 1999). No information was given on the costs or concentrations of the reagents. Additional case studies are summarized below. In all the cases, APEG dehalogenation was the technology employed.

Site:	Montana Pole, Butte, MT
Contaminant:	Dioxin/Furans < 84 mg/kg
Media:	Soil
Results:	Dioxin/Furans < 1 □g/kg
Cost per kg:	Unknown

Site:	Economy Products, Omaha, NE
Contaminant:	Dichlorodibenzo-dioxin: 17,800 mg/kg; trichloro-dioxin: 2800 mg/kg
Media:	Soil
Results:	Dichlorodibenzo-dioxin: 334 mg/kg; trichloro-dioxin: 55 mg/kg
Cost per kg:	Unknown

Site:	Signo Trading International, Inc., NY
Contaminant:	Dioxin: 135 mg/L
Media:	Sludge: 15 gallons
Results:	Dioxin: 1 □g/L
Cost per kg:	Unknown

Solidification/Stabilization

Unlike other remedial technologies, solidification/stabilization does not seek to remove contaminants. Rather, the goal of both is to reduce the potential mobility of contaminants, trapping them in the soil matrix. The treatment can be performed *in situ* or it may be performed *ex situ* for subsequent disposal in a landfill. Typically, solidification/stabilization technologies have been used for inorganic contaminants, but limited effectiveness for semivolatile volatile organic compounds (SVOCs), including PCBs, has been demonstrated. Due to the maturity of this technology, a cost estimate of $0.011 per kilogram is considered to be relatively accurate (FRTR, 1999).

Solidification involves physically binding or encapsulating a contaminant within a solidified mass. In addition to reducing the mobility of the entrapped contaminant, the solidified material has improved handling characteristics. Historically, Portland cement has been used to bind hazardous materials. When water is added, the cement components, primarily silicates, react chemically to form a solid matrix. Often pozzolanic materials, such as fly ash, blast furnace slag or kiln dust, are added to the cement to improve its durability and strength. Cement and pozzolan matrices have been employed principally to immobilize inorganic contaminants. Organic contaminants, even table sugar, may prevent the cement from hardening.

FRTR (1999) has described several innovative stabilization materials and technologies, most of which involve some degree of processing of the contaminated soil:

- *Modified sulfur cement* can be melted at temperatures below 150°C (300°F) and mixed with contaminated soil to form a molten slurry. The slurry can be poured into containers, cooled and disposed of in a landfill.
- *Emulsified asphalt* is used to encapsulate hydrophilic wastes. Emulsions are formed by stabilizing fine droplets of asphalt in water with the use of chemical dispersants. The resulting emulsions are either cationic or anionic, depending on the dispersant used, and are mixed with liquid wastes. Once mixed the emulsion breaks, releasing the water, and the organic asphalt entraps the solid contaminants in a water-resistant matrix.
- *Bitumenization*, also appropriate for hydrophilic wastes, entails mixing molten bitumen with contaminated material in a heated extruder. Once water has been allowed to evaporate to a point of about 0.5% moisture content by weight, the mixture is extruded and allowed to harden.
- *Polyethylene extrusion* is similar to the bitumenization process with polyethylene being used rather than bitumen to bind dry wastes. Polyethylene forms a very stable solid.

Stabilization differs from solidification in that mobility is reduced by chemical rather than physical means. It is the process of inducing chemical reactions of contaminants with stabilizing agents. The most common example of this process is the immobilization of heavy metals by converting them to their most insoluble forms. Sludges may be

stabilized by adding reagents which transform hazardous constituents into their least soluble or least toxic state. Stabilization does not necessarily result in a hardened substance. For example, the soluble phosphates process entails adding phosphates to a contaminated soil to complex with heavy metals such as lead. The resulting molecules have a low solubility, effectively immobilizing them without encapsulating them. *Advantages:* Solidification/stabilization has been used extensively and is well-documented. It is also a relatively inexpensive technology, as low as $0.011 per kg of soil. In addition, the equipment and processes employed are not technologically complex.

Disadvantages: One of the major drawbacks of this technology is the potential for leaching of the contaminants from the solid matrix over long time periods. Weathering of the matrix due to wind or water erosion, acid precipitation, or freeze-thaw cycles may remobilize contaminants. Furthermore, physical disturbance by uncontrolled future land use could result in significant releases from the stabilized mass. Therefore, long-term monitoring of the site, whether a cleanup site or a landfill, is required, and the technology should only be used at low-occupancy sites.

Other drawbacks associated with solidification are that the encapsulating material must be tailored to the contaminant and that its effectiveness may be influenced by soil properties and moisture condition. While inorganic co-contaminants may be effectively bound, organic co-contaminants may interfere with the chemical reactions that take place between the cement components and thereby prevent effective binding. In the case of *in situ* solidification, confirmatory sampling is more difficult than for *ex situ* treatment because of the danger of disturbing the solidified matrix with sampling equipment.

Site:	EPA *SITE* Demo, Hialeah, FL (1988-1990)
Technology:	*In situ* solidification
Contaminant:	PCBs, organic and inorganic co-contaminants
Media:	Wet and dry soil, sludge, and sediment
Results:	PCB immobilization likely but not confirmed
Cost per kg:	$0.12

Site:	EPA Demo, Douglassville, PA (10/87)
Technology:	*In situ* solidification and stabilization with "Chloranan"
Contaminant:	Organic compounds, oils and grease, heavy metals
Media:	Soil and sludge
Results:	Material hardened into a concrete-like mass
Cost per kg:	Unknown

Site:	Envirocare of Utah, Inc., Salt Lake City, UT (1996)
Technology:	Encapsulation with low-density polyethylene
Contaminant:	Radioactive waste
Media:	Lead bricks (500,000 lbs)
Cleanup goal:	RCRA Land Disposal Restrictions for debris
Results:	Treatment goals met
Cost per kg:	$0.28-0.31/kg lead for encapsulation + $4.32/kg for disposal

Site:	EPA *SITE* Demo, Robins AFB, Warner-Robins, GA (8/91)
Technology:	*Ex situ* solidification with proprietary binding agents
Contaminant:	Organic and inorganic compounds
Media:	Soil, sludge, and liquid
Results:	Non-leaching high-strength solid
Cost per kg:	Unknown

Site:	EPA *SITE* Demo, Selma Pressure Treating, Selma, CA (11/90)
Technology:	*Ex situ* solidification with silicate compounds
Contaminant:	Organic and inorganic compounds
Media:	Soil, sludge, groundwater
Results:	PCP leachate concentration reduced up to 96%, As, Cr, Cu immobilized
Cost per kg:	Unknown

Site:	Installation Restoration, Site 2, NAS Northern Island, San Diego, CA
Technology:	*Ex situ* stabilization by mixing with clay at high temps.
Contaminant:	VOCs, metals
Media:	Soil
Results:	VOCs removed; metals fixed as silicates by reaction with clay
Cost per kg:	Unknown

Vitrification

Vitrification is actually a specific type of solidification and stabilization technology which can be performed either *in situ* or *ex situ*. *In situ* vitrification (ISV) uses an electric current to melt soils to form glass at temperatures ranging from 1600 to 2000°C (2900 to 3600°F). One method entails driving electrodes into the soil and then passing a strong electric current between them. The resistance of the soil particles generates heat. An alternative method employs a plasma arc. The plasma arc is lowered into a drilled hole and used to melt the soils directly (Figure 12).

The resulting glass and crystalline material entraps inorganic contaminants whereas organic contaminants are destroyed by oxidation and/or pyrolysis. Pyrolysis is the decomposition of materials by heat in the absence of oxygen. The byproducts resulting from pyrolysis of organic materials are coke, a solid residue containing carbon and ash, and combustible gases such as methane, carbon monoxide, hydrogen and hydrocarbons. The production of these gases necessitates the use of an off-gas collection hood which can condense or trap the gases. The gases will require further treatment, such as combustion. EPA's cost estimates for the treatment of PCBs by ISV include $30,000 for treatability tests plus analytical fees, as well as $200,000 to $300,000 for equipment mobilization and demobilization. These costs are fixed and independent of the amount of soil to be treated. The remainder of the costs will be influenced by electricity costs, the water content of the soil and the depth to which ISV is required. Tests of ISV technology have yielded cost estimates for the vitrification process itself of $0.16 to $0.47 per kg of soil treated.

Ex situ vitrification involves heating waste materials to 1200°C (2200°F), converting contaminated soil to glass and crystalline materials. In the same manner as ISV, inorganics are entrapped in glass and crystalline material and organics are destroyed by oxidation or pyrolysis. If contaminated soils are vitrified, the silicon in the soil particles

will form glass. Contaminated materials other than soil, such as sludges, can be vitrified as well, but glass-forming materials such as borosilicate or soda-lime must be added to provide the crystalline matrix to entrap the inorganic contaminants.

Advantages. The principal advantage of vitrification, particularly with respect to other forms of solidification, is that PCBs are destroyed. Inorganic co-contaminants are immobilized in a material that is easily handled and resistant to leaching. Unlike other solidification technologies, there is no increase in volume of contaminated materials. In addition, if the treatment is performed *in situ,* potential added exposure to workers or others during excavation or transport can be avoided.

Disadvantages. While vitrification destroys PCBs, the off-gases still require treatment. In addition, vitrification is subject to the same limitations of other solidification technologies. In the long term, inorganic contaminants may be remobilized through leaching, due to environmental conditions such as freeze/thaw fracturing or pH changes. Future land use may be restricted by the presence of the solidified material. The depth of treatment is generally limited to approximately 20 feet (6 m) below the surface.

A major drawback of ISV is its sensitivity to soil moisture content; a high moisture content will require a greater energy input and may prevent a solidified mass from forming. Like other *in situ* treatments, the effectiveness of the treatment is often difficult to quantify and verify without disturbing the solid. In addition, because of the lower temperatures at the fringe of the treatment area, there exists the potential that contaminants at the fringe will be converted to more toxic forms, such as dioxin, rather than be destroyed or entrapped.

Case studies. ISV has been tested at sites containing PCBs. A demonstration of ISV treatment of a PCB contaminated soil at a private Superfund site in Washington was reported by the Federal Remediation Technologies Roundtable (FRTR). A total of 2,812,000 kg of soil were treated. The technology had a destruction and removal efficiency (DRE) of greater than 99.9999%, although original PCB concentrations were not reported. Costs were $0.41 to $0.47 per kilogram for actual vitrification plus an additional $0.11 per kilogram for treatability and pilot tests, mobilization and demobilization. The FRTR (1999) also reported an incident at an unspecified site involving a high moisture content soil, in excess of 20 weight percent, which resulted in excessive water vapor which caused the off-gas hood to overheat.

The Parsons Chemical/ETM Enterprises Superfund Site in Grand Ledge, Michigan, did not contain PCBs but was contaminated with pesticides (up to 340 mg 4,4'-DDT /kg), metals (up to 150 mg zinc/kg) and dioxin (up to 1.13 μγ/kg) along with other heavy metals and pesticides. ISV was performed on 4,898,000 kg of silty clay with a moisture content of 17 weight percent, with the overall project running from May 1993 to May 1994. Cleanup requirements were met for both soil and off-gases, including a final treated soil concentration of 4 mg 4,4'-DDT/kg. In addition, core sampling showed that the vitrified materials met the RCRA requirements for disposal in a landfill. Direct costs

for vitrification were $800,000 or $0.16 per kilogram. Cases of *ex situ* vitrification are summarized below.

Site:	DOI Demo, Salt Lake City Research Center
Technology:	*Ex situ* vitrification
Contaminant:	Copper byproducts, arsenic, heavy metals
Media	Slags, dusts, sludges
Results	Non-reactive glass-like material; vitrification of arsenic sulfide
Cost per kg:	Unknown
Site:	Albany Metallurgy Research Center, OR
Technology:	*Ex situ* vitrification
Contaminant:	Residues from incineration of municipal waste
Media	Solid residues
Results	Dense glassy slag and dense metallic phase
Cost per kg:	Unknown
Site:	Component Development & Integration Facility, MT
Technology:	*Ex situ* vitrification
Contaminant:	Organic and metals
Media	Soils and sludges
Results	Metals trapped in vitrified mass; organics removed in vapor stream
Cost per kg:	$0.83-$2.09

Soil Washing and Soil Flushing

Soil washing is an *ex situ* process that entails excavation of contaminated soils and literally washing them in an aqueous solution which may contain surfactant or cosolvent, leaching or chelating agents, or some type of pH adjustment. The process differs from *in situ* soil flushing because the soil particles are mechanically agitated and separated. This technology is based on the idea that, since organic contaminants sorb more readily to silt and clay particles and to particles of organic material than to larger sand particles, the majority of the contaminant is concentrated in the smaller particles. During washing, the larger, denser particles settle out more quickly than the finer particles, so that a particle size gradient is formed, in the same manner that a particle size gradient is formed in multimedia filters during backwashing.

Two separate mechanisms are, therefore, involved in the removal of hydrophobic organic contaminants during soil washing. The first mechanism is the dissolution of some of the contaminant into the aqueous phase and its removal with the wastewater. The second mechanism is the particle size separation. The fine particles can be removed for further treatment while the large particles, assuming the contaminant has been sufficiently removed from them, can be returned to the site as fill. EPA estimates costs for the soil washing itself at $0.19 per kilogram of soil.

Soil washing can also be performed *in situ,* but this process is often more generally referred to as soil flushing. A surfactant or cosolvent solution is injected directly into the soil. If the contaminant is located in the saturated zone, the solution is pumped through the contaminated area and removed by extraction wells. In the unsaturated zone, the solution is typically delivered to the contaminated area by gravity drainage or by a vacuum pump system. The surfactant increases the apparent solubility of the

contaminant, increasing its desorption from the soil particles and removal along with the aqueous solution. EPA offers a widely ranging cost estimates of $0.019 to $0.19 per kilogram for soil flushing. Costs are highly dependent on the type and concentration of surfactant used.

Advantages. Soil washing reduces the volume of soil or sediment that must be treated. In addition, there are no off-gases to contend with. Soil washing is more effective in soils with high percentage of coarse particles. Perhaps most advantageous is the fact that soil washing can also be effective for removing inorganic co-contaminants. Soil flushing, because it is performed *in situ*, offers the advantage of reduced exposure of workers and the environment to the contaminant. It also can have lower mobilization costs in addition to avoiding excavation costs.

Disadvantages. Soil washing does not destroy PCBs, so the resulting contaminated water must be treated in addition to the fines. The method is sensitive to ambient temperature. For some contaminants the kinetics of dissolution or desorption may be too slow to permit sufficient cleaning of the coarse soil particles so that it may be not be possible to return these particles to the site as fill. In addition, when low-solubility contaminants are present in mixtures such as Aroclors, the more soluble congeners may be removed preferentially. Very hydrophobic compounds, such as PCBs, may be so strongly sorbed to the soil particles that solubility enhancement is minimal. Therefore, large volumes of solution and long treatment times may be required to reach remediation goals.

In situ soil flushing suffers from these same disadvantages, with the added difficulty of needing to remove the contaminant from the fine particles as well as the coarse particles. Thus, soils that contain a high amount of organic matter or fine particles may require flushing with a large number of pore volumes to obtain satisfactory results, particularly when the contaminant has low solubility. Another major drawback of *in situ* flushing is the risk of mobilizing the contaminant.

Case studies. A full-scale cleanup of the King of Prussia Technical Corporation site in Winslow Township, NJ, was performed using an *ex situ* soil washing system. Soils and sludges were contaminated with high concentrations of heavy metals. Eleven metals were identified for cleanup and goals for all of them were met in a four month period from June 1993 to October 1993. Total cost for treating 19,200 tons was $7,700,000 including off-site disposal. Costs per kilogram were $0.44.

In situ surfactant flushing has been evaluated as a means of treating PCB contaminated soil. A surfactant solution was injected into a test plot containing PCBs and oils (Abdul *et al.*, 1992). PCBs were removed from the captured leachate by an activated carbon system. After 70 days, only about 10% of the initial mass had been removed. Later bench scale studies showed removal of more than 85% of PCBs by surfactant flushing but only after 105 pore volumes were injected (Abdul and Ang, 1994). The following are summaries of case studies in which *ex situ* soil washing was used to treat contaminated soils. The solutions used and their concentrations were not provided (FRTR, 1999).

Site:	EPA Demo, Santa Maria, CA
Contaminant:	PCBs and PAHs: concentrations up to 15,000 mg/kg soil
Media:	Soil
Results:	99% removal
Cost per kg:	Unknown

Site:	Escambia Wood Treating Co. Superfund Site, Pensacola, FL
Contaminant:	PAHs: 550-1700 mg/kg soil; PCP: 48-210 mg/kg soil
Media:	Soil
Results:	PAHs: 45 mg/kg soil; PCP: 3 mg/kg soil
Cost per kg:	$0.15

Site:	Toronto Port Industrial District, Toronto, ON
Contaminant:	Naphthalene: 52 mg/kg soil; benzo(a)pyrene: 10 mg/kg soil
Media:	Soil
Results:	Naphthalene: <5 mg/kg soil; benzo(a)pyrene: 2.6 mg/kg soil
Cost per kg:	Unknown

Site:	MacGillis & Gibbs Superfund Site, MN
Contaminant:	PCP and PAHs: concentrations unknown
Media:	Soil
Results:	89% removal of PCP; 88% removal of PAHs
Cost per kg:	Unknown

Site:	Small arms range, Fort Polk, LA
Contaminant:	Lead: 3500 mg/kg soil
Media:	Soil: 9,000,000 kg
Results:	Lead: 200mg/kg soil. TCLP: 2 mg/L
Cost per kg:	$0.19

Chemical extraction

The chemical extraction process is quite similar to *ex situ* soil washing but is distinguished by its use of a pure extractive organic solvent, or an acid in the case of heavy metal contamination, as opposed to an aqueous solution containing an organic solvent or surfactant. The goal of the treatment is to reduce the volume of contaminated soil by effectively removing the contamination from coarse particles, leaving only the fine particles to require further treatment. Solvent extraction tends to be less efficient for high molecular weight organic molecules which may be bound more strongly to clay particles or organic matter in the soil. The process is also less effective on hydrophilic contaminants which will not readily partition into the organic solvent phase.

EPA provides cost estimates of $0.11 to $0.44 per kilogram of soil for treatment by solvent extraction. Due to relatively high costs for equipment, mobilization and demobilization and other capital costs, this technology is considered to be more economical at larger sites. Means (1999), for example, reports a cost of $361,250 for site preparation and mobilization of the smallest extraction unit (1500 cubic yard/month). The use of pure solvents is also expensive relative to the aqueous solutions used in soil washing. However, increased extraction efficiency and lower volume of extraction fluid to be treated may result in solvent extraction being more economical than soil washing.

Recent studies have focused on the use of supercritical fluids to extract PCBs from contaminated soils and sediments. A supercritical fluid is substance that has been heated and pressurized beyond the point that it would ordinarily be in a gaseous form. Because

the substance takes on properties of both liquids and gases, it is considered to be a "fluid". Supercritical fluids expand to fill a contained space while retaining the density of a liquid and therefore serve as very effective solvents. Contaminants dissolve in the fluid and are transported with it into a separation vessel where pressures are lowered, allowing the contaminant to be condensed and collected.

Researchers at Syracuse University have developed a supercritical fluid extraction (SFE) system that uses carbon dioxide and methanol operating at pressures of 80 to 350 atm and temperatures of 40 to 60°C. In one study, the SFE system was used on sediments contaminated PCBs at a concentration of 2200 mg/kg. After applying the supercritical fluid for 45 to 60 minutes, 99.8% of the contaminate was removed from the sediment.

Experimental studies have also been conducted to evaluate the use of supercritical water oxidation for treatment of PCB contaminated sits. In this process, water is supercritically heated to 450 to 550°C at a pressure of 250 atm. When oxygen is added to the fluid, a series of oxidation reactions is initiated that is capable of breaking down organic compounds into carbon dioxide and water or other harmless products. When 2-monochlorobiphenyl and 3,3',4,4'-tetrachlorobiphenyl were subjected to this process, the compounds were completely oxidized. When the process was implemented on a solution of Aroclor 1248 at a concentration of 5245 ppm, 99.99% of the PCB congeners were oxidized. The maximum fluid residence time in the experiments was 54 seconds. An economic analysis of the SFE and supercritical water oxidation systems operated in sequential stages suggested costs for implementing both stages of this technology would be approximately $0.12 to $0.19 per kg of soil.

Advantages: The advantages of conventional chemical extraction are the same as those of soil washing. Both processes reduce the volume of soil or sediment that must be treated. There may be off-gases to contend with, but these will be volatilized solvent rather than contaminants or contaminant by-products. Solvent extraction is more effective in soils with a high percentage of coarse particles. If treatment with supercritical fluids is found to be a viable remediation option, it may be possible to remove contaminant from all of the soil rather than only the coarse particles.

Disadvantages: As with many other technologies, high moisture content and high clay and organic content reduces extraction efficiency. In addition, organic-bound metals may be removed along with the target contaminant which introduces restrictions for residuals handling. Furthermore, the toxicity of the solvent must be considered because solvent traces may remain in the treated soils. The same is true of any co-solvents, such as methanol, used in conjunction with supercritical fluids.

Case studies: Tillmann *et al.* (1995) performed a pilot-scale study of PCB removal using liquefied propane as the solvent. The researchers were unable to achieve the remedial action standard of 1 mg/kg soil; however, in some cases, they were able to reach levels of 1.8 mg/kg soil. The PCB concentration levels attained indicated an average removal of approximately 98% of the mass. At the Sparrevohn Long Range Radar Station in

Alaska, solvent extraction was used to treat 288 cubic yards (approximately 443,000 kg) of PCB-contaminated soil from June to August, 1996. Initial PCB concentrations ranged from 13 to 346 mg/kg with an average of 80 mg/kg. The target level for cleanup was 15 mg/kg but average concentrations of 3.27 mg/kg were achieved. Total costs were $828,179 or $1.87 per kg of soil. However, this figure includes $602,530 for mobilization and demobilization by air to the remote location. Actual solvent extraction costs were $225,649 or $0.51 per kg of soil. It should be noted, however, that the soil was mostly gravel with fines and little or no clay. Other sites using solvent extraction are summarized below.

Site:	New Bedford Harbor, MA and O'Connor Site, ME
Contaminant:	PCBs: 300-2500 mg/kg soil
Media:	Soil , sludge, wastewater
Solvent:	Unknown
Results:	Removal: 90-98%
Cost per kg:	Unknown

Site:	General Refining Co., GA
Contaminant:	PCBs: 5 mg/L; lead: 10,000 mg/L
Media:	Sludge
Solvent:	Unknown
Results:	PCBs: undetectable; lead concentrated in solids
Cost per kg:	Unknown

Site:	Naval Air Station, North Island Site 4, San Diego, CA
Contaminant:	PCBs (Aroclor 1260): 170 mg/kg
Media:	Soil
Solvent:	Triethyl amine
Results:	PCBs: <2 mg/kg. Average removal efficiency: 98.39%
Cost per kg:	Unknown

Bioremediation

Bioremediation involves the use of microorganisms to mineralize contaminants or to reduce them to innocuous compounds as part of a natural process (Exner, 1994). PCB treatment can be performed either *in situ* or *ex situ*. Nutrients are usually added to the soil matrix to provide conditions that favor growth of biodegrading organisms and thereby to increase the degradation rate (biostimulation). In addition, the soil may be inoculated with nonindigenous microorganisms which have been proven capable of degrading the contaminant (bioaugmentation). Bioremediation offers a method of PCB destruction which entails substantially less energy and overall costs than incineration and which potentially has no hazardous byproducts, though this is not the case with all contaminants. The success of biodegradation may be hindered by low bioavailability of the contaminant, by mass transfer limitations of the electron acceptor and by low temperatures.

To date, bioremediation has not consistently proven to be a reliable and cost- effective remediation technique. Although costs for bioremediation are often much lower than incineration and other alternatives, treatment choices are driven for the most part by regulatory criteria, and bioremediation alone has usually been insufficient to reduce contaminants to specified cleanup levels. Exner (1994) described a site contaminated

with phenanthrene where concentrations were reduced to 25 mg/kg within 10 days through bioremediation with an aerobic slurry-phase treatment. However, to reach the required cleanup concentrations of 1.5 mg/kg with the slurry-phase treatment, an estimated 60 to 100 days would have been required at a cost of approximately $10,000 per pound of contamination. In such cases, alternative treatments may be more cost-effective.

The biological activity observed under the optimtal conditions of the laboratory cannot be assumed to exist in the field (Glaser, 1997). Among the many environmental factors that must be controlled are temperature, salinity, and pH, all of which may vary over time. In a batch reactor system, these factors may also vary from batch to batch. Nonetheless, these factors are much more easily controlled in bioreactors than in *in situ* treatment systems (Christodoulatos and Agamemnon, 1998). Salinity and pH can be measured and adjusted in a bioreactor relatively easily. Temperature can be controlled by integrating heating or cooling systems into the water recirculation system.

A lag period in which microorganisms acclimate to the new conditions and nutrient availability can be expected after startup. In systems in which microorganisms specifically intended to degrade the contaminant are used, the lag period can often be shortened by acclimating the microbial culture to the contaminant prior to inoculation. Microorganisms intended to degrade PCBs are often acclimated using biphenyl, because it has the same basic structure as chlorinated biphenyls. Theoretically, growth on biphenyl induces production of enzymes that are used in the degradation of both biphenyl and substituted biphenyls. Lu *et al.* (1997) found that Aroclor 1242 is degraded more readily by microbes acclimated on Aroclor 1242 rather than biphenyl. However, the additional expense incurred by acclimating organisms with chlorinated compounds is probably not justified by the reduction in the lag period. Three types of bioremediation most amenable to PCB treatment are discussed in greater detail in the following sections: slurry-phase bioremediation, landfarming and composting.

Slurry-phase bioremediation

The principal concept behind slurry reactors is to increase the contact of the microorganisms with the contaminants, as well as with oxygen and the other nutrients necessary for microbial growth. This contact is increased by completely mixing the slurry in the reactor. Bioreactors are therefore able to decrease contaminant levels at a much faster rate than composting, land treatment or other solid-phase forms of bioremediation. FRTR (1999) offers treatment cost estimates of $160 to $210 per cubic meter (approximately $0.10 to $0.12 per kg) for slurry treatment including off-gas treatment for volatile compounds.

Slurry-phase biological treatment is usually an *ex situ* process. Soils are excavated and placed in a batch reactor along with water. Slurry reactors generally have a solids content of 10-40 weight percent. The greater the amount of solids, the more economical the system, since more soil can be treated per batch. However, as solids increase, more

energy is required to suspend particles and to achieve the same degree of mixing. Castaldi (1994), working with PAHs in soil wash concentrates, reported that approximately 40 weight percent of solids was optimal. Nutrients and microbial cultures are often added to the slurry. If aerobic conditions are desired, the reactor will need to be aerated with air jets or bubble diffusers.

Mixing is usually accomplished with mechanical agitators, such as augers, with water jets and with air diffusers. This portion of the design is difficult and many configurations have been proposed. Air jets are almost always necessary to ensure that enough dissolved oxygen is available for aerobic reactions. However, air bubbles may interfere with and reduce the efficiency of the mechanical agitators.

Efficient mixing is difficult to achieve and to monitor and is a subject of continuing study. Four mixing regimes have been described: nonsuspension, semisuspension, off-bottom suspension and complete suspension (Glaser *et al.*, 1995). Ideally, all particles will remain suspended at all times to maximize contact of microorganisms with the contaminant and nutrients. However, in cases where the degradation kinetics are controlled by factors other than aqueous mass transfer, other regimes may be more efficient. For example, if the microbial transformation kinetics are slower than mass transfer rates, it may uneconomical to mix constantly. This is true as well if the rate of desorption of the contaminant from the solid phase to the aqueous phase is the controlling factor of the degradation rates. In these cases, intermittent periods of agitation to resuspend the soil may be sufficient to achieve adequate mixing.

As with incineration, most contaminated soils require some degree of size reduction prior to bioslurry treatment. Particles must be small enough (usually less than ¼ inch in diameter) that they can be suspended by the reactor's agitators, and gravel may jam agitator blades. A good option for size reduction is soil washing since the wash effluent can be treated in the reactor. It should be noted that the abrasive wearing of metal parts by sand may lead to less efficient mixing, so reduction of the amount of sand size particles is desirable if it is not impractical. The organic carbon content of soils also has a major impact on reactor performance since hydrophobic contaminants will sorb more strongly and be less bioavailable in high carbon soils.

PCBs possess many properties that make remediation with biological processes a challenge. Their low aqueous solubilities, ranging from 1.2 mg/L for monochlorobiphenyl down to approximately 1.2 E –6 mg/L for decachlorobiphenyl, result in a low bioavailability of the contaminant to microorganisms. Bioavailability is often enhanced by the addition of surfactants to the bioremediation system. Care must be taken to use surfactants that do not inhibit microbial growth, and which do not act as a preferred substrate to the contaminant. In addition, high concentrations of the contaminant may also be inhibitory.

Another distinguishing characteristic of PCBs are the two distinct, congener-specific processes that are involved in their degradation by microorganisms. More highly

chlorinated congeners, those having five to ten chlorine substitutions, are degraded under anaerobic conditions through the process of reductive dechlorination, whereas lightly chlorinated congeners are degraded under aerobic conditions through a cometabolic process. The transformation of the heavily chlorinated congeners results in an increase in the concentrations of the more lightly chlorinated congeners. Thus, anaerobic degradation, a relatively slow process, will progress only to a certain degree at which point aerobic conditions become necessary for complete mineralization. Slurry reactors are an excellent technology for coping with this challenge.

Advantages. Bioremediation results in dechlorination of PCBs and possibly even in mineralization of the contaminant. Energy costs are lower than other forms of treatment. Slurry phase treatment usually requires less time than solid-phase biological treatment due to the increased rates of contaminant mass transfer. Furthermore, it is relatively simple to maintain either aerobic or anaerobic conditions in the reactor and to switch between the two conditions. As noted above, this characteristic is especially beneficial when treating PCBs. Anaerobic conditions can be maintained to allow for reductive dechlorination of the highly-chlorinated congeners. After this stage is complete, aerobic conditions can be established to allow the resulting lightly-chlorinated congeners to be metabolized aerobically.

Disadvantages. To date biological treatment with slurry reactors has not proven to be reliable or cost-effective, because many of the factors impacting performance are still poorly understood (Glaser *et al.*, 1995). The success of biodegradation may be hampered by low bioavailability of the contaminant, by mass transfer limitations of the electron acceptor and by low temperature. In addition, most feed soils will require some degree of size reduction, usually to a greater extent than that required with incineration. Particles must be small enough that they can be suspended by the reactor's agitators, and gravel may jam agitator blades.

Soils with large amounts of oily or greasy waste have been found to be problematic in slurry treatments. Because these wastes are hydrophobic, dispersants must be used to keep agglomerations of the waste from forming. Even so, the oils are often in large droplets with fairly low surface area with respect to their volume so that microbial attack is hindered. In addition, the oils will adhere to the sides of the reactors and to the agitation equipment, often blocking air nozzles.

Case studies. OHM Remediation Services Corporation completed a full-scale cleanup of 9600 cubic meters of PAH-contaminated wastes using slurry-phase treatment. Batch reactors were able to meet treatment criteria in about 12 days a cost of about $200 per ton or $0.22 per kg (Jerger and Woodhull, 1995). An aerobic bioslurry was used at the Joliet Army Ammunition Plant in Illinois to treat soil contaminated with TNT, HMX and RDX. Bioremediation resulted in a removal rate over 99%. A feasibility study for use of slurry-phase bioremediation has begun at the Iowa Army Ammunition Plant. No cases of a bioslurry being used to treat PCBs have been documented.

Landfarming

Landfarming is a long-term form of enhanced biological treatment. Contaminated soils and sediments are spread over a large area and mixed with native soil, usually by tilling, to allow the contaminants to be degraded or transformed by microorganisms. Cultures of microorganisms known to degrade the contaminant may be added to the soil. Though climate conditions cannot be controlled, microbial degradation rates are optimized to the extent possible by controlling soil moisture content by irrigation or spraying, aeration by tilling, and pH by lime addition. Bulking agents, such as vermiculite or wood chips, are often added to the soil to increase soil porosity and ensure adequate aeration of the soil in order to maintain aerobic conditions. Nutrient amendments such as phosphates or nitrates are often added as well to ensure that there are no nutritional limitations on microbial growth.

EPA reports landfarming treatment costs of less than $0.06 per kilogram of contaminated soil, excluding costs for laboratory and pilot studies. Harmsen *et al.* (1994) proposed using "extensive" landfarming, as opposed to "intensive" landfarming, which could lower these costs. Extensive landfarming involves much less intervention in soil conditions; after the initial spreading and mixing of the contaminated soil with native soil, further mixing may be performed only once or twice year if at all. This type of landfarming would be used in cases where most of the bioavailable portion of the contaminant has been degraded and microbial kinetics are limited by desorption of the contaminant into the aqueous phase. In this case, landfarming becomes a low-cost means of allowing time for mass transfer from the solid phase to the liquid phase to occur. However, decades may be required for the contaminant to transformed to an extent that meets remediation goals (Harmsen *et al.*, 1997).

Advantages. Landfarming requires relatively little maintenance and capital input. As a result, costs can be extremely low with respect to other technologies. The equipment required for landfarming is not technologically complex and is commercially available.

Disadvantages. Adequate environmental safeguards and monitoring must be in place to ensure that there is no contamination of the food chain, air or water. Runoff collection facilities must be constructed, and dust control, particularly during tilling, may be required. Landfarming is not suitable for wastes with volatile co-contaminants that would evaporate, nor for wastes with inorganic co-contaminants that are not biodegradable. Metals in particular could inhibit microbial growth. A large area of land is required, but this may not be a problem for some sites, such as military bases. Due to the long timeframe involved, landfarming would not be a suitable treatment option for sites that needed to be converted quickly to other uses.

Case studies. Most investigations of landfarming have concentrated on nonhalogenated contaminants. However, Smith *et al.* (1997) were able to achieve significant reductions in PCB contaminant levels in a pilot scale study. Comparisons were made using the Toxicity Characteristic Leaching Procedure (TCLP), a standard method of determining the amount of mobile contaminants in soils. Three of four land

treatment units showed TCLP extract concentrations of 500 ppb or less after 800 days of land treatment. Other measurements indicated that losses due to volatilization or leaching were less than 0.02% and 1%. These findings suggest that PCB contaminated soils may be good candidates for this form of treatment. Other studies are listed below.

Site:	Petroleum Products Terminal
Contaminant:	Petroleum hydrocarbons – 1000 mg/kg soil
Media:	Soil
Time:	3 years (seasonally operated)
Results:	Total petroleum hydrocarbons – 100 mg/kg soil
Cost per kg:	Unknown

Site:	Fuel oil spill
Contaminant:	Petroleum hydrocarbons – 6000 mg/kg soil
Media:	Soil
Time:	120 days
Results:	Total petroleum hydrocarbons – 100 mg/kg soil
Cost per kg:	Unknown

Site:	Wood treatment plant
Contaminant:	Pyrene: 135 mg/kg soil; pentachlorophenol: 132 mg/kg soil
Media:	Soil
Time:	Ongoing
Results:	Pyrene: <7.3 mg/kg soil; pentachlorophenol: 87 mg/kg soil
Cost per kg:	Unknown

Site:	Pesticide storage facility
Contaminant:	Pesticides: 86 mg/kg soil
Media:	Soil
Time:	5 months
Results:	Pesticides: 5 mg/kg soil
Cost per kg:	Unknown

Composting or biopiles

Composting is a form of biological treatment that seeks to enhance the natural rate of microbial degradation of contaminants by improving environmental conditions. The composting process entails excavating contaminated soil and mixing it with bulking agents to increase porosity and with organic amendments, such as manure, wood chips or vegetative wastes, to provide a carbon and nutrient source for microorganisms. Compost piles are irrigated to maintain appropriate moisture conditions and are aerated, usually by turning, to keep the piles aerated. In addition, heat produced during microbial degradation of the organic material may lead to thermophilic conditions, i.e. 54 to 65°C (130 to 150°F), which may further enhance degradation rates as well as desorption of contaminants from soil particles.

There are three principal composting process designs:

- Aerated static piles or biopiles, in which the compost is placed into piles and aerated with vacuum pumps;
- Mechanically agitated in-vessel composting, in which the compost is mixed in a reactor;

- Windrow composting, in which the compost is formed into long piles and mixed periodically with mobile equipment.

Windrow composting has been found to be the most cost-effective for large sites, although this method may also result in the highest amount of fugitive emissions. Because they are aerated with less frequency, (e.g., one time per week), windrows are also more likely to develop anaerobic zones. These anaerobic zones may not be desirable; however, some contaminants, such as highly-chlorinated PCBs, are more readily degraded under anaerobic conditions. Soils with SVOC contaminants may require off-gas control. EPA estimates costs at $0.15 per kilogram for windrow composting and $0.18 and $0.23 per kilogram, respectively, for static pile and in-vessel composting (FRTR, 1999). These figures are based on treatment of approximately 26 million kg (15,300 m^3) of soil, though no contaminant, duration or initial contaminant concentration are specified. Estimates given elsewhere in the same document for static pile composting range from only $0.08 to $0.15 per kg.

Advantages. Composting may result in the biodegradation of PCBs, and those compost piles containing both anaerobic and aerobic zones may be able to transform the more recalcitrant highly-chlorinated PCB congeners. In addition, composting is technically simple in terms of both equipment and operation. Though composting has not been tested extensively for halogenated organic compounds, it has proven successful in treating TNT and other recalcitrant compounds (FRTR, 1999).

Disadvantages. The disadvantages of composting include the large space requirement and the increase in volume due to the addition of amendments. In addition, metal co-contaminants will not generally be treated and may be toxic to the desired microorganisms. In some cases, off-gases may require monitoring and treatment. Treatability tests are generally necessary to ascertain the correct amount of aeration and nutrient amendment as well as the correct moisture content. Bioavailability of the contaminant may be limited by slow desorption kinetics. Therefore, adequate transformation of the contaminant may require a long period of time. Furthermore, if a biopile design is employed, biodegradation of the contaminant may be less uniform than with designs that require mixing of the compost.

Case studies. Composting has not been frequently employed to treat PCBs or other halogenated organic compounds. However, the technology has been employed at several sites involving soil contaminated with fuel or non-halogenated organics. At the Umatilla Depot, for example, windrow composting was demonstrated on explosives-contaminated soil. In 40 days of operation, TNT reductions as high as 99.7% were observed. In addition, RDX had a maximum removal efficiency of 99.8% and HMX, 96.8%. Other cases are summarized below.

Site:	Marine Corps Air Ground Combat Center, Twenty-Nine Palms, CA
Technology:	Biopiles
Contaminant:	Fuel – Total petroleum hydrocarbons (TPH): 702 mg/kg soil
Media:	Soil
Time:	Unknown
Results:	TPH: 234 mg/kg soil
Cost per kg:	$0.02

Site:	UMDA, Hermiston, OR
Technology:	Windrow composting
Contaminant:	TNT: 1563 mg/kg soil; RDX: 953 mg/kg soil; HMX: 156 mg/kg soil
Media:	Soil
Time:	Unknown
Results:	TNT: 4 mg/kg soil; RDX: 2 mg/kg soil; HMX: 5 mg/kg soil
Cost per kg:	$0.21 (based on 20 million kg)
Site:	Badger Army Ammunition Plant, WI
Technology:	Biopiles
Contaminant:	[TNT + HMX + RDX]: 17,000 mg/kg
Media:	Lagoon sediments
Time:	Unknown
Results:	Reductions: TNT, 99.6%; RDX, 94.8%; HMX, 86.9%
Cost per kg:	Unknown
Site:	Dubose Oil Products Co., Cantonment, FL
Technology:	Windrow composting
Contaminant:	PCP: up to 51 mg/kg soil
Media:	Soil – 17,900,000 kg
Time:	Unknown
Results:	PCP: up to 49.9 mg/kg soil. Clean-up goals met.
Cost per kg:	$0.29

Other treatment technologies

Due to the properties of polychlorinated biphenyls, certain remedial technologies have not been considered as viable options. For example, conventional soil vapor extraction, while an economical means of removing volatile organic compounds (VOCs) from soil, would be ineffective for removing PCBs from soil. The volatility of PCBs is so low that only minute quantities would be recovered with the extracted vapor.

Landfilling has also not been considered as a remedial treatment due to the fact that PCB-contaminated soil is subject to land disposal restrictions (Blackman, 1996). Solid wastes contaminated with PCBs may only be disposed of in a RCRA chemical waste landfill. As noted in the introduction, under the original provisions of CERCLA, PCB-contaminated and other contaminated soils were, more often than not, either landfilled or the sites were capped to prevent water intrusion, these being the least expensive alternatives. Unfortunately, since these techniques did nothing to destroy the compounds, landfilling had the effect of creating additional Superfund sites (Hesketh *et al.,* 1990). When Congress passed SARA, Congress specifically stated that landfilling and capping were undesirable without first permanently reducing the toxicity, volume or mobility of the contaminant (Hesketh *et al.,* 1990).

However, landfilling can still be used with some of the technologies discussed here, most notably *ex situ* solidification. Excavation and off-site disposal is the simplest of all remediation techniques, involving no special equipment and having well-defined procedures as determined by RCRA. The major drawback of this procedure is the increased potential exposure of workers and the public to the contaminant during excavation and transport. Nonetheless, excavation is necessary for all *ex situ* technologies regardless of the treatment. Cost estimates for excavation, transport and disposal of contaminated waste at a RCRA facility range from about $0.30 to $0.51 per

kg. Costs vary depending on the nature of the waste, the depth of contamination and the distance the waste must be transported.

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Attachment D-3.
Guidance Manual for the Use of GEMS

DESCRIPTION OF GENETICALLY ENGINEERED ORGANISMS (GEMs) TO BE USED IN PCB BIOREACTOR STUDIES
January 26, 2001

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Microorganism Identity Information

Burkholderia sp. strain LB400 was isolated from PCB-contaminated soil in New York state, by enrichment on biphenyl-containing medium (Bopp, 1986; 1989). *Rhodococcus* sp. strain RHA1 was isolated from a soil contaminated with hexachlorocyclohexane by enrichment on biphenyl medium (Seto et al., 1995). Both strains were taxonomically identified by morphology, physiology, Gram reaction, oxygen requirement and 16S rRNA sequence. The 16S rDNA sequences are available from GenBank, Accession Nos. U86373 (LB400) and U16312 (RHA1).

Descriptions of Both Recipient Microorganism and New Microorganism

Strain LB400 was originally described as a *Pseudomonas* but the identification was later changed to *Burkholderia cepacia* (Gene Bank Accession No. U86373). Recent recA gene sequencing indicated that this strain does not belong to pathogenic *Burkholderia cepacia* and was suggested to form a new species – *B. fungorum* (Dr. Eshwar Mahenthiralingam, Cardiff University, UK, personal communication). Strain LB400 can uniquely be identified by its ability to grow on both biphenyl and some ortho-chlorinated PCB congeners such as 2-, 2-2'-, and 2,4-2'-CBs (Bedard et al., 1986; Maltseva et al., 1999). The recombinant variant of LB400 has been constructed by introduction of the recombinant plasmid pRO41 which carries oxygenolytic *ortho*-dechlorination *ohbABCR* genes originally acquired from donor *Psequdomonas aeruginosa* strain 142 (Hrywna et

al., 1999; Tsoi et al., 1999). The GEM LB400(pRO41) can uniquely be identified as simultaneously growing on and mineralizing biphenyl and *ortho*-substituted chlorobiphenyls such as 2-, 2-2'-, 2-4'-, and 2,4-2'-chlorobiphenyls and 2-, 2,3-, and 2,5-chlorobenzoates. Nearly the entire genome of LB400 has now been sequenced by the Joint Genome Institute, and annotated by the Oak Ridge National Laboratory's Informatics group. The sequence contigsare available at: http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html (click on the image titled "*Burkholderia cepacia*"), and the gene annotation is available at http://genome.ornl.gov/microbial/bcep/

Strain RHA1 is a gram-positive non-sporulating, nonmotile organism with dual morphology of rods and cocci and containing meso-diaminopimelic acid and mycolic acids, and appears to belong to *Rhodococcus opacus-percolatus* cluster based on its 16S r DNA sequence (GenBank Accession No. U16312). It can uniquely be identified as simultaneously growing on biphenyl and ethylbenzene (Seto et al., 1995; Masai et al., 1995). The recombinant variant of strain RHA1 was constructed by introduction of recombinant plasmid pRHD34 which carries hydrolytic para-dechlorination *fcb*ABC genes originally acquired from *Arthrobacter globiformis* strain KZT1 (Rodrigues et al., 2001). The GEM RHA1(pRHD34) can uniquely be identified as simultaneously growing on both 4-chlorobenzoate and 4-chlorobiphenyl, as well as on biphenyl and ethylbenzene.

Genetic Construction of the New Microorganism

Data Substantiating Taxonomy of the Donor Microorganism to the Level of Strain

The donor of the hydrolytic para-dechlorination *fcb*ABC genes was *Arthrobacter globiformis* strain KZT1. KZT1 was isolated from vegetable garden soil in Moscow region, Russia by enrichment on 4-chlorobenzoate medium (Zaitsev and Karasevich, 1981). The organism had dual rod and cocci (in aging culture) morphology, was nonmotile and non-sporulating, gram-positive aerobe. It contained catalase and hydrolyzed starch but did not have nitrate reductase and did not form acid from glucose during growth on peptone, and therefore was identified as *Arthrobacter globiformis* (Zaitsev and Karasevich, 1981)

Donor of the oxygenolytic ortho-dechlorination *ohb*ABCR genes was *Pseudomonas aeruginosa* strain 142 (Tsoi et al., 1999). Strain 142 was isolated from PCB-contaminated soil in Moscow region, Russia, by enrichment on 2-chlorobenzoate-containing medium (Romanov et al., 1993). The organism was gram-negative, motile, nonsporulating rod, strict aerobe that formed fluorescin on KingA medium, reduced nitrate to nitrite and had other activities identifying it as P. aeruginosa (Romanov et al., 1993; Romanov and Hausinger, 1994).

Both Arthrobacter globiformis and Pseudomonas aeruginosa species are common in soils.

Description of the Traits for which the New Microorganism Has Been Developed

Strain LB400(pRO1) was developed to mineralize and grow on both *ortho*-substituted PCB congeners and *ortho*-chlorobenzoates. Strain RHA1(pRHD34) was developed to grow on *para*-chlorobiphenyl and *para*-chlorobenzoate.

Other Traits Known to Have Been Added or Modified

Both GEMs, LB400(pRO41) and RHA1(pRHD34), now also carry resistance to tetracycline encoded by the vector pRT1which was used to clone the dehalogenation genes. Naturally occurring variants resistant to rifampicin were selected to aid in tracking the organisms in soil.

Detailed Description of the Genetic Construction of the New Microorganism

The GEMs, LB400(pRO41) and RHA1(pRHD34), were constructed by electrotransformation of recipient cells with the respective plasmids. Plasmid pRO41 carries the oxygenolytic *ortho*-dechlorination *ohbABCR* genes and confers to the biphenyl-degrading host the ability to grow on *ortho*-halobenzoates and *ortho*-PCBs. The plasmid was constructed by recloning of the *ohb* gene region into universal shuttle vector pRT1. Plasmid pRHD34 carries the hydrolytic *para*-dechlorination *fcbABC* genes and confers to the biphenyl-degrading host the ability to grow on 4-chlorobenzoate and 4-chlorobiphenyl. The plasmid was constructed by recloning the *fcb* gene region into universal shuttle vector pRT1. The universal shuttle vector pRT1 was constructed by inserting the *Rhodococcus*-specific replicon from plasmid pRC1 into plasmid pSP329, a vector for gram-negative bacteria.

Description of genes, plasmids and strategy used for genetic modification of LB400 and RHA1 was published in peer-reviewed journals, as follows:

Tsoi TV, Zaitsev GM, Plotnikova EG, Kosheleva IA, Boronin AM. 1991. Cloning and expression of the Arthrobacter globiformis KZT1 fcbA gene encoding dehalogenase (4-chlorobenzoate-4-hydroxylase) in Escherichia coli. FEMS Microbiol Lett. 1991 Jun 15; 65(2): 165-9.

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Tsoi TV, Plotnikova EG, Cole JR, Guerin WF, Bagdasarian M, Tiedje JM. 1999. Cloning, expression, and nucleotide sequence of the Pseudomonas aeruginosa 142 ohb genes coding for oxygenolytic ortho dehalogenation of halobenzoates. Appl Environ. Microbiol. 65(5): 2151-62.

Rodrigues, J.L.M., O.V. Maltseva, T.V. Tsoi, R.R. Helton, J.F. Quensen, M. Fukuda, J.M. Tiedje. 2001. Development of a Rhodococcus recombinant strain for degradation of products from anaerobic dechlorination of PCBs. Environ. Sci. Technol. (in press).

Technique Used to Modify the Microorganism, e.g. Electroporation, Fusion of Cells

Electroporation with plasmid DNA was used to modify both the LB400 and RHA1 recipient organisms

Description of Introduced Genetic Material, Including Regulatory Sequences, Structural Genes and Products of the Genes

The *fcb* genes recruited from *A. globiformis* strain KZT1 were described previously by Tsoi et al. (1991), Zaitsev et al. (1991), Hrywna et al. (1999), and Rodrigues et al. (2001). The *fcb* sequence is available from GenBank, Accession No. AF304300. Structural *fcbABC* genes encode 3 enzymes, chlorobenzoyl-CoA ligase (FcbA), dehydratase/para-dehalogenase (FcbB) and thioesterase (FcbC), and are preceded by the *fcb*-specific promoter region and vector-encoded *lac*-promoter of *Escherichia coli* origin.
The *ohbABCR* genes recruited from *P. aeruginosa* strain 142 were described previously (Hrywna et al., 1999, Tsoi et al., 1999). The structural *ohbAB* genes encode terminal oxidoreductase large and small subunits, while the *ohbC* gene encodes putative transport protein, and the *ohbR* gene encodes putative regulatory protein. The *ohbABCR* nucleotide sequence is available from GenBank, Accession No.121970.

Expected Behavior of the Recipient Due to Introduced Genes

As a result of acquisition of the *ohbABCR* genes, the GEM LB400(pRO1) is now capable of growth on 2-, 2,5-, 2,4-, and 2,6-dichlorobenzoates and consequently mineralization of 2-2'-, 2,4-2'-, and 2,6-chlorobiphenyls. Similarly, the GEM RHA1(pRHD34) that acquired the *fcbABC* genes is now capable of growth on 4-chlorobenzoate and consequently mineralization of 4-chlorobiphenyl.

Expression, Alteration and Stability of the Introduced Material

The genes *fcb* and *ohb* were successfully expressed and stably maintained in the respective recipient hosts RHA1 and LB400, as verified by PCR amplification with gene-specific primers and by selective plating on the respective chlorobenzoate substrates, even after prolonged exposure to soil environment and to non-selective conditions.

Methods for Vector Construction and Introduction

Standard gene manipulation methods such as restriction endonuclease digestion, DNA gel-purification, DNA ligation and electroporation were used for vector construction.

Genetic Maps of Introduced Sequences

Universal shuttle vector for gram-negative and gram positive bacteria pRT1 has been constructed to maintain dehalogenation *fcb* and *ohb* genes as described and illustrated in Rodrigues et al. (2001). Vector pRT1 consists of vector plasmid pSP329 for gram-negative bacteria (Schmidhauser et al., 1985) and plasmid pRC1 with the origin or replication for Rhodococcus species (Hashimoto et al., 1992). Plasmid pSP329 consists of plasmid pTJS75a (Schmidhauser et al., 1985) that is a deletional variant of plasmid RK2/RP4 and a white-blue selection/expression block from plasmid pUC18 (M13MP18) (Yanish-Perron et al., 1985).
Complete genome sequence of the parental plasmid RP4/RK2 that was used to derive cloning vectors pSP329 and pRT1 is available from GenBank, Accession No. L27758.
Complete sequence of M13MP18 phage (pUC18/19 vectors) that was used in construction of cloning vectors pSP329 and pRT1 (white-blue selection/expression block containing *lac*-promoter) is available from GenBank, Accession No. X02513.

Genetic map of the *ohb* genes sequence (GenBank, Accession No. 121970) was published in Tsoi et al. (1999) and Hrywna et al. (1999). Recombinant plasmid pRO41 is comprised of the *ohbABCR* genes cloned into vector pRT1 (Rodrigues et al., 2001)

Genetic map of the *fcb* genes (GenBank, Accession No. AF304300) and the recombinant plasmid pRHD34 carrying *fcb* genes on pRT1 was published in Tsoi et al. (1999) and Rodrigues et al. (2001).

Phenotypic and Ecological Characteristics

Habitat, Geographical Distribution and Source of the Recipient Microorganism

The strain LB400 was isolated from PCB-contaminated landfill soil in Moreau,New York (Bopp, 1986; 1989). The strain was obtained from the USDA Culture Collection, and is described in U. S. Patent No. 4,843,009.

The strain RHA1 was isolated from hexachlorocyclohexane contaminated agricultural soil in Japan (Seto et al., 1995; Masai et al., 1995). The strain is a co-property of Michigan State University and Japan Science and Technology Co., Institute of Physical and Chemical Research (Japanese Patent Appl. No. 155069/1994, Ref. No. MI-5-2).

Members of both taxonomic clusters are very common in soil world wide. Members of these taxa are among the most common heterotrophic organisms in soil.

Survival and Dissemination Under Relevant Conditions, Including:

Method for Detecting the New or Recipient Microorganism in the Environment

Detection of the GEMs in soils was tested and verified by both selective colony plating and real-time quantitative PCR.

Limit of Detection for the Technique

At least 10^4 cells/g soil detection limit using selective colony plating or real-time PCR.

Anticipated Biological Interactions With and Effects on Other Organisms:

a. Target organisms
b. Competitors
c. Prey
d. Hosts
e. Symbionts
f. Parasites
g. Pathogens

None expected for these two taxonomic groups of common soil organisms.

Description of Host Range

Not known to be associated with hosts. Normal habitat is free living.

Pathogenicity, Infectivity, Toxicity, Virulence or Action as a Vector of Pathogens

Not expected.

Capacity for Genetic Transfer Under Laboratory and Relevant Environmental Conditions

Not detected under laboratory conditions.

Anticipated Involvement in Biogeochemical or Biological Cycling Processes, Mineral or Nutrient Cycling or Inorganic Compound Cycling

Anticipated improvement of environmental conditions via removal of toxic pollutants. Normal biogeochemical activity of these microorganisms is to recycling soil organic matter.

Proposed Research and Development Activity

Objectives, Significance and Rationale for Release

The GEMs will not be released to the environment, rather they will be utilized in contained vessels (bioreactors) located at the U.S. Army US Army Engineer Research Development Center (ERDC), 3909 Halls Ferry Road, Vicksburg, MS. The purpose of the experiment is a pilot-scale test of whether these modified organisms will enhance the rate and extent of PCB degradation in contaminated soil at three soils loading levels.

Number of Microorganisms to be Released and Method of Release, Including

Viability per Volume

We intend to add populations densities of 10^6 to 10^7 organisms per gram of soil.

Test Site Characteristics

Location, Geography, Physical, Chemical and Biological Features

As noted above, the GEMs will not be released to the environment. The bioreactors are housed at the U.S. Army US Army Engineer Research Development Center (ERDC), 3909 Halls Ferry Road, Vicksburg, MS. The laboratory facility has restricted access and the test will be conducted in controlled reactors.

Proximity to Human Habitation or Activity

The laboratory is a secure facility. Access is restricted to authorized personnel only. The facility is used exclusively for research and development activities.

Characteristics Influencing Dispersal or Confinement

The bioreactors are contained vessels, and therefore, no leaching or animal vectors can mobilize the microbes from the test vessel.

Target Organism, if Applicable

None; PCBs are the target.

Planned Start Date and Duration

The estimated (earliest) start date for the project is March 1, 2001. The GEMs will not be added to the bioreactors until the aerobic phase commences, which will be approximately six months after the start of the project (August 1, 2001). The PCB congener distribution in each bioreactor will be monitored on a monthly basis to determine change from anaerobic to aerobic conditions.

Evidence of Notification of State or Local Governments, if Applicable

The PCB-contaminated material will be excavated from a General Electric site located in Rome, GA. This effort has been coordinated with Jennifer Kaduck, Head of the Hazardous Waste Management Branch, Georgia Environmental Protection Division. Contact information is provided below:

Jennifer Kaduck
Hazardous Waste Management Branch
Environmental Protection Division
GA Department of Natural Resources
205 Butler Street, SE
Suite 1154, East Tower
Atlanta, GA 30334
Phone: (404) 656-7802

Fax: (404) 651-9425

The State of Mississippi, Department of Environment has been notified about the PCB R&D activity being carried out at US Army Engineer Research and Development Center, Vicksburg, MS.

Information on Monitoring and Control Measures

Confinement Procedures and Access and Security Measures

Pilot-scale test will be conducted in a confined place with restricted access.

Procedures for Routine Termination

For termination we propose to use agrimycin, which is an antibiotic (streptomycin) routinely used in soil for agricultural purposes. Both strains LB400 and RHA1 were found sensitive to this compound at concentrations of 60 mg/L. The EPA has waived all toxicological requirements for this product since it is a well-known antibiotic. (http://pmep.cce.cornell.edu/profiles/fung-nemat/febuconazole-sulfur/streptomycin/fung.

Mitigation and Emergency Procedures

In addition to the routine termination procedures described above in IV-B, the laboratory facility at ERDC is equipped with emergency spill control kits. The incidental spill kits include patch and plug material for container spills and leaks; and sorbent materials (pillows, socks, rolls or sheets) for absorbing the spills. However, if the spill is larger than a reportable quantity of a hazardous material (MSDS), the containment is treated as an emergency response within the scope of the Contingency Plan for Hazardous Materials Spills at US Army ERDC.

Measures to Detect and Control Potential Adverse Effects

No adverse effects of these common organisms and their degradative genes are known or expected.

Principal Investigator and Site Person in Charge of Emergency

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F. Personnel Protective Equipment, Engineering Controls and Procedures to Minimize Dispersal by People, Equipment and Machinery

All the test reactors are leak tested with built-in leachate collection system. The equipment/ machinery used in this pilot-test is restricted to that area. All the contaminated equipment will be properly decontaminated for storage or future use to avoid any dispersal of GEMs from the test reactors. Proper spill control procedures will be followed for the containment of any incidental spills, as described in IV-C above.

G. Procedures for Disposal of Contaminated Articles

The vessels containing inoculum, sample jars, contaminated gloves, and other equipment will be thoroughly autoclaved at 121 °C for 20 min, for decontamination. The disposable contaminated equipments and auxiliaries will be stored separately in "Bio-Hazard" containers and finally shipped for disposal to the facility under contract with US Army ERDC for the disposal of biohazard waste.

H. Inactivation Procedures and Disinfection Procedures

See IV-B above.

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General Electric Company, Schenectady, NY

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Federal Integrated Biotreatment Research Consortium (Flask to Field) represented a 7-year Strategic Environmental Research and Development Program (SERDP)-funded effort by several research laboratories to develop bioremediation technologies for contaminated U.S. Department of Defense (DoD) sites. The project was proposed as a new 6.2-level research program to enhance, but not duplicate, the existing funded efforts in the DoD. It also complemented both the U.S. Environmental Protection Agency and U.S. Department of Energy short- and long-term research strategies. The primary objective of this project was to develop the most promising biotreatment processes at the bench, intermediate, and pilot scale in each of four thrust areas: polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, energetics and explosives, and polychlorinated biphenyls (PCBs).

The consortium structure consisted of a lead principal investigator or director and four thrust area coordinators. Lead researchers at various government installations and academic institutions carried out the individual projects in each thrust area. Engineering groups worked closely with scientists in evaluating the potential of the resulting technologies and in the transfer of technologies from bench scale to field. A technical advisory committee consisting of leading biotechnology specialists in academia, industry, consulting, and government assisted and advised the director and reviewed individual projects for technical merit.

15. SUBJECT TERMS

Bioremediation
Chlorinated solvents
Explosives
Polychlorinated Biphenyls (PCBs)
Polycyclic Aromatic Hydrocarbons (PAHs)

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