

## **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. 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 evidence of the processes and rates of the degradation. Our proposal aims to provide that evidence 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 aims to 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

Candidate cultures of SRPA methanotrophs have been maintained in culture for several months. These methane-consuming cultures often contained a co-occurring heterotroph, a finding that is consistent with that of others. Two candidate cultures are from filtered groundwater derived from well ANP-9 and two are from basalt substrates that were kept in well ANP-10 for a six month colonization period. To identify the methanotrophs and co-occurring heterotrophs present, DNA was extracted from one of the SRPA cultures and a culture of a known methanotroph (*Methylosinus trichosporium* OB3b ) and amplified using polymerase chain reaction (PCR) with universal bacterial primers 8F and 1492R. The PCR products were extracted from the agarose gel and purified. The purified product was then cloned and the clones from each sample were screened by restriction fragment length polymorphism (RFLP). Clones with unique RFLP patterns have been sent for sequencing of the 16S rRNA insert. Following sequencing the identity of the methanotrophs and the co-occurring heterotrophs can be determined.

Experiments were conducted in a biomass recycle reactor (BRR) (Figure 1) in order to determine the amount of methane consumed when the cells exist at maintenance level activities. The low activities exhibited by cells surviving in the BRR are believed to be representative of cells living in the subsurface. The model methanotroph, *Methylosinus trichosporium* OB3b, was inoculated into the BRR at a starting cell density of approximately  $7.7 \times 10^6$  cells/ml (Figure 2) as determined by acridine orange direct counts. Initial BRR experiments suggest that this organism reaches maintenance level activity after one to two weeks in the BRR when the numbers of cells in the reactor stabilize (Figure 2). The reactor has operated continuously for over 150 days and the numbers of cells present have remained stable at just greater than  $1 \times 10^9$  cells/ml. These cells continue to oxidize coumarin indicating the presence of active methanotrophs. Fluorescence in situ hybridization (FISH) directed towards methanotrophs in the BRR have estimated that the heterotroph population in the BRR is about 20-30 % of the total cell population. However, heterotrophic plate counts suggest that the level of co-occurring heterotrophs maybe as low as  $1.23 \times 10^6$  cells/ml within the BRR.

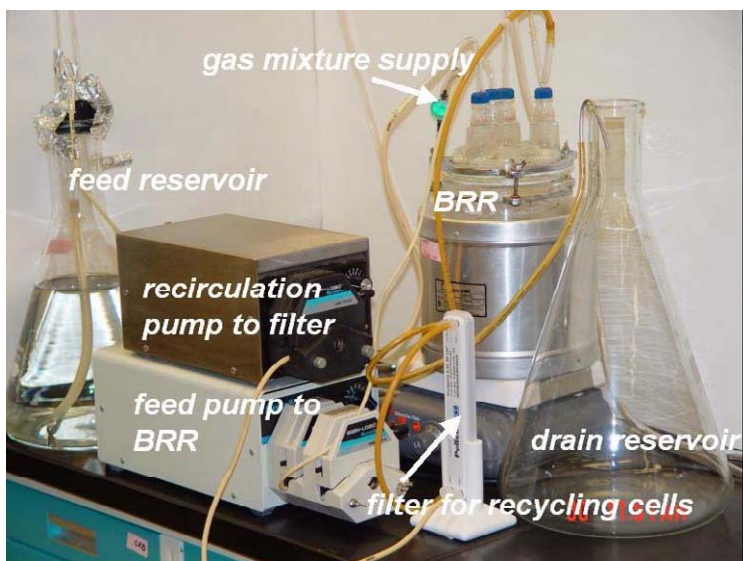


Figure 1. Biomass recycle reactor (BRR) showing basic elements of the system used for growing methanotrophs under nutrient limiting conditions

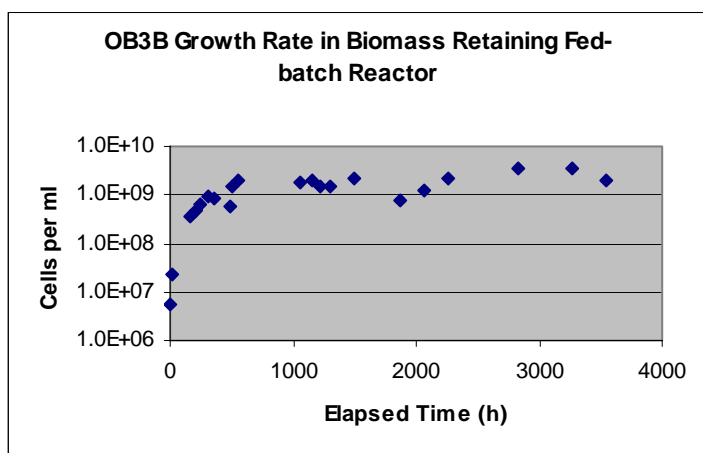


Figure 2. Direct count data for continuously operating biomass recycle reactor containing the model methanotroph *Methylosinus trichosporium* OB3b showing roughly stable cell numbers for last 125 days of operation.

Preliminary determinations of the maintenance level methanotrophic activities exhibited by these starved cells indicate that the cells use between  $2.4$  and  $12.0 \times 10^{-19}$  moles of methane/cell/day. Published values for specific methane consumption rates by methanotrophs as determined by conventional (i.e., non-BRR systems) appear to be several orders of magnitude higher. For example, Svernig et al. (2003, FEMS Microbiol Ecol. 44: 347-354) derived methanotrophic rates of approximately  $1.3 \times 10^{-11}$  moles of methane/cell/day. This suggests that the BRR effectively maintains these cells in a low growth condition in contrast to the typical strategies used by microbiologists to culture environmental microbes.

SRPA microbial community characterization at the genomics level of the *in situ* methanotroph and ammonia oxidizing microbial populations was performed by PCR of environmental DNA samples, PCR product cloning, RFLP analyses to classify clones into related groups, and sequencing of representative cloned inserts from the observed groups. To obtain environmental DNA, planktonic microorganisms were collected using water (17,300 L) from well ANP-9 using hollow fiber filtration. Biofilms (attached microbial populations) were obtained well ANP-10 by suspending sterile substrate columns containing several kilograms of basalt chips into the aquifer for six months. These chips were then retrieved and DNA was extracted from the biofilms that had formed on their surfaces. The wells were sampled at approximately 200 meters depth. The close proximity of the wells to each other (ca. 200 m) suggests that their respective water chemistries and microbial community structure should be similar. Previous water chemistry analysis has shown comparable characterization between the two wells.

A functional gene library was produced using PCR primers that specifically target the *pmoA* gene of the particulate form of MMO, the *amoA* gene of ammonia monooxygenase (AMO), and the *mmoX* gene of the soluble form of MMO. Five archives were produced from whole community (metagenomic) DNA recovered from communities attached to the fractured basalt as well as free-living communities located within a pristine well outside of TAN. Whole Genome Amplification (WGA) was used to amplify metagenomic DNA from each community and used in the production of five additional functional gene archives. This library of ten

archives was used to conduct a molecular assessment of the potential TCE degrading community structure within the SRPA.

The majority of clone sequences in each archive were found to be Type II-like methanotrophs highly related to the genus *Methylocystis*. A small subset of Type I clones was found only in planktonic communities and grouped nearest to the genus *Methylobacter* and the known TCE-co-metabolizing *Methylomonas* sp. LW15. Sequences similar to the *amoA* gene associated with ammonia oxidizing bacteria (AOB) were seen in this library and are most closely related to the uncultured bacterium BS870 with no substantial alignment to any cultured AOB. Metagenomic DNA amplified by WGA showed similar characteristics to un-amplified samples. Archives produced from WGA samples were similar in structure to their un-amplified counterparts. This observation indicates that it will be possible to use WGA techniques to study even minute quantities of metagenomic DNA obtained from extremely oligotrophic environments such as the SRPA that contain only about 1000 microbial cells per ml of groundwater. Overall, our studies of methane and ammonia oxidizing communities in the ESRPA showed that conditions are present in the pristine region of the aquifer sampled that promote the growth of sMMO producing bacteria, all of which have been previously associated with high rates of TCE co-metabolism. These observations support the conclusion that intrinsic processes are responsible for the observed removal of TCE from the groundwater at the INEEL TAN site.

In addition to the above studies, indigenous suspended and planktonic microbial populations present in the same samples were investigated by performing analyses of PCR-amplified 16S rDNA markers to determine the microbial diversity and the potential of the indigenous SRPA microorganisms to degrade TCE. DNA prepared from the planktonic and biofilm communities were used as templates for 16S rDNA amplification using universal 16S rDNA primers 27f and 907r. The resultant amplicons were cloned and picked into 96 well format plates. Plasmid isolations were performed using robotic equipment. Restriction enzyme digests of the purified plasmid DNA were analyzed by agarose gel electrophoresis. Clones were grouped according to their restriction patterns. Amplicons from representative clones from each group were sequenced. Results showed that the microbial community present in the SRPA contains many previously undescribed and uncultured bacteria as well as new species in known genera such as *Arthrobacter*, *Janthinobacterium*, *Sphingomonas*, *Afipia*, *Caulobacter*, and *Pseudomonas*. Some of these genera are known to contain representatives that degrade TCE. These observations also support the conclusion that intrinsic processes are responsible for the observed removal of TCE from the groundwater at the INEEL TAN site. The identification of microorganisms present in the SRPA will aid in elucidation of the role that intrinsic bioremediation might play when chlorinated solvents contaminate deep basalt aquifers at other DOE locations.

Another component of this study is to identify DOE sites where existing data suggest that TCE is being naturally attenuated under aerobic conditions. A survey of 127 plumes at 24 DOE sites was performed to identify TCE plumes in aerobic groundwater that had characteristics suitable for assessing the rate of TCE attenuation due to degradation. TCE attenuation due to processes in addition to dispersion was evaluated by comparing concentrations of TCE to concentrations of a conservative co-contaminant that is thought to have a similar source function

as TCE. Plumes at four DOE sites (Brookhaven National Laboratory, Paducah Gaseous Diffusion Plant, Rocky Flats Environmental Technology Site, and Savannah River Site – A/M Area) were identified, and the rate of TCE attenuation (in addition to dispersion) was determined for each of these plumes. There is evidence that TCE is being attenuated in 8 of the 12 plumes evaluated, and at all four of the DOE sites examined. Degradation half-lives ranged from less than one year to 12 years. The details of the TCE attenuation evaluation are provided in *EMSP Annual Report: Coupling of Realistic Rate Estimates with Genomics for Assessing Contaminant Attenuation and Long-Term Plume Containment – Task 4 – Modeling*, by Robert C. Starr, Michael C. Koelsch, and Kent S. Sorenson, Jr. NWE-ID-2004-062. North Wind Inc., Idaho Falls, Idaho.

### **3. Planned Activities**

In the near future, approximately 20 clones will be sequenced in order to identify the 16S rRNA gene affiliation of microbes present in the BRR and the SRPA cultures from ANP-9. In addition, following the stabilization period for cells within the BRR we are prepared to make the first determinations of how maintenance level methane consumption rates by these cells changes when TCE is introduced to the BRR. We will use concentrations of TCE that are consistent with the levels present at the edge of the TCE plume at TAN and calibrate the system with respect to the numbers of methanotrophs in the BRR relative to the numbers present in the SRPA. The major activity under Task 4 (Modeling) will be to revise the existing flow and transport model developed for assessing natural attenuation of TCE in the aerobic portion of the aquifer at the INEEL Test Area North plume. The model will be modified to simulate TCE degradation using the kinetics (both the type of kinetic model and kinetic parameter values) determined from the laboratory studies performed at INEEL under Task 1 of this project.

### **4. Information Access**

Part of the research sponsored by this EMSP project has been depicted in an animation. The animation was developed jointly by PI Colwell and Tanya Atwater of the University of California – Santa Barbara and her NSF-funded Environmental Multimedia Visualization Center. This animation (entitled: Natural Attenuation of a Trichloroethylene Plume in a Basalt Aquifer) describes the natural attenuation of TCE that is occurring at TAN. The animation can be viewed by downloading the animation and the software Flashplayer at the EMVC website (<http://www.geol.ucsb.edu/projects/emvc/> follow the links to “Downloads”). EMSP research funds were not used to create the animation.

### **5. Optional Additional Information**

1. Brinkman, C., F. Colwell, R.L. Crawford, and M.E. Delwiche. 2003. *Estimating realistic rates of methanotrophy and TCE co-metabolism using a biomass recycle reactor*. Subsurface Science Symposium. Inland Northwest Research Alliance. Salt Lake City, UT.
2. Brinkman, C., F. Colwell, R.L. Crawford, M.E. Delwiche, and D. Erwin. 2004. *Estimating realistic rates of methanotrophy and TCE co-metabolism using a biomass recycle reactor*. Annual Meeting of the Intermountain Branch of the American Society for Microbiology, Idaho Falls, ID.

3. Erwin, Daniel P. 2004. *Molecular Characterization of Methanotroph Communities within the Eastern Snake River Plain Aquifer*. Master of Science, Microbiology, Molecular Biology and Biochemistry, University of Idaho.
4. Strap, Janice L., Amy L. Torguson, F. S. Colwell and Ronald L. Crawford. 2004. *Characterization of the Microbial Diversity Associated with Planktonic and Biofilm Populations in a Deep-Water Well from the Snake River Plain Aquifer*. Presented at the 2004 Annual Meeting of the American Society for Microbiology, New Orleans, LA.
5. Starr, Robert C., Michael C. Koelsch, and Kent S. Sorenson. 2004. *EMSP Annual Report: Coupling of Realistic Rate Estimates with Genomics for Assessing Contaminant Attenuation and Long-Term Plume Containment – Task 4 – Modeling*. NWE-ID-2004-062. North Wind Inc., Idaho Falls, Idaho.

## **6. Optional Proprietary Information**

None to report.