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Advancement of Nucleic Acid-Based Tools for Monitoring *In Situ* Reductive Dechlorination

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Final Report – October 2006

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1.0 RESEARCH OBJECTIVES

This project addressed the following high priority technical needs for Monitored Natural Attenuation and Enhanced Attenuation (MNA/EA): (i) design of nucleic acid-based site assessment tools, (ii) development of correlations between phylogenetic and functional gene information and chlorinated ethenes reductive dechlorination activity, and (iii) further characterization of chlorinated ethenes-dechlorinating enrichment cultures.

The current state of knowledge allows, at best, short-term predictions on plume development during MNA or implementation of more aggressive treatment(s) including biostimulation and bioaugmentation (EA). Limited data are available on the sustainability of natural attenuation processes, and predictions on whether MNA or EA can be used with confidence for long-term plume control are elusive. Hence, ***the overall goal of this research effort was to design tools and provide information for guidance protocols for assessment and monitoring of the key microbial populations*** responsible for chlorinated ethenes removal in plumes undergoing MNA or EA, and to make meaningful predictions on long-term plume development. The availability of rigorously tested tools and protocols will demonstrate whether MNA and EA are viable approaches that can be used with confidence at high-risk DOE waste sites. With the increased knowledge and understanding of the dechlorination process, along with appropriate assessment tools, site managers and regulators will have the means to convincingly argue that MNA and EA are viable, cost effective approaches for plume control and long-lasting risk reduction. This work was conducted in two phases.

The aims of **Phase 1** were:

1. to refine the 16S rRNA gene-based approach and define its limitations.
2. to design PCR-based approaches that target reductive dehalogenase (RDase) genes implicated in chlorinated ethene reductive dechlorination, and to environmental samples for the presence of RDase genes involved in chlorinated ethene reductive dechlorination.

3. to establish a link between the presence (and expression) of a particular gene (or set of genes) with an observable trait (e.g., reductive dechlorination of VC to ethene).
4. to explore microarray technology for identification of novel reductive dechlorination biomarker genes.
5. to further characterize chlorinated solvent-dechlorinating enrichment cultures and obtain additional dechlorinating isolates.

The aim of **Phase 2** was:

6. to test and validate the nucleic acid-based tools designed in Phase 1 using samples collected at field sites.

2.0 RESULTS AND DISCUSSION

2.1 Phase 1 Results:

A. Refinement of 16S rRNA gene-based PCR approaches.

Laboratory and field evidence linked complete reductive dechlorination of chlorinated ethenes to environmentally benign ethene to the presence of *Dehalococcoides* bacteria. Hence, the focus was on 16S rRNA gene-based approaches to distinguish *Dehalococcoides* strains capable of efficient vinyl chloride (VC) to ethene formation from those *Dehalococcoides* strains that cannot dechlorinate chlorinated ethenes or co-metabolize VC, which is a slow and incomplete reaction.
Based on 16S rRNA gene sequence differences, *Dehalococcoides* group was originally divided into the Cornell, Victoria and Pinellas groups. *Dehalococcoides ethenogenes* strain 195 belongs to the Cornell group and co-metabolizes VC to ethene. In contrast, *Dehalococcoides* sp. strain BAV1, a member of the Pinellas group, grew with VC as electron acceptor and produced ethene more efficiently. Hence, we explored if 16S rRNA gene-based analysis is useful to distinguish *Dehalococcoides* strains belonging to different groups and whether or not this grouping reflects physiological capabilities (i.e., reductive dechlorination of chlorinated ethenes). The alignment of 16S rRNA gene sequences from the few *Dehalococcoides* isolates and environmental clone sequences available in GenBank allowed us to design 16S rRNA gene-targeted primers that distinguish members of the Cornell, Victoria and Pinellas groups. These tools distinguished the VC-respiring strain BAV1 from the strain 195, which cannot grow with VC. Three other Pinellas isolates, strain CBDB1, strain FL2 and strain GT share identical 16S rRNA gene sequences, yet each strain has distinct dechlorination capabilities. Strain CBDB1 fails to dechlorinate chlorinated ethenes, strain FL2 grows with trichloroethene (TCE) and *cis*-1,2-dichloroethene (*cis*-DCE) as electron acceptors but dechlorinates VC only co-metabolically, whereas strain GT respires TCE, *cis*-DCE and VC and efficiently produces ethene. These finding demonstrate that the *Dehalococcoides* 16S rRNA gene carries insufficient information to infer physiological properties (i.e., reductive dechlorination activity).

Nevertheless, the 16S rRNA gene-based approach is a valuable tool to detect *Dehalococcoides* bacteria in environmental samples. A first round PCE with bacterial 16S rRNA gene-targeted primers followed by a second round of PCR with *Dehalococcoides* 16S rRNA gene-targeted primers (i.e., nested PCR) allows detection of *Dehalococcoides* organisms in environmental samples with unsurpassed sensitivity. This approach allows the detection of as few as 100 cells per liter of groundwater. We further designed, optimized and validated a quantitative real-time PCR approach to enumerate *Dehalococcoides* 16S rRNA genes in laboratory and environmental samples. Despite its limitations to distinguish individual *Dehalococcoides* strains, the 16S rRNA gene approach provides relevant information on *Dehalococcoides* presence and abundance. To gain an understanding of the relative abundance of *Dehalococcoides* organisms in a given sample, we designed qPCR protocols to

enumerate total Bacteria and Archaea. The combined application of these quantitative tools provides information on the relative abundance of *Dehalococcoides* 16S rRNA genes in mixed cultures or environmental samples. Despite the limited resolution of the 16S rRNA gene-based approach, these tools are useful for monitoring the *Dehalococcoides* community at MNA/EA sites over temporal and spatial scales. The application of these 16S rRNA gene-based approaches have been optimized and validated with field samples, and protocols for their application have been published (see section 5.0).

B. RDase-targeted PCR approaches.

To overcome the limitations of the 16S rRNA gene-based approach, we designed PCR approaches to specifically detect the *tceA*, *vcrA* and *bvcA* genes implicated in chlorinated ethene reductive dechlorination. The *tceA* gene was detected in *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain FL2 and is implicated in TCE-to-ethene-dechlorination. The RDase genes *vcrA* and *bvcA* were identified in *Dehalococcoides* sp. strain VS and strain BAV1, repectively, and are involved in VC-to-ethene dechlorination. qPCR approaches using linear hybridization (i.e., TaqMan) probe reporter systems were designed to detect and quantify the *tceA*, *vcrA* and *bvcA* genes in laboratory cultures and environmental samples. This approach was productive and useful to monitor individual RDase genes, and hence specific *Dehalococcoides* strains in environmental samples.

The combined application of 16S rRNA gene- and RDase gene-targeted approaches provides additional information about the chlorinated ethenes-dechlorinating *Dehalococcoides* population. Figure 1 shows the relative abundance of *Dehalococcoides* bacteria following bioaugmentation with BioDechlor INOCULUM, a commercial PCE-to-ethene dechlorinating culture containing *Dehalococcoides* sp. strain FL2 (possessing *tceA*), strain BAV1 (possessing *bvcA*) and strain GT (possessing *vcrA*) at a site where no native *Dehalococcoides* had been detected. The *Dehalococcoides* community was maintained for at least 14 months following inoculation, and the quantitative assessment of the *tceA*, *vcrA* and *bvcA* RDase genes suggested temporal dynamics of individual *Dehalococcoides* populations with distinct dechlorination properties.

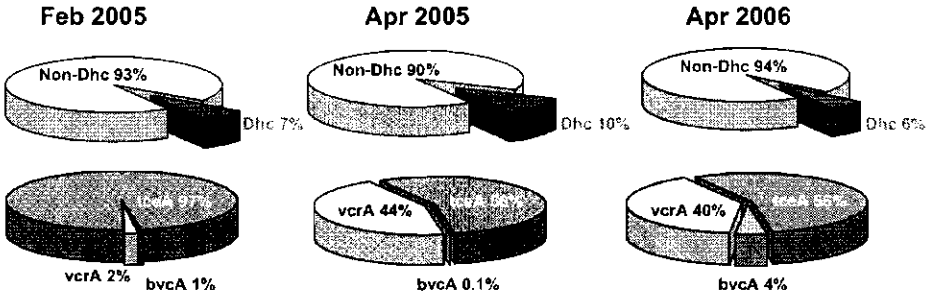

Figure 1: Relative abundance of *Dehalococcoides* bacteria following bioaugmentation with a PCE-to-ethene dechlorinating culture (BioDechlor INOCULUM) at a site where no native *Dehalococcoides* had been detected. The bottom part of the figure shows the relative abundances of the *tceA*, *vcrA* and *bvcA* genes.

C. Linking RDase gene presence and expression with dechlorination activity.

Targeting *Dehalococcoides* 16S rRNA genes and RDase genes implicated in chlorinated ethene reductive dechlorination provides valuable information about their presence and abundance; however, DNA-based tools cannot provide information about gene expression and dechlorinating activity. To

assess gene expression, different RNA extraction procedures were tested on *Dehalococcoides* pure cultures and *Dehalococcoides*-containing mixed cultures. These efforts formed the basis to expand these efforts to RNA extraction from groundwater samples from field sites. A protocol for RNA extraction from groundwater is in its final stages of testing, and we anticipate that the procedures developed will serve as a foundation for the design of standard procedures and protocols for RNA analyses of groundwater samples. Our efforts with pure and mixed cultures demonstrated that the expression of specific RDase genes (e.g., *tceA*, *vcrA* and *bvcA*) can be detected and quantified using a reverse transcriptase (RT)-qPCR approach.

The applicability of the RT-qPCR approach was tested on groundwater samples collected from a site undergoing EA including biostimulation with lactate and bioaugmentation with BDI. Groundwater samples (1 L each) were collected for biomass concentration and nucleic acid extraction. The biomass of duplicate groundwater samples was harvested *on-site* by vacuum filtration, and the membrane filters were flash frozen in liquid nitrogen. Two 1-L bottles were transported on blue ice to the laboratory and stored for 12 hours prior to biomass collection by vacuum filtration. RNA extraction from all samples was performed simultaneously. Figure 2 demonstrates that target 16S rRNA and *tceA*, *vcrA* and *bvcA* mRNAs were successfully detected and quantified in groundwater samples collected from the site undergoing EA. DNA was extracted from the same samples and the *Dehalococcoides* 16S rRNA genes and *tceA*, *vcrA* and *bvcA* genes were quantified.

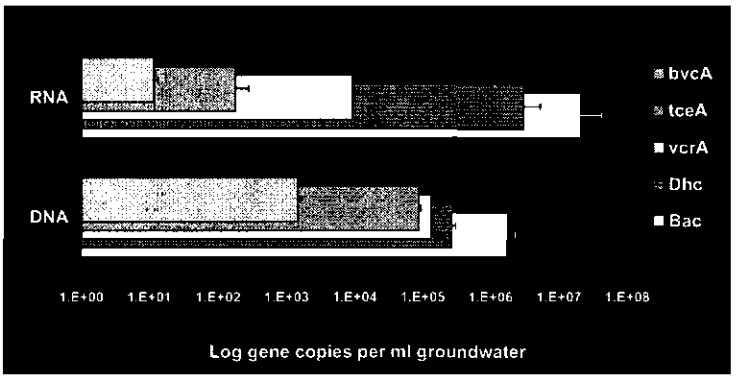

Figure 2: Quantification of *Dehalococcoides* 16S rRNA and RDase mRNA in total RNA extracts from groundwater (top). Also shown is the quantification of *Dehalococcoides* 16S rRNA genes and RDase genes (bottom).

Similar results were obtained with samples that were flash frozen immediately after collection and groundwater samples that were stored at 4°C for 24 hours. These preliminary findings suggest that RNA analysis of groundwater samples is feasible, and can provide information on overall *Dehalococcoides* activity and the expression of specific RDase genes. Further development and optimization is required to advance this approach, and ultimately link target transcript (i.e., mRNA) abundance to activity and provide practitioners with estimates on *in-situ* dechlorination rates.

PCR primers were also developed for four distinct *Dehalobacter*-type RDase genes from cultures that dechlorinate 1,2-dichloroethane, 1,1,2-trichloroethane and 1,1,1-trichloroethane (see below). These cultures dechlorinate PCE and TCE to *cis*-DCE as final product. Transcription of these RDase genes was verified by testing cDNA samples obtained from enrichment cultures exposed to different chlorinated ethanes and ethenes provided as electron acceptors and these genes appear to be specific to the chlorinated ethanes tested. More work is required to determine how effective this screening will be on environmental samples.

D. Identification of additional biomarker genes for reductive dechlorination.

To identify new biomarker genes involved in reductive dechlorination, several approaches were taken, including culture-based experiments and preliminary microarray experiments. Microarray experiments are described below, while the results of culture-based experiments are summarized in section 5.

Microarray experiments. Two types of microarrays were designed for screening for identification of chlorinated ethene biomarker genes: 1) a shotgun DNA microarray array and 2) a reductive dehalogenase (RDase) array.

The shotgun DNA microarray is made from many random DNA fragments from dechlorinating mixed culture KB-1 spotted onto a glass microscope slide. A small number of prototype arrays were fabricated (printed) with several thousand random DNA fragments from KB-1 to optimize the printing process and array protocols. Using the prototype microarrays, we completed the refinement and optimization of microarray experimental protocols adapted to anaerobic mixed cultures and samples containing FeS and other groundwater constituents. All steps including RNA extraction, reverse transcription, labeling, hybridization and analysis have been optimized. RNA samples extracted from KB-1 cultures during dechlorination of different chlorinated ethenes (i.e., TCE, VC) and 1,2-DCA were compared to RNA extracted from KB-1 cultures grown in the absence of chloro-organic compounds. Analysis of the arrays showed that multiple RDase genes were transcribed simultaneously during dechlorination of a single chlorinated compound, and that some RDase genes were transcribed under all conditions tested. In particular, *vcrA* was found to be most highly transcribed when KB-1 was grown on vinyl chloride. Reverse transcriptase qPCR experiments confirmed the microarray results (Waller et al. 2005). In addition to genes of known sequence, such as the RDase genes, we also identified several genes of currently unknown function that were up-regulated during dechlorination. Some of these up-regulated genes were not from *Dehalococcoides*, providing an avenue to explore the synergistic relationships between acetogens, methanogens and *Dehalococcoides* organisms in this culture. More work is required to identify and validate these results.

Unlike the shotgun DNA microarray, the RDase array targets *known* DNA sequences (i.e., all known RDase genes). As such, it will be useful to explore the diversity and function of these RDase genes, but unknown genes will not be discovered. All RDase gene sequences available in public databases and the numerous *Dehalococcoides* RDase sequences we have discovered in our laboratories were aligned, compared and combined in an Excel spreadsheet. Also included were hydrogenase, regulatory and housekeeping genes detected in the available *Dehalococcoides* genomes. The final version of the spreadsheet combines all unique sequences and provides detailed information on the origin of each sequence (e.g., which culture, culture conditions, dechlorinating activity, etc.). In total, we have so far identified 302 unique RDase gene sequences, 104 hydrogenase gene sequences and 58 "control" gene sequences (e.g., select housekeeping genes and 16S rRNA genes). The probe design was completed and three distinct oligonucleotides were synthesized per target gene and spotted onto glass slides. Prototypes of the spotted oligoarrays are now available to explore the expression of RDase, hydrogenase and regulatory genes in *Dehalococcoides* pure culture and *Dehalococcoides*-containing consortia grown with different chlorinated electron acceptors.

E. Characterization of chlorinated solvent-dechlorinating cultures and isolation efforts.

Several PCE-to-ethene-dechlorinating mixed cultures were obtained from contaminated and pristine sites, as well as enrichment cultures capable of dechlorinating 1,2-DCA, 1,1,2-TCA, 1,1,1-TCA and 1,1,-DCA were established.

The PCE-to-ethene-dechlorinating enrichment cultures yielded two novel isolates, *Dehalococcoides* sp. strain GT and *Geobacter lovleyi* strain SZ. Strain GT is the first *Dehalococcoides* isolate that grows with TCE, *cis*-DCE and VC as electron acceptor (Sung et al. 2006a). Cultures of strain GT provided with acetate as the carbon source, hydrogen as the electron donor and TCE as the electron acceptor, reduced TCE to ethene with very little intermediate formation of *cis*-DCE and VC (Figure 3).

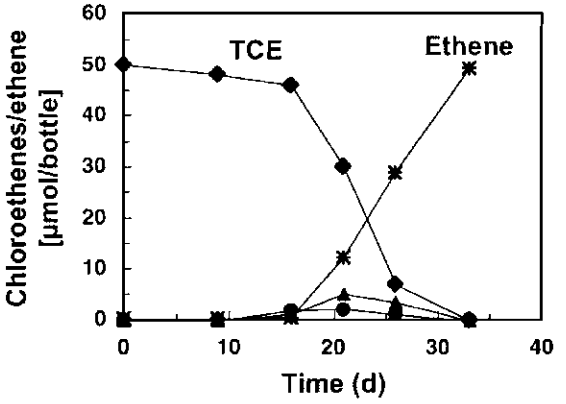

Figure 3: Reductive dechlorination of TCE to ethene by isolate *Dehalococcoides* sp. strain GT. Note that very little formation of *cis*-DCE (circles) and VC (triangles) occurred during TCE dechlorination to ethene suggesting that this type of organism may avoid the "*cis*-DCE and VC stall" often observed at chlorinated ethene contaminated sites.

Another isolate obtained from a PCE-to-ethene-dechlorinating enrichment culture was identified as the first *Geobacter* species capable of PCE-to-*cis*-DCE reductive dechlorination. The new isolate was designated *Geobacter lovleyi* strain SZ (Sung et al. 2006b). Strain SZ also reduced soluble and mobile hexavalent uranium, U(VI), to immobile U(IV) (uraninite). Figure 4 shows that both electron acceptors, PCE and U(VI), were reduced concomitantly indicating that *Geobacter lovleyi* has promising characteristics for bioremediation applications at mixed waste sites.

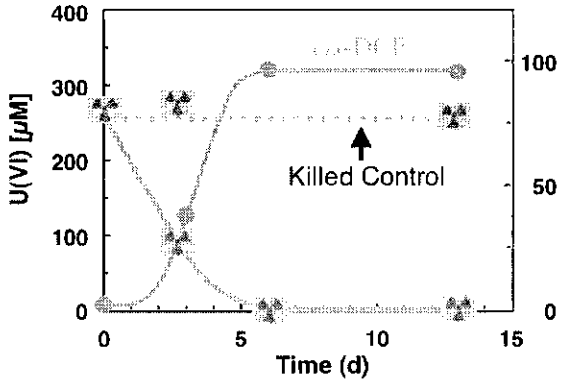

Figure 4: Simultaneous reduction of PCE to *cis*-DCE and U(VI) to U(IV) by *Geobacter lovleyi* strain SZ.

An enrichment culture capable of 1,1,2-TCA and 1,2-DCA transformation was developed from a site contaminated with both chlorinated ethenes and ethanes. This culture was maintained with 1,1,2-trichloroethane as electron acceptor and ethanol as electron donor (Figure 5).

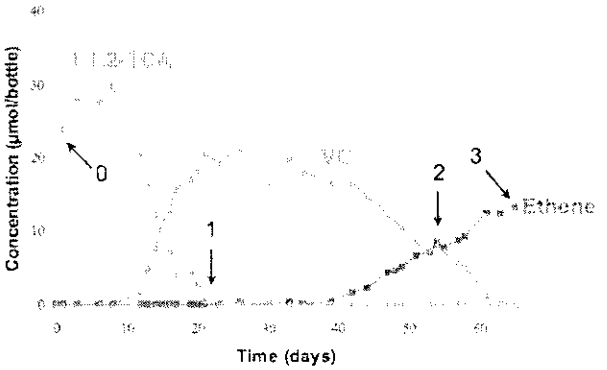

Figure 5: Dechlorination of 1,1,2-TCA in an enrichment culture. DNA was extracted at the time points indicated with arrows (0, 1, 2, 3).

This culture was determined to be dominated by *Dehalobacter* and *Dehalococcoides*. The growth of *Dehalobacter* and *Dehalococcoides* during different steps of dechlorination was determined using qPCR targeting the 16S rRNA gene specific for these two genera. Using this method, we were able to show that *Dehalobacter* grew only during the dihaloelimination step from 1,1,2-TCA to VC, while *Dehalococcoides* grew only during the reductive dechlorination of VC to ethene (Figure 6 and Grostern and Edwards, 2006a). These data illustrate that both genera are required for complete dechlorination of 1,1,2-TCA, as summarized schematically in Figure 7.

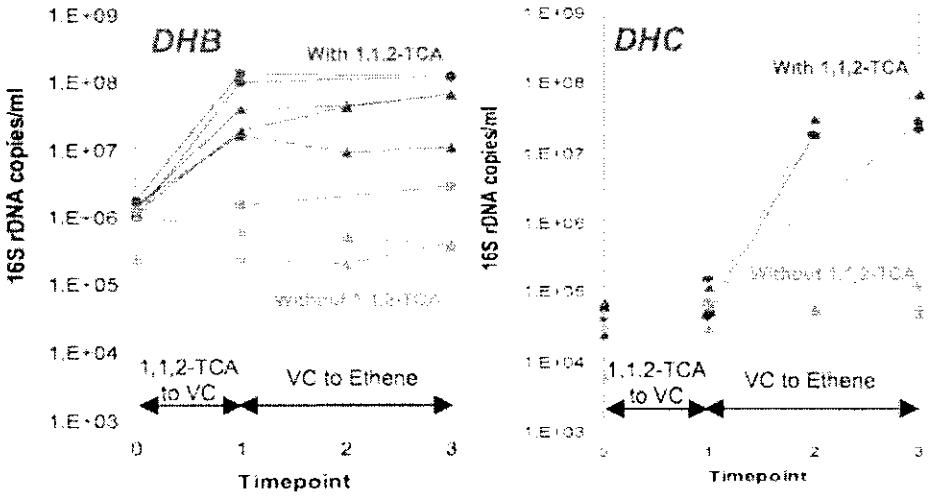

Figure 6: Growth of *Dehalobacter* (*DHB*) and *Dehalococcoides* (*DHC*) during dechlorination of 1,1,2-TCA via VC to ethene. Each line represents a different culture bottle (blue lines with 1,1,2-TCA, red lines without).

A similar approach was taken with cultures enriched from a 1,1,1-TCA-contaminated site. These enrichment cultures dechlorinate 1,1,1-TCA via 1,1-DCA to chloroethane and no further. It was shown that dechlorination was metabolic, and that one or more organisms belonging to the genus *Dehalobacter* were responsible for reductive dechlorination of 1,1,1-TCA (Figure 7).

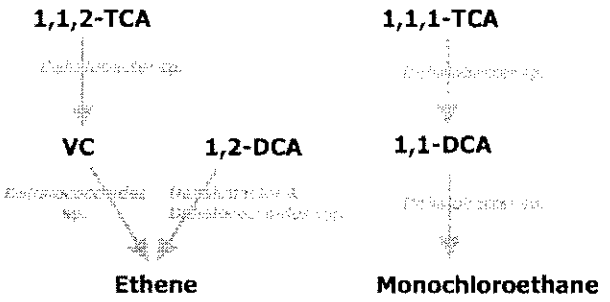

Figure 7: Involvement of *Dehalobacter* and *Dehaloccocoides* in dechlorination of chlorinated ethanes (Grostern and Edwards, 2006b)

In addition, given that 1,1,1-TCA is a frequent co-contaminant with TCE and is a known inhibitor of methanogenesis and reductive dechlorination of chlorinated ethenes, we investigated the effects of degradation of mixtures of TCE and 1,1,1-TCA. For example, TCE dechlorination by KB-1 normally proceeds rapidly to ethene. However, when KB-1 is incubated with both TCE and 1,1,1-TCA, dechlorination stalls at *cis*-DCE (Figure 8a).

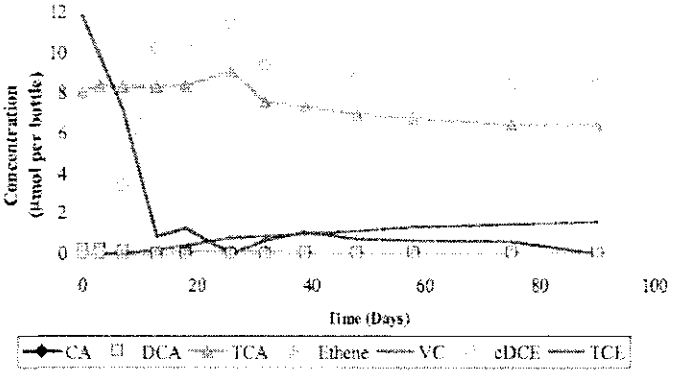

Figure 8a: Dechlorination of TCE only as far as *cis*-DCE in KB-1 cultures amended with both TCE and 1,1,1-TCA (30 mg/l each)

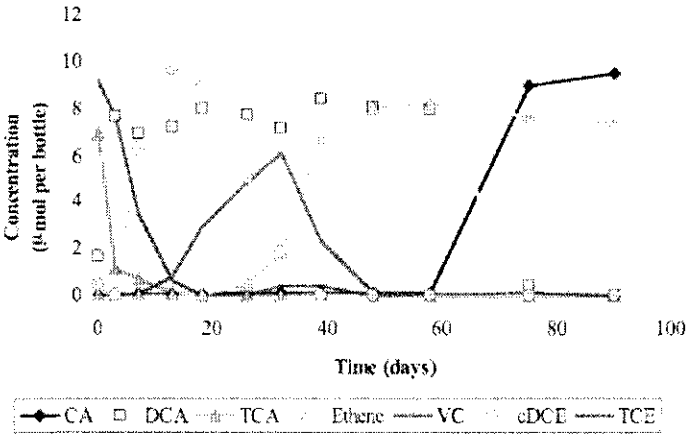

Figure 8b: Complete dechlorination of TCE and 1,1,1-TCA in a culture containing both KB-1 and a 1,1,1-TCA dechlorinating enrichment culture.

However, if KB-1 and the 1,1,1-TCA enrichment culture are mixed and amended with both TCE and 1,1,1-TCA, complete dechlorination of TCE to ethene and 1,1,1-TCA to chloroethane (CA) was observed, in a sequential manner (Figure 8b). These results suggest that inhibition of TCE dechlorination by 1,1,1-TCA is reversible, and can be avoided if active 1,1,1-TCA dechlorination can be stimulated (Grostern and Edwards, 2006b).

2.2 Results for Phase 2 – Field samples

Application of nucleic acid-based tools to field samples.

At all stages of tools development, we tested the 16S rRNA gene- and RDase gene-targeted approaches with groundwater samples collected from field sites undergoing enhanced treatment (i.e., biostimulation and bioaugmentation). These samples were provided by GeoSyntec, Regenesis and HSW Engineering. The validation of the DNA-based tools with field samples is ongoing, and current efforts focus on standardizing the procedures to ultimately develop a guidance document for analytical laboratories.

We had tentatively scheduled a visit from Dr. Chris Yeager from Savannah River National Laboratory (SRNL) to Dr. Löffler's laboratory at the Georgia Institute of Technology for 2006. The plans included training for Dr. Yeager in the qPCR approach and to analyze field samples from the Savannah River Site (SRS). Due to scheduling conflicts, these activities were postponed but we will try to reschedule or find alternative options to include SRS site samples in the analyses.

We successfully demonstrated that the application of RNA-targeted approaches to estimate general (i.e., 16S rRNA-targeted) and specific dechlorination (i.e., RDase gene-targeted) activity is feasible with groundwater samples. The RNA-targeted approach requires further development of procedures to assess biomarker RNA integrity during sampling, transport and storage and quantify RNA loss during biomass collection and

3.0 SUMMARY AND FUTURE WORK

This collaborative effort between Dr. E. Edwards at the University of Toronto and Dr. F. Löffler at the Georgia Institute of Technology developed new qualitative and quantitative tools for site assessment and bioremediation monitoring. The value and the limitations of *Dehalococcoides* 16S rRNA gene-targeted approaches were evaluated and described. PCR approaches targeting specific RDase genes implicated in chlorinated ethene reductive dechlorination were designed and optimized for quantitative assessment. Application of these tools to groundwater samples demonstrated their applicability for site assessment and bioremediation monitoring. New dechlorinating isolates were obtained and chlorinated ethane-dechlorinating cultures were characterized.

Future efforts should focus on obtaining more enrichment cultures and isolates to comprehensively describe the diversity of chlorinated solvent-dechlorinating bacteria. A major shortcoming of the current tools is the limited knowledge of reductive dechlorination biomarker genes. The RDase gene microarray designed in this effort will provide a valuable platform to assign function to many more RDase genes, and design (RT)-qPCR tools to more comprehensively describe the reductively dechlorinating *Dehalococcoides* community.

4.0 COLLABORATIVE EXCHANGES AND VISITS TO OTHER LABORATORIES

July 2004

A project kickoff meeting via conference call was held on July 7th 2004 between Drs. Edwards (University of Toronto), Löffler (Georgia Tech), Bagwell (SRNL), and Brigmon (SRNL). Project funding became available in August (Georgia Tech) and September (University of Toronto).

November 2004
Dr. Löffler and one of his students visited Dr. Edwards' laboratory at the University of Toronto for 2 days. The goals were to coordinate efforts, exchange expertise, plan future efforts, and enhance student interactions. Dr. Edwards' students working on the project gave presentations followed by intensive discussions, and we toured the microarray facilities at the University of Toronto. One outcome of these discussions was to begin to compile all the available information on reductive dehalogenase gene sequence, and to generate an Excel spreadsheet, in which to store existing sequences, and new sequences as they are discovered.

March 2005:
Dr. Ruth Richardson (Cornell University) and four students and postdocs from her group visited Dr. Elizabeth Edwards and her group at the University of Toronto on March 11-12. The purpose was to share ideas about quantitatively measuring RNA in cultures and inferring dechlorination rates. Dr. Richardson also obtained a sample of consortium KB-1 and growth medium in order to test a culture that metabolically dechlorinates VC to ethene, as compared to strain 195, which reduces VC cometabolically.

June 2005: Annual ASM Meeting in Atlanta
This opportunity was used to enhance interactions between students from the University of Toronto and Georgia Tech, and discuss and plan future experiments. Drs. Edwards (University of Toronto) and Löffler (Georgia Tech) also met with Drs. Brigmon (SRNL) and Yeager (SRNL) to discuss results and the current status of the project, and plan future efforts. A work plan was drafted.

August 2005:
August 9-11, 2005, Dr. Löffler (as keynote speaker) and Dr. Edwards (as a rapporteur) participated in the SERDP/ESTCP Molecular Biology Tools Workshop, in Charlottesville, Virginia. A report was prepared and is available at: http://docs.serdp-estcp.org/viewfile.cfm?Doc=MBT%20Workshop%20Report%2Epdf

January 2006:
Dr. Löffler spent one week in January, 2006, at the Technical University of Munich to explore the feasibility to apply FISH technology for the detection and quantification of *Dehalococcoides*. Initial experiments were promising and we will continue to explore this approach. Although qPCR will remain the quantification approach of choice, a second, PCR-independent procedure is desirable for validation of qPCR results.

February 2006:
Two of Dr. Edwards' students (A. Waller and C. Washer) attended a microarray training course offered by the University of Toronto Microarray Centre.

March 2006:
A student from Dr. Löffler's laboratory (Elizabeth Padilla) spent 4 days in Toronto learning optimized microarray protocols.

May 2006:
Together with Karen Vangelas from SRNL, Dr. Löffler organized a 2-day workshop at Georgia Tech with all principal and co-principal investigators funded through DOE-SRS program. In attendance were also Claire Sink who is with the Department of Energy (DOE) Headquarters in the Office of Cleanup Technologies and serves as the Project Manager for the MNA/EA project; Karen Adams who is the SRS Project Manager for the MNA/EA project; and Karen Vangelas who is with the Savannah River National Laboratory and is the Operations Lead for the MNA/EA project.
Drs. Edwards and Löffler and one student from each group attended the Fifth International Conference on Remediation of Chlorinated and Recalcitrant Compounds in Monterrey, CA, to present research findings (two oral presentations and two poster presentations).

5.0 PAPERS PUBLISHED, IN PRESS, OR IN PREPARATION

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2. **Duhamel, M. and E.A. Edwards.** 2006. Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*. FEMS Microbiol. Ecol. 58: 538-549
3. **Grostern, A., and E. A. Edwards.** 2006a. Growth of *Dehalobacter* and *Dehalococcoides* spp. during degradation of chlorinated ethanes. Appl. Environ. Microbiol. **72:**428-436.
4. **Grostern, A. and E.A. Edwards.** 2006b. A 1,1,1-trichloroethane-degrading anaerobic mixed culture enhances biotransformation of mixtures of chlorinated ethenes and ethanes. Appl. Environ. Microbiol. In press.
5. **He, J., Y. Sung, R. Krajmalnik-Brown, K. M. Ritalahti, and F. E. Löffler.** 2005. Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe. Environ. Microbiol. 7:1442-1450.
6. **Krajmalnik-Brown, Y. Sung, R., K. M. Ritalahti, F. Michael Saunders, and F. E. Löffler.** 2006. Environmental distribution of the trichloroethene reductive dehalogenase gene (*tceA*) suggests lateral gene transfer among *Dehalococcoides*. FEMS Microbiol. Ecol. In Press.
7. **Löffler, F. E., and E. A. Edwards.** 2006. Harnessing microbial activities for environmental cleanup. Current Opinion in Biotechnology. 17:274-284.
8. **Löffler, F. E.**, R. A. Sanford, and K. M. Ritalahti. 2005. Enrichment, cultivation, and detection of reductively dechlorinating bacteria. Methods Enzymol. Vol. 397:77-111.
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6.0 CONFERENCE PRESENTATIONS

1. Löffler, F. E. Molecular Tools Supporting Efficient Bioremediation. Interstate Technology & Regulatory Council (ITRC) Fall Meeting, October 23-27, Scottsdale, AZ.
2. Edwards, E.A. Genomes to Solutions - Bioremediation in the Genomics Era. Gordon Research Conference on Environmental Sciences: Water. June 25-28, 2006.
3. Löffler, F. E. 5/2006. Molecular Biological Tools to Support Bioremediation Applications: The Road Ahead, The 5th International Conference on Remediation of Chlorinated Compounds, Monterey, May 21-25, 2006, Monterey, CA.
4. Amos, B. K., E. J. Suchomel, K. D. Pennell, and F. E. Löffler. 2006. Microbial Distribution and Microbial Reductive Dechlorination Performance in the Immediate Vicinity of a NAPL Source Zone. Fifth International Conference on Remediation of Chlorinated and Recalcitrant Compounds. May 21-25, 2006, Monterey, CA.
5. Seguiti, F., C. Drummond, E. Petrovskis, F. E. Löffler, and K. M. Ritalahti. 2006. Management/site closure strategies for a trichloroethene (TCE) impacted aquifer following bioaugmentation. Fifth International Conference on Remediation of Chlorinated and Recalcitrant Compounds. May 21-25, 2006, Monterey, CA.
6. Waller, A. S., K. Mo, D. Zhang, C. Washer, B. Higgins, B. Curtis, S. Bowman, and E. A. Edwards. 2005. Microbial Community Genome Sequencing of Biodegrading Consortia. *In* Abstracts from the International Conference on Microbial Genomes; Halifax, Nova Scotia.
7. Waller A.S. K. Mo, C. Washer, and E.A. Edwards. 2005. Microarray Detection of Gene Expression During Dechlorination of a *Dehalococcoides*-Containing Mixed Culture - Presented at the 55th Canadian Chemical Engineering Conference, Toronto, ON, Canada. October 16-19.
8. Krajmalnik-Brown. R., F. M. Saunders, F. E. Löffler, K. M. Ritalahti. 2005. A Reductive Dehalogenase (RDase) Gene is Shared Among *Dehalococcoides Species*, abstr. R-039. American Society for Microbiology - General Meeting. June 5-9, Atlanta, Georgia.
9. Waller, A. S., K. Mo, D. Zhang, R. Krajmalnik-Brown, K. M. Ritalahti, F. E. Löffler, E. A. Edwards. 2005. Identification of Genes Involved in Anaerobic Dechlorination in a *Dehalococcoides*-Containing Mixed Consortium Using Microarray Analysis, abstr. Q-378. American Society for Microbiology - General Meeting. June 5-9, Atlanta, Georgia.
10. Sung, Y., K. M. Ritalahti, F. E. Löffler. 2005. Complete Detoxification of Trichloroethene (TCE) to Ethene by a New *Dehalococcoides* Species Designated Strain H10, abstr. Q-038. American Society for Microbiology - General Meeting. June 5-9, Atlanta, Georgia.
11. Grostern, A., E.A. Edwards. 2005. Investigation of Community Dynamics in Anaerobic Chlorinated Ethane-Degrading Microbial Cultures. American Society for Microbiology - ASM 105th General Meeting, June 5-7, Atlanta, GA
12. Duhamel, M., A. Waller, K. Mo, E.A. Edwards, P.C. Dennis, S. Dworatzek, X. Druar, E.R. Hendrickson, and D. Major. 2005. Importance of non-*Dehalococcoides* organisms in reductive dechlorinating cultures. Battelle In Situ and On-Site Bioremediation Symposium, June 6-9, Baltimore, MD.

Note that other federal funding agencies and industry sponsors, in addition to the DOE-SRS funding, contributed resources to support the research on reductive dechlorination in Drs. Edwards and Löffler's laboratories. However, the scope and objectives of the DOE-SRS funding had significant impact on the research productivity and direction.

7.0 RELATED PROPOSAL SUBMISSIONS

A large **Genome Canada** grant application, entitled "Microbial Environmental Genome Alliance (MEGA)", to further pursue identification of metabolic and regulatory genes in dechlorinating cultures via genome analysis was submitted on January 28, 2005. This SRNL project (Drs. Edwards & Löffler) and Dr. Ruth Richardson's SRNL project were included as co-funding for the Genome Canada grant application, which required 1:1 matching funds. This application solidified ongoing collaboration between Dr. Edwards and Ruth Richardson and Steve Zinder at Cornell University. The application made it through two stringent cuts, but was unfortunately eliminated in the final round of the selection process. The reviews, however, were very encouraging about the bioremediation metagenomic section of the application.

Dr. Edwards resubmitted a component of the Genome Canada application to Canada's National Science and Engineering Research Council (**NSERC**) and the application is pending.

A Proposal to the Joint Genome Institute (**DOE-JGI**) for community sequencing of the KB-1 culture was submitted March 2006 and was accepted June 2006.

In addition, *Dehalococcoides* strain GT will be sequenced by **DOE-JGI**; this complements genome sequences of *Dehalococcoides* strains 195, CBDB1, BAV1 and VS that are available or sequencing is in progress.

A proposal to **SERDP** entitled "BioReD: Biomarkers and Tools for Reductive Dechlorination Site Assessment, Monitoring, and Management" was submitted in March 2006. This proposal builds on the current results to expand the biomarker identification efforts, to develop a relative quantification approach and establish activity correlation factors to estimate *in situ* reductive dechlorination activity, and to perform field demonstration/validation. This proposal was successful and will enable the continuation of the work described herein.