EXECUTIVE SUMMARY

Developing and Field-Testing Genetic Catabolic Probes for Monitored Natural Attenuation of 1,4-Dioxane

SERDP Project ER-2301

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ACRONYMS AND ABBREVIATIONS

Dioxane	1,4-Dioxane
DoD	Department of Defense
DOE	Department of Energy
EPA	Environmental Protection Agency
GC/MS	Gas Chromatograph / Mass Spectrometer
MNA	Monitored Natural Attenuation
MO	Monooxygenase
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SDIMO	Soluble Di-iron Monooxygenase
SERDP	Strategic Environmental Research and Development Program
THF	Tetrahydrofuran

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1.0 INTRODUCTION

Remediation of aquifers contaminated with 1,4-dioxane can be complex because dioxane is highly mobile in groundwater, recalcitrant to biodegradation, and is not easily removed by volatilization or adsorption. Monitored natural attenuation (MNA), which relies primarily on contaminant's removal by biodegradation, is often the most cost-effective approach to manage large and dilute groundwater plumes such as those encountered in sites impacted by dioxane contamination. However, the burden of proof that MNA is an appropriate solution lies on the proponent, which requires site-specific demonstration of contaminant's removal with strong evidences to support the presence of expression of biodegradation capabilities.

2.0 OBJECTIVES

In this project, we *developed and validated catabolic gene probe(s) to quantify the presence and expression of dioxane biodegradation genes*, to support decisions to select or reject MNA and assess its performance at dioxane-impacted sites. Specific tasks included:

1. Identification of genes responsible for initiating dioxane biodegradation, development of a primer/probe biomarker to target the identified genes, and assessment of reliability (selectivity/sensitivity) of gene biomarker to evaluate the presence and activity of dioxane degraders at a broad range of contaminated sites.
2. Statistical analyze to determine how the abundance of this catabolic biomarker (and associated degradation activity) is influenced by geochemical factors and contamination scenarios.
3. Identification (and perhaps isolation) of dioxane degraders from these sites, using enrichment and dilution to extinction or fluorescence activated flow cytometry with cell sorting techniques.
4. Characterization of catabolic gene sequences involved in dioxane metabolism in isolated or sorted cells using DNA and messenger RNA (mRNA) sequencing approaches to obtain more sequences for possible adjustment/optimization of our previous biomarker design.

3.0 TECHNICAL APPROACH

This project required a multidisciplinary multi-scale approach. The first step was to identify genes responsible for initiating dioxane biodegradation. We identified the *dxmADBC* operon in the archetype dioxane metabolizer, *Pseudonocardia dioxanivorans* CB1190, using bioinformatics and transcriptomic analyses. Then, we developed a primer/probe set to quantify a putative dioxane monooxygenase gene using PCR and *Taqman* chemistry. This process involved multiple sequence alignment of the four *thmA/dxmA* genes available on the National Center for Biotechnology Information (NCBI) database. Then we used our developed *thmA/dxmA* biomarker and quantitative polymerase chain reaction (qPCR) assays to conduct a broad survey of the presence of dioxane degraders and assess their activity at numerous, diverse dioxane-impacted sites (Task 1). This was critical to determine if our biomarker was selective, sensitive, as well as reliable as a tool to assess dioxane biodegradation potential with potential to infer on site-specific biodegradation rates.

The data obtained during Task 1 was statistically evaluated (multivariate analysis of variance) in conjunction with site-specific geochemical characteristics and contaminant concentrations to heuristically discern general conditions that are amenable for MNA, which served to identify factors that may broadly affect biodegradation activity (Task 2). Furthermore, we developed a method to isolate and identify the phylogenetic and metabolic diversity of indigenous dioxane degraders using gene probes to target mRNA from catabolic genes (i.e., actively degrading bacteria) and fluorescence activated flow cytometry with cell sorting (Task 3). The catabolic genes in isolated bacteria were sequenced and annotated to obtain additional information about dioxane metabolic pathways, and to optimize the design of catabolic biomarkers for further evaluation of the performance of dioxane MNA and bioremediation (Task 4).

4.0 RESULTS AND DISCUSSION

Through multiple alignment analyses, the project team found that critical nucleotide sequences of key catabolic genes coded for the active site of the enzymes are highly conserved. These sequences were used to develop the *thmA/dxmA* probe capable of targeting putative dioxane degrading monooxygenase. We demonstrated that this primer-probe set was both highly selective (no false positives) and sensitive (7,000 ~ 8,000 copies/g soil) when tested against a dioxane-degrading reference strain (CB1190). A significant correlation between biodegradation rates and the abundance of *thmA/dxmA* genes were observed. Contrariwise, nonspecific 16S rRNA gene copy numbers were neither sensitive nor reliable as indicator of dioxane biodegradation activity (Task 1).

While many geochemical factors (pH, temperature, nutrients) and the abundance of specific degraders may be important for dioxane biodegradation, these factors may not exert as strong of an influence on the potential biodegradation activity as the concentration of dioxane. Unequivocally, *in situ* dioxane concentrations significantly influenced the observed dioxane biodegradation rates with higher dioxane concentrations. Whereas auxiliary carbon sources may temporarily enhance dioxane biodegradation, they may also exert counterproductive long-term consequences. Non-inducing growth substrates can boost the overall microbial biomass but inhibit the indigenous dioxane degraders (Task 2).

Fluorescently labeled oligonucleotide probes and flow cytometry is a cultivation-free strategy to separate and concentrate genus-specific subpopulations of interest. This will expedite discovering novel *Pseudonocardia* strains and discerning the molecular basis for specific metabolic traits of interest. Besides, using dilution to extinction method, two dioxane degrading consortia were enriched from uncontaminated environments. These have comparable dioxane degradation capabilities with two archetype dioxane degraders. *Mycobacterium* was the dominant genus and group 5 and group 6 soluble diiron monooxygenase (SDIMOs) was prevalent after enrichment (Task 3).

Through whole-genome sequencing, mRNA sequencing and real-time polymerase chain reaction, a group-6 propane monooxygenase (*prmABCD*) was identified in *Mycobacterium dioxanotrophicus* PH-06. Accordingly, a primer/probe set was developed and validated through four dioxane-degrading consortia and strong correlation was found between dioxane degradation rates and the abundance of biomarkers. This new biomarker is highly selective and sensitive and would minimize the false negatives of our previous biomarker (*thmA/dxmA*). Furthermore, the preliminary results of construction of a reporter strain to monitor dioxane degradation activities would facilitate future development (Task 4).

5.0 BENEFITS

This project supports the rational selection of MNA over other remediation approaches that are usually more complex and costly (e.g., pump-and-treat with advanced chemical oxidation treatment). It also provides a scientific basis for the interruption of remediation strategies when these are no longer removing dioxane faster than MNA. Together, these findings could aid decision-makers to more appropriately select for cost-effective clean up strategies, saving the industry, state, and federal government from multi-billion-dollar risk penalties. This work also responds to urgent calls for more reliable risk assessment and management approaches for hazardous wastes that have come from environmental advocacy groups, industry, and the regulatory community.