Microbial Mechanisms Controlling the Fate of Fuel Component in Soil

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A 3-year effort has been completed that examined the microbiological processes governing environmental fate of fuel components. Laboratory assays examining the potential biodegradation of quadracycline by aerobic soil microorganisms from a variety of contaminated habitats revealed that this proposed fuel additive was resistant to microbial attack. A combination of biochemical and molecular biological approaches were used to examine how microorganisms in a coal tar waste-contaminated field site have adapted to metabolize the fuel component, naphthalene. A unique intermediary metabolite, cis-1,2-dihydroxy-1,2-dihydronaphthalene, was extracted from groundwater and identified by GC/MS. This proved that naphthalene catabolism by indigenous microorganisms was in progress at the time of sampling. In addition, naphthalene catabolic mRNA, transcribed from the nahAc gene, was extracted from groundwater, reverse transcribed, amplified by PCR, and sequenced. These nucleotide sequences were compared to one another and to sequences from pure cultures; new genetic diversity for contaminant metabolism was discovered. Horizontal transfer of naphthalene catabolic genes was documented in site bacteria by discovering a highly conserved nahAc allele among diverse bacterial hosts. The mobile genetic element responsible for metabolic adaptation was found to be a plasmid approximately 80 kb in size that was highly homologous to plasmid pDTG1 originally described in a bacterium isolated from soil in Bangor, Wales, UK, decades ago.
FINAL TECHNICAL REPORT

SUBMITTED TO

U.S. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
DIRECTORATE OF CHEMISTRY AND LIFE SCIENCES
801 NORTH RANDOLF ST., ROOM 732
ARLINGTON, VA 22203-1977

TO THE ATTENTION OF: Dr. Walter J. Kozumbo
For AFOSR Project – F49620-95-1-0346

MICROBIAL MECHANISMS CONTROLLING THE FATE OF FUEL COMPONENTS IN SOIL

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EXECUTIVE SUMMARY

Objectives:

The project research was designed to utilize a combination of laboratory and field studies to identify physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of fuel components. The major focus was on factors governing the fate of naphthalene in a contaminated field study site. Quadracyclane, a potential jet fuel additive, was also examined.

In accordance with this broad design, the three objectives were:

1. Explain the presence of highly conserved naphthalene metabolism genes at the field study site by documenting the presence of mobile genetic element(s) in both isolated bacteria and DNA directly extracted from contaminated sediments;
2. Use analytical chemistry and both biochemical and molecular tools for exploring the diversity of naphthalene catabolic pathways at the field site; and
3. Measure the susceptibility of quadracyclane to microbial attack and its metabolic pathways in both culture media and soil.

MAJOR ADVANCEMENTS:

Quadracyclane. A variety of assays [Gas chromatography/Mass spectrometry (GC/MS) analyses monitoring of loss from soil and spectrophotometric measures of microbial growth in soil suspensions] examining the susceptibility of the fuel additive, quadracyclane, indicates that this compound is highly resistant to microbial metabolism. Regardless of the type of inoculum used (soil and sediment from a diversity of climatic and prior contamination histories), no evidence for use of quadracyclane by microorganisms as a carbon and energy source was obtained. Efforts exploring possible cometabolic reactions using cyclohexane, cycloheptane, and norbornadiene as co-substrates also failed to demonstrate quadracyclane biodegradation. Thus, should AFOSR use quadracyclane as a fuel additive, little biodegradation of this material in soil should be expected.

Naphthalene. Insights into the expression of biodegradation genes and their transfer and dissemination between microorganisms at the contaminated study site were obtained. A metabolic pathway for bacterial naphthalene oxidation (established decades ago) was used as a
guide for selecting 1,2-dihydroxy-1,2-dihydronaphthalene (1,2-DHDN) as a unique transient intermediary metabolite whose presence in samples from a contaminated field site would indicate real time *in situ* naphthalene biodegradation. When surface waters emerging from the site were processed in a manner designed to avoid sample-handling artifacts, the 1,2-DHDN intermediate was successfully concentrated, extracted, and identified by gas chromatography/mass spectrometry. This is an example of how knowledge and techniques of biochemistry, microbial physiology, and analytical chemistry can be focused toward measuring transformations catalyzed *in situ* by naturally occurring microbial communities in real time.

A procedure for extracting and stabilizing mRNA-transcripts of naphthalene-dioxygenase genes (*nahAc*) expressed during microbial metabolism in the contaminated study site, was developed and applied to groundwater. The naphthalene catabolism mRNA transcripts were sequenced and compared to one another and related genes. The resultant phylogenetic analysis revealed the genetic diversity of naphthalene metabolism. This type of information has never been produced from a contaminated site before.

Our molecular characterization of *nahAc* detoxification genes actually expressed in groundwater was a novel approach to exploiting genetic diversity. The findings of field site-derived mRNA sequences that differ from those of cultivated microorganisms suggest that previously undiscovered genes operate in detoxification processes in natural microbial communities.

We have identified the location of naphthalene catabolism genes (on chromosomes and plasmids of our isolated bacteria). Furthermore, mating assays have verified the transferal of the mobile genes between bacteria. Restriction fragment length polymorphism (RFLP) and Southern analyses have proven that the plasmids carried by bacteria in our site are very closely related to one carried by a bacterium isolated decades ago from Bangor, Wales, UK. These results provide insights into the global distribution of biodegradation genes. Mating experiments with microorganisms extracted directly from site soil have proven this plasmid to be mobile and the likely mechanism by which the community adapts to contaminant exposure. These findings have important implications for AFOSR and other agencies that need to predict the fate of fuel components in soil.

Documenting the exchange of catabolic and other genes among bacteria inhabiting soil, sediment, and aquatic habitats is of ecological significance and poses substantial methodological
challenges. Using a population genetics approach to the distribution of naphthalene metabolism genes in the study site, a diversity of isolates capable of metabolizing naphthalene have been isolated from 2 spatially distinct locations at the field site (the contaminated seep area and an uncontaminated adjacent hillside soil). The isolated bacteria have been characterized taxonomically. These possessed PCR-amplifiable homologs of nahAc, the gene which codes for a key component of naphthalene dioxygenase. DNA sequencing was utilized to investigate relationships between these amplifiable nahAc genes and the 16S rRNA genes of the same bacteria. It was found that the naphthalene catabolic gene sequence was highly conserved between the taxonomically diverse hosts of the gene. We have isolated the plasmids and using pulsed field gel electrophoresis and southern hybridization characterize them. These mobile genetic elements (78 to 88 kb in size) are carried by individual bacteria and have been retrieved directly from the microbial community native to site soils. Thus, we have documented how naturally-occurring microbial communities can adapt to contaminant exposure and the mobile genetic element responsible for that adaptation. These findings provide evidence for the horizontal transfer of nahAc among the lineages of the naphthalene-degrading populations represented by our isolates. Genetic transfer of catabolic genes may play an important role in the evolution and adaptation of biodegradative bacterial populations to contaminants.

The information from horizontal gene transfer studies suggests that both (intracellular) transposon-mediated and (intercellular) plasmid-mediated transfer occurs in situ at our field study site. These findings are fundamental for advancing a mechanistic understanding of microbiologically-based intrinsic bioremediation technology – essential for both AF and society’s environmental clean-up needs.

Overall, these findings are fundamental for advancing a mechanistic understanding of microbiologically-based intrinsic bioremediation technology – essential for both AF and society’s environmental clean-up needs.
LIST OF PERSONNEL:

Eugene L. Madsen, Assistant Professor, Cornell University
Time devoted represented project cost share (no salary received)

Sharon E. Best, Laboratory Coordinator and Technician
Maintained continuity in all laboratory operations. Contributed data and technical assistance to all projects.

Mark S. Wilson, Ph.D. awarded January 1998. Graduate Field of Microbiology with minors in Environmental Toxicology and Genetics.
Title: Transient molecular markers of \textit{in situ} naphthalene biodegradation of a coal tar-contaminated field site.
Present position: Lecturer in cell biology and environmental science, Humboldt State University, Arcata, CA.

Karen G. Stuart, Ph.D. awarded August 1998. Graduate Field of Environmental Toxicology with minors in Microbiology and Civil/Environmental Engineering.
Title: Horizontal transfer of naphthalene catabolic genes among bacteria indigenous to a coal tar-contaminated field site.
Present position: On maternity leave prior to post-doctoral studies.

Corien Bakermans, Ph.D. candidate. Graduate Field of Microbiology with minors in Biochemistry and Civil/Environmental Engineering.

Amy Hohnstock, Ph.D. candidate. Graduate Field of Microbiology with minors in Biochemistry and Environmental Toxicology.

Undergraduates:

Peter Nguyen, Premedical Student. Cloning of naphthalene catabolic genes.

Eric Osborn, Presently enrolled in the Environmental Engineering Graduate Program at Oregon State University. Project: Isolation of mRNA from groundwater.

Kevin Drees, Presently enrolled in Environmental Engineering Graduate Program at University of Arizona. Project: Characterization of plasmids involved in horizontal gene transfer.


PUBLICATIONS:

Ph.D. Theses:


Corien Bakermans. (In progress). Diversity and cellular expression of naphthalene catabolic genes in a contaminated groundwater microbial community.


Peer-Reviewed Publications:


**RESULTS:**

Project results are summarized in the Executive Summary and publications listed above.

**INTERACTIONS/TRANSITIONS:**

A. Participation at Meetings:


Madsen, E. L. “Molecular and physiological strategies for understanding the fate of environmental pollutants in field sites” Invited speaker, Institute for Bioremediation and Detoxification, Penn State University (1995).


B. Consultation
Invited speaker and consultant with the Los Alamos National Laboratory, Chemical and Environmental Research and Development Group (1995). Contact: James Brainard.

Armstrong Environmental Research Laboratory, Tyndall Air Force Base, Panama City, FL (1996). Contact: Jim Spain.

C. Transitions:
Information listed above has led to no New Patents
HONORS/AWARDS TO E. L. MADSEN:

Member of editorial board of *Applied and Environmental Microbiology* (since 1987)
Professional Profile appears in *Who’s Who in Science and Engineering* (1992 to present)
National Academy of Sciences (NRC) Panel on *In Situ* Bioremediation, Committee Member and
Rapporteur (1992), Author of first two drafts of the NRC book entitled, “*In situ*
Gordon Research Conference, Invited Discussion Leader, Applied and Environmental
Microbiology (1997)
National Academy of Sciences Panel on Intrinsic Bioremediation of Contaminants in the
Subsurface Environment (1997-1999)

PROCEDURES:

All procedures utilized in this AFOSR project are reported in the peer reviewed publications
listed above.

APPENDIX:

All collected data are reported in the above-listed peer reviewed publications.