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13. ABSTRACT (Maximum 200 words) 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.				
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**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**

Submitted by:

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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 *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.

Topic: Diversity and cellular expression of naphthalene biodegradation genes in a contaminated groundwater community. Graduation expected December 1999.

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

Topic: Mechanisms of horizontal gene transfer in a coal tar waste-contaminated field site. Graduation expected May 2000.

Undergraduates:

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

Eric Osborn, Presently enrolled in the Environmental Engineering Graduate Program at Oregon Sate 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.

- Nai Zhou, New York University. Biology Graduate student. Project: Characterization of naphthalene metabolic pathway.
- Tom Dispenza, Cornell University. Biochemistry Major. Project: Characterization of naphthalene metabolic pathway.
- Erin Krull. Cornell University. Biology major. Project: Microscopic identification of naphthalene-degrading microorganisms in groundwater.
- Rebecca Lorang. Cornell University. Biology major. Project: Horizontal transfer of biodegradation genes.

#### PUBLICATIONS:

##### Ph.D. Theses:

- Mark S. Wilson. January 1998. Transient molecular markers of *in situ* naphthalene biodegradation of a coal tar-contaminated field site. Ph.D. Thesis. Cornell University, Ithaca, NY.
- Karen G. Stuart-Kiel. August 1998. Horizontal transfer of naphthalene catabolic genes among bacteria indigenous to a coal tar-contaminated field site. Ph.D. Thesis. Cornell University, Ithaca, NY.
- Corien Bakermans. (In progress). Diversity and cellular expression of naphthalene catabolic genes in a contaminated groundwater microbial community.
- Amy Hohnstock. (In progress). Mechanisms of horizontal gene transfer in a coal tar waste-contaminated field site.

##### Peer-Reviewed Publications:

- Wilson, M.S., C. Bakermans, and E. L. Madsen. 1998. In situ, real time catabolic gene expression: Extraction and characterization of catabolic mRNA transcripts from groundwater. *Appl. Environ. Microbiol.* (In Press)
- Stuart-Keil, K.G., A.M. Hohnstock, K.P. Drees, J.B. Herrick. and E. L. Madsen. 1998. Plasmids responsible for horizontal transfer of naphthalene catabolic genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *Pseudomonas putida* 9816-4. *Appl. Environ. Microbiol.* 64: 3633-3640.
- Madsen, E. L. 1998. Verification of Bioremediation. In: J. J. Valdes (ed.) *International Society for Environmental Biotechnology: Environmental Monitoring and Biodiagnostics*. Kluwer Academic Publ. (In Press).
- Madsen, E. L. 1998. Theoretical and Applied Aspects of Bioremediation: The Influence of Microbiological Processes on Organic Compounds in Field Sites. In: R. Burlage, R. Atlas, D. Stahl, G. Geesey, G. Saylor (eds.) *Techniques in Microbial Ecology*. Oxford University Press. pp.354-407.
- Madsen, E. L. 1998. Epistemology of environmental microbiology. *Environ. Sci. Technol.* 32: 429-539.
- Madsen, E. L. 1997. Themes and variations of aromatic hydrocarbon catabolism by microorganisms in a contaminated field site. In: *Progress in Microbial Ecology. Proceedings of the 7th International Symposium on Microbial Ecology*. M. T. Martins *et al.*, (eds), pp. 587-596.

- Herrick, J. B., K. G. Stuart-Keil, W. C. Ghiorse, and E. L. Madsen. 1997. Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native a contaminated field site. *Appl. Environ. Microbiol.* **63**:2330-2337.
- Herrick, J. B., D. N. Miller, E. L. Madsen, and W. C. Ghiorse. 1997. Extraction, purification, and PCR amplification of microbial DNA from sediments and soils. *In*: J. Burke (ed.) *PCR: Essential Techniques*. Bios Scientific Publishers, Oxford.
- Madsen, E. L. 1997. Methods for determining biodegradability. *In*: C.J. Hurst, *et al.* (ed.). *Manual of Environmental Microbiology*. American Society for Microbiology, Washington, DC, pp. 709-720.
- Sandoli, R. S., W. C. Ghiorse, and E. L. Madsen. 1996. Regulation of microbial phenanthrene mineralization in sediments by sorbent- sorbate contact time, inoculum, and gamma irradiation-induced sterilization artifacts. *Environ. Toxicol. Chem.* **15**:1901-1907.
- Madsen, E. L., C. L. Mann, and S. Bilotta. 1996. Oxygen limitations and aging as explanation for the persistence of naphthalene in coal-tar contaminated surface sediments. *Environ. Toxicol. Chem.* **15**:1876-1882.
- Madsen, E. L., C. T. Thomas, M. S. Wilson, R. L. Sandoli, and S. B. Best. 1996. *In situ* dynamics of aromatic hydrocarbons (AHs) and bacteria capable of AH metabolism in a coal tar waste-contaminated field site. *Environ. Sci. Technol.* **30**:2412-2416.
- Madsen, E. L. 1996. A critical analysis of methods for determining the composition and biogeochemical activities of soil microbial communities *in situ*. *In*: G. Stotzky and J.-M. Bollag (eds.). *Soil Biochemistry*, Vol. **9**:287-370.
- Wilson, M. S., and E. L. Madsen. 1996. Field extraction of a unique intermediary metabolite indicative of real time *In situ* pollutant biodegradation. *Environ. Sci. Technol.* **30**:2099-2103.
- Ghiorse, W. C., J. B. Herrick, R. L. Sandoli, and E. L. Madsen. 1995. Natural selection of PAH-degrading bacterial guilds at coal tar disposal sites. *Environmental Health Perspectives* **103**(5):107-111.
- Madsen, E. L. 1995. Impacts of agricultural practices on subsurface microbial ecology. *In*: D. Sparks (ed.). *Advances in Agronomy* Vol. **54**, pp. 1-67.
- Madsen, E. L., S. E. Bilotta-Best, and W. C. Ghiorse. 1995. Development of a rapid <sup>14</sup>C-based field method for assessing potential biodegradation of organic compounds in soil and sediment samples. *J. Microbiol. Meth.* **21**:317-327.

## **RESULTS:**

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

## **INTERACTIONS/TRANSITIONS:**

### **A. Participation at Meetings:**

- Wilson, M. S., and E. L. Madsen. 1995. Recovery of 1,2-dihydroxy-1,2-dihydronaphthalene from a contaminated field site indicates *in situ* naphthalene biodegradation. Abstract, *Amer. Soc. Microbiol. Annu. Meet.*, Washington, DC. Q126, p. 421.
- Wilson, M. W., J. B. Herrick, and E. L. Madsen. 1995. Analysis of intermediary metabolites from diverse bacteria indicate a highly conserved pathway for naphthalene catabolism. Abstract, *Amer. Soc. Microbiol. Annu. Meet.*, Washington, DC. Q-411, p. 472.



- Madsen, E. L., J. B. Herrick, M. S. Wilson, K. Stuart, R. S. Sandoli, and W. C. Ghiorse. 1995. Molecular and biochemical microbial ecology of a coal tar-contaminated waste site undergoing intrinsic bioremediation. Abstract, the Third International Bioreclamation Symposium, San Diego, CA.
- Madsen, E. L. "A view toward bioremediation based on microbiological studies of a contaminated aquifer". Opening speaker for private industrial bioremediation symposium sponsored by Merck Co., Inc., Whitehouse Station, NJ (1994).
- 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).
- Madsen, E. L. Speaker and participant in U.S. Dept. Defense Tri-Service Workshop on Bioavailability of Organic Contaminants in Soils and Sediments, Monterey, CA (1995).
- Madsen, E. L. Participant and Panel Member responsible for reviewing progress and status of a Lawrence Livermore Laboratory-sponsored Bioremediation Technology Development Program. U.S. Department of Energy, Contaminant Plumes Focus Area, Pleasonton, CA (1995).
- Stuart-Keil, K. G., J. B. Herrick, and E. L. Madsen. 1996. Preliminary characterization of plasmids that may be responsible for *In situ* horizontal transfer of a naphthalene catabolic gene. Abstract, Amer. Soc. Microbiol. Annu. Meet., New Orleans, LA.
- Miller, D. N., E. L. Madsen, and W. C. Ghiorse. 1996. Evaluation and optimization of soil DNA extraction and purification techniques. Abstract, Amer. Soc. Microbiol. Annu. Meet., New Orleans, LA.
- 7<sup>th</sup> International Symposium on Microbial Ecology. Santos, Brazil (1995). Madsen served as chairperson for Oral Session on Ecology and Bioremediation; Invited speaker contributing to Symposium on the Ecology of Bioremediation Lecture title: "Themes and variations of aromatic hydrocarbon catabolism by microorganisms in a contaminated field site".
- Madsen, E. L., C. L. Mann, S. Bilotta, R. Yager. 1997. In situ attenuation of chlorinated ethenes by naturally-occurring microorganisms in a fractional dolomite aquifer near Niagara Falls, NY. Abstract, American Society for Microbiology. Annu. Meet., Miami, FL.
- Wilson, M. S. and E. L. Madsen. 1997. RT-PCR detection of naphthalene catabolic gene expression serves as real-time evidence for biodegradation in a coal tar waste-contaminated field site. Abstract, American Society for Microbiology. Annu. Meet., Miami, FL.
- Bakermans, C., and E. L. Madsen. 1997. Progress towards direct cloning of soil DNA. Abstract American Society for Microbiology. Annu. Meet., Miami, FL.,
- 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)  
Gordon Research Conference, Invited Speaker, Applied and Environmental Microbiology (1991)  
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*  
bioremediation : When Does It Work?". 1993.  
Gordon Research Conference, Invited Speaker, Environmental Science: Water (1994)  
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.