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# Coupling of Realistic Rate Estimates with Genomics for Assessing Contaminant Attenuation and Long-Term Plume Containment

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## *1. Research Objective*

Dissolved dense nonaqueous-phase liquid plumes are persistent, widespread problems in the DOE complex. While perceived as being difficult to degrade, at the Idaho National Engineering and Environmental Laboratory, dissolved trichloroethylene (TCE) is disappearing from the Snake River Plain aquifer (SRPA) by natural attenuation, a finding that saves significant site restoration costs. Acceptance of monitored natural attenuation as a preferred treatment technology requires direct proof of the process and rate of the degradation. Our proposal aims to provide that proof for one such site by testing two hypotheses. First, we believe that realistic values for in situ rates of TCE cometabolism can be obtained by sustaining the putative microorganisms at the low catabolic activities consistent with aquifer conditions. Second, the patterns of functional gene expression evident in these communities under starvation conditions while carrying out TCE cometabolism can be used to diagnose the cometabolic activity in the aquifer itself. Using the cometabolism rate parameters derived in low-growth bioreactors, we will complete the models that predict the time until background levels of TCE are attained at this location and validate the long-term stewardship of this plume. Realistic terms for cometabolism of TCE will provide marked improvements in DOE's ability to predict and monitor natural attenuation of chlorinated organics at other sites, increase the acceptability of this solution, and provide significant economic and health benefits through this noninvasive remediation strategy. Finally, this project will derive valuable genomic information about the functional attributes of subsurface microbial communities upon which DOE must depend to resolve some of its most difficult contamination issues.

## *2. Research Progress and Implications*

This report summarizes work after eight months of a three-year project. In the fall of 2002 two wells were identified at the INEEL near the trichloroethylene plume at Test Area North. These wells were selected based on their construction and proximity to TAN. From well ANP-9 we concentrated the free-living microbial cells present in approximately 3500 L of groundwater using an Amicon hollow fiber filter apparatus (Figure 1). The final concentrate, reduced to 700 mL (ca. 5000-fold concentration) and containing approximately $3.5 \times 10^{10}$ cells, was frozen at -80° C and shipped to the University of Idaho to be used in the construction of the cosmid library for the free-living component of the aquifer microbial community. Also in the fall we initiated a three-month downhole incubation of basalt chips in well ANP-10. In March 2003 these basalt chips were retrieved for use in the construction of the complementary cosmid library for the attached component of the aquifer microbial community. In April 2003 a new set of basalt chips were admitted to the aquifer in ANP-10 for a six-month incubation to derive additional attached biomass.


Figure 1. Retrieval of basalt chips from the SRPA following a three-month downhole incubation and collection of water samples (shown) occurred in March 2003. Biomass on the chips will be used to construct a cosmid library for attached communities in the aquifer. Water samples were used for ongoing isolation of methanotrophs, microbes that are believed to be responsible for the natural attenuation of TCE in the aquifer. Cindi Brinkman (left) and Dan Erwin are shown, both students at the University of Idaho.

Methanotrophs have been isolated from both ANP-9 and ANP-10. Two isolates were obtained from basalt substrates incubated in the well (ANP-10) and two from three liters of groundwater filtered onto a 0.2-µm membrane filter (ANP-9). Samples from the wells were incubated at 30° C in small serum bottles (10-25ml volume) containing nitrate mineral salts (NMS) media under an 80% air, 20% methane atmosphere, with ca. 2% $CO_2$ added to the gas mix. Solid NMS media was used to obtain methanotroph colonies and these were incubated as for the liquid cultures.

Pure cultures of methanotrophs were obtained by picking isolated colonies from a previous transfer plate and streaking onto NMS agar, R2A agar, and into sterile NMS liquid. After incubation for ca. one week liquid vials were checked for. Cultures were believed to contain isolated methanotrophs if methane was consumed (as determined by gas chromatography) after ca. 1 week of incubation and heterotrophic growth was not detected on R2A agar. Isolated colonies on NMS plates have been transferred repeatedly to new media and colony morphology remains the same with each transfer.

A biomass recycle reactor (BRR) has been constructed for determining the realistic rates of methanotrophy in a constrained subsurface environment. Initial experiments have been conducted to identify potential sources of contamination of the reactor and approaches to minimize contamination. Because of frequent fungal contamination the reactor medium contains 100 mg/L cyclohexamide. Preliminary studies indicate that this antibiotic concentration eliminates the fungal contaminant without inhibiting methanotrophy.

With respect to the metagenomic library of the attached and planktonic microbial communities of the SRPA, this library will be probed for the presence of genes in the microbial communities that may be used in the cometabolism of chlorinated aliphatics such as trichloroethylene (TCE). Our approach proceeds through four phases: sample collection, DNA processing, clone production, and library screening. As noted above, by suspending sterile substrate columns in the aquifer and planktonic organisms were concentrated from the water via hollow fiber filtration. The cells from these collections will be purified and suspended in agarose plugs for subsequent lysis and endonuclease treatment. Pulsed Field Gel Electrophoresis (PFGE) will be used to isolate DNA fragments of 100 kb-300 kb in size. These fragments will be electroeluted, inserted into copy control vectors, and used in the production of our metagenomic library. The libraries will then be screened for genomic markers for such enzymes as methane monooxygenase and other alkane monooxygenases present in the indigenous microorganisms.

During the past few months, efforts have been made to achieve three goals: community sampling, optimization of DNA extraction from lab cultures, design of a protocol to process the DNA resulting in large (100 kb-350 kb) fragments and cell purification from community samples. The first goal has been met through the limited bias collection of planktonic as well as attached cellular communities. Both collections resulted in slurries consisting of mostly inorganic impurities (basalt sediments). This has made it necessary to establish a method for cell isolation from these slurries for further processing. In order to optimize DNA extraction, large fragment genomic DNA has been isolated from mixed lab cultures. Gram positive and Gram negative organisms were subjected to agarose plug lysis followed by subsequent endonuclease digestion to produce DNA fragments >100 kb.

Because the quantity of high-quality DNA that we can isolate from our environmental samples is limited, we have examined a novel PCR procedure for amplifying total genomic DNA without bias. The method employs the REPLI-g whole genome amplification kit (Molecular Staging, Inc). We have confirmed that

this kit will amplify all DNA in a sample without bias, and thus will be able to generate sufficient DNA for a metagenomic library even from a very limited amount of community DNA. We have recently used this approach to amplify community DNA obtained from microbial communities that reside on basalt exposed to the atmosphere as a preparatory step to conducting this amplification on samples from the aquifer (Figure 2).

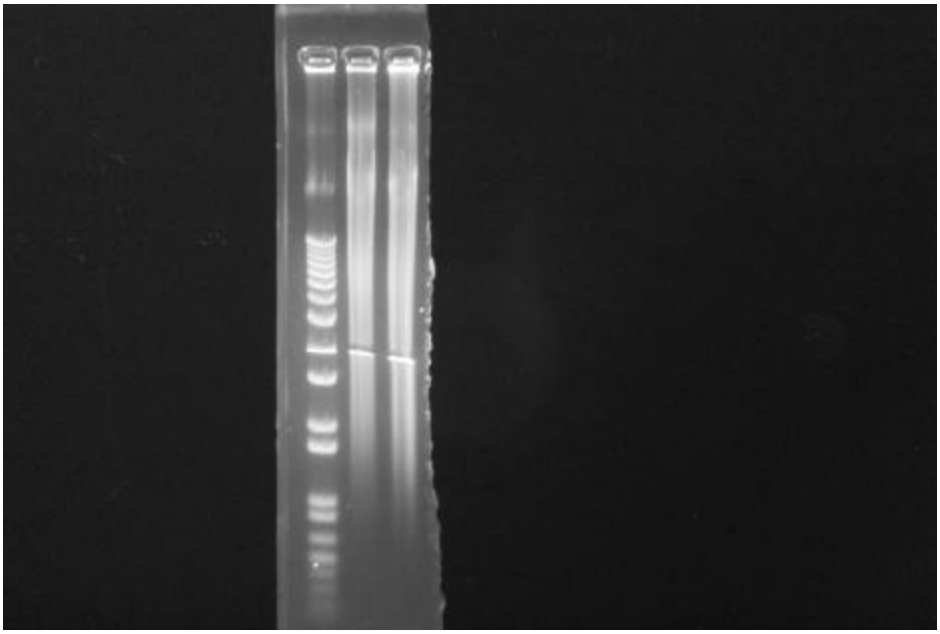

Figure 2. Agarose gel electrophoresis of DNA isolated from environmental samples of desert rock varnish and amplified by Whole Genome Amplification (WGA). Lane 1: 1kb Plus DNA ladder. Lane 2: Positive control supplied with kit. Lane 3: 10 ng of template DNA amplified using the REPLI-g 625S kit from Molecular Staging Inc. The 1% gel was run at 5V/cm for 1hr followed by staining with ethidium bromide.

Finally, we are determining the microbial diversity associated with the planktonic and biofilm microbial populations collected from the SRPA. Polymerase chain reaction (PCR) will be used to amplify the *eubacterial* 16s rDNA from the microbial community present in the hollow-fiber filtered water sample. Following amplification, the PCR product will be subjected to denaturing gradient gel electrophoresis (DGGE) to determine the number of different microorganisms present in the water sample. After DGGE analysis, the PCR fragments will be cloned and sequenced. Database comparison using Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP) will be used to determine the identity of the microorganisms. This will allow us to determine if there are organisms indigenous to the SRPA that are related to cells known to co-metabolize TCE. 16s rDNA analysis of both the planktonic and biofilm microbial communities will allow us to compare the two populations to determine where potential TCE degraders exist in the subsurface (attached or free-living). The identity of the microorganisms present in the SRPA will help us to determine the role that intrinsic bioremediation might play when a region of the aquifer is unexpectedly contaminated by chlorinated solvents such as TCE.

To ensure that our experimental approach will work on the hard to obtain and valuable aquifer samples, pure cultures of difficult to amplify bacterial isolates were used to optimize the procedure. Four actinomycete isolates (high G + C Gram-positive bacteria) isolates were obtained. They included strains R19B, R25A, SB11, and SB12 that had been isolated in the Crawford laboratory. These isolates were grown on Yeast-Dextrose Agar and non-sporulating colonies were used for PCR. To extract the DNA necessary for PCR, individual colonies were added to 100uL of TE containing 1% Triton X-100 and boiled

at 96°C for 10 minutes to lyse the cells. The cell suspension was then centrifuged at 13,000 rpm for 2 min to pellet cell debris. The resulting supernatant was used in PCR reactions using the following protocol:

| | Volume (µL) |
|---|---|
| 10X PCR Buffer | 5 |
| dNTPs (10 mM) | 1 |
| bovine serum albumin (2 ug/uL) | 1 |
| $MgCl_2$ (25 mM) | 4 |
| forward primer (10 pmol) | 2 |
| reverse primer (10 pmol) | 2 |
| Taq polymerase (Fisher) | 0.25 |
| cell supernatant | 1 |
| TOTAL | 50 |

PCR Cycle

1. 95° C (5min)
2. 95° C (1 min)
3. 55° C (1min)
4. 72° C (2min)
5. Cycle to STEP 2 (30X)
6. 72° C (7min)
7. 4° C (hold indefinitely)

Two *Eubacterial* primer sets will be used to amplify 16s rDNA genes: 1) 27F-907R and 2) 338F-907R.

27F
AGAGTTTGATCMTGGCTCAG
338F
ACTCCTACGGGAGGCAGC
907R
CCGTCAATTCMTTTRAGTTT

Three of the four DNA samples, from strains R19B, R25A, and SB12, amplified with the primer set 338F-907R. The amplified products were then run on a 40%-80% DGGE gel, and as expected from a pure culture, only single bands were obtained. To determine whether the primer sets and reaction conditions would work on Gram-negative organisms, several were grown in pure culture and mixed together to simulate a mixed population such as what might be found in the environment. On hundred µL of the mixed culture was centrifuged and lysed in the manner described above. PCR was carried out using both primer sets and amplified products were obtained with both primer sets. The products of each were run on a 40-80% DGGE gel and multiple bands resulted from the Gram-negative mixed bacterial culture as expected.

Once successful PCR conditions were established for both Gram-positive and Gram-negative organisms, we obtained 25 L of water from a shallow, local farm well to confirm that our method would work with natural water samples. Cells were removed from the water by centrifugation (30 min at 7,500 rpm). Cell pellets were resuspended in 2 mL of the well water supernatant. Two different conditions were used to lyse cells and liberate the DNA. For lysis, 100 µL and 500 µL were centrifuged at 13,000 rpm for 2 min to pellet the cells. Each pellet was resuspended in 100 µL of the TE/Triton X-100 (1% v/v). Both samples were then lysed and treated as described above. PCR reactions were performed using 1 µL of each cell lysate. An amplified product was obtained from the 100-µL sample using the 338F-907R primer set. Use of a 50-80% gradient DGGE gel indicated that at least nine different dominant microbial species were present in the water.

### *3. Planned Activities*

Activities planned for the immediate future regarding the methanotroph isolates include extraction of DNA from the cultures followed by amplification of 16S rRNA genes using bacterial primers and subsequent cloning, sequencing and identification of the isolates. We anticipate preliminary determinations of

methanotroph maintenance level energy requirements once the Snake River Plain aquifer methanotroph isolate that we are working with has stabilized in the BRR. This will be followed by addition of TCE to the reactor and the associated re-establishment of (higher) maintenance level energy requirements for the isolate.

With respect to future efforts to isolate attached aquifer DNA, additional substrate tubes have been placed into the well as the first set only resulted in a small amount of biomass. These tubes will be allowed to colonize for six months before removal and extraction of biomass as before. Currently, we are collecting bacterial isolates from the well samplings so that large fragment DNA can be harvested from them.

Because the aquifer samples are relatively clean (lacking contaminating organics) we are considering direct isolation methods to remove the metagenomic DNA from the samples. Various groups have extracted high quality DNA of greater than 40 kb from soils. Fragments of this size could be used in a cosmid library. There are benefits of using a cosmid library over the BAC library (originally proposed). First, a cosmid library uses smaller DNA fragments. Second, cosmid libraries can be prepared using randomly sheared DNA, which is then end-repaired to produce blunt ends prior to ligation. This bypasses the yield reducing digestion and subsequent repurification steps necessary with BAC inserts.

For microbial community characterization using 16S rRNA genes, once we are satisfied with the reproducibility of our field sample data we will extract DNA and amplify the 16s rDNA genes from SRPA water filtrate. We anticipate some problems with contaminating substances; so will consider alternate ways of lysing the cells, possibly using a soil extraction kit. Alternatively, we may need to add a step to our procedure to isolate the DNA away from substances that may interfere with PCR reactions.

The modeling for this project will build on previous modeling of natural attenuation processes at the TAN site in support of the CERCLA cleanup. The GMS platform will be used because of its straightforward user interface and the fact that it allows the use of several different codes. MODFLOW will be used to generate flow fields because it is widely accepted in the field. Transport and attenuation will initially use MT3D-MS, which allows tracking of multiple species and first-order degradation processes. Laboratory attenuation rates will be approximated as first-order processes. If laboratory data indicate that the process is not first order, then a reactive transport code such as RT3D will be used to generate a more representative kinetic model to be coupled with the flow model. Attenuation will be modeled for the TAN site first because of the abundance of data for this site. Modeling of a second DOE site (to be determined) is planned to take advantage of the lessons learned from modeling that TAN site

Figure 3. Timeline depicting progress towards completion of the project.

### *4. Information Access*

Not applicable at this time.

### *5. Optional Additional Information*

Not applicable at this time.

### *6. Optional Proprietary Information*

Not applicable at this time.