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Coupled Biogeochemical Process Evaluation for Conceptualizing Trichloroethylene Co-Metabolism

Team Members:
Idaho National Laboratory - Oregon State University
Rick Colwell, Corey Radtke, Mark Delwiche, Deborah Newby, Lynn Petzke
Lawrence Berkeley National Laboratory
Mark Conrad, Eoin Brodie
North Wind Inc.
Hope Lee, Bob Starr, Dana Dettmers
University of Idaho
Ron Crawford, Andrzej Paszczynski, Nick Bernardini, Ravi Paidisetti, Tonia Green

Research Objectives

Chlorinated solvent wastes (e.g., trichloroethene or TCE) often occur as diffuse subsurface plumes in complex geological environments where coupled processes must be understood in order to implement remediation strategies. Monitored natural attenuation (MNA) warrants study as a remediation technology because it minimizes worker and environment exposure to the wastes and because it costs less than other technologies. However, to be accepted MNA requires different "lines of evidence" indicating that the wastes are effectively destroyed. We are studying the coupled biogeochemical processes that dictate the rate of TCE co-metabolism first in the medial zone (TCE concentration: 1,000 to 20,000 μg/L) of a plume at the Idaho National Laboratory's Test Area North (TAN) site and then at Paducah or the Savannah River Site. We will use flow-through in situ reactors (FTISR) to investigate the rate of methanotrophic co-metabolism of TCE and the coupling of the responsible biological processes with the dissolved methane flux and groundwater flow velocity. TCE co-metabolic rates at TAN are being assessed and interpreted in the context of enzyme activity, gene expression, and cellular inactivation related to intermediates of TCE co-metabolism. By determining the rate of TCE co-metabolism at different groundwater flow velocities, we will derive key modeling parameters for the computational simulations that describe the attenuation, and thereby refine such models while assessing the contribution of microbial co-metabolism relative to other natural attenuation processes. This research will strengthen our ability to forecast the viability of MNA at DOE and other sites contaminated with chlorinated hydrocarbons.

Research Progress and Implications

Aquifer sampling: In order to conduct coupled biogeochemical investigations to determine what controls rates of TCE co-metabolism in the aquifer at TAN both free-living and attached microbial communities from the aquifer must be collected for analysis

by the research team. To collect free-living (planktonic) cells we are using a Kros-Flo hollow fiber filtration apparatus (0.01 μm pore-size cutoff) that permits the rapid concentration of cells in aquifer water such that their physiological capabilities can be readily analyzed. For attached communities, we have developed a flow-through *in situ* reactor (FTISR) that can be immersed in the aquifer for extended periods. This reactor contains basalt fragments derived from cores that were collected from the TAN TCE plume and, when operating, transmits aquifer water through the replicate FTISR in order to collect naturally colonized biofilms consisting of aquifer communities. During 2006, the cells present in approximately 4000 L of water from well TAN-29 and also from well TAN-35 were concentrated for analysis. Also this year, the FTISR was designed and then autonomously operated and tested at an uncontaminated site (well UP-1) in preparation for full deployment at the TAN site in November 2006. A prototype of the reactor was incubated for seven months in well TAN-35 in the medial zone of the TCE plume. The colonized basalt chips obtained when this reactor was removed from the TAN aquifer in September 2006 have served as the source biomass for several of the studies explained in the following sections wherein different methods are used to characterize the physiological characteristics of the methanotrophs in the aquifer.

TCE co-metabolism: To establish co-metabolic degradation rates for TCE, separate microcosm experiments were conducted using suspensions of *Methylosinus trichosporium* (OB3b) and cells concentrated from TAN-29 groundwater incubated at aquifer temperature (12°C). Co-metabolic rates from the microcosms will be used to estimate the rate of natural attenuation due to TCE co-metabolism in the aquifer, specifically as it relates to methanotrophs. The small-scale experiment, using 2-mL ampules, permitted replication and destructive sampling for TCE at selected time points over several weeks. Three variations of microcosm headspace were used: air, air with 1% methane (a methanotroph energy source and a competitive inhibitor of TCE co-metabolism), and air with 0.03% acetylene (an inhibitor of methane oxidation by the enzyme soluble methane monooxygenase, sMMO). A control with no cells added to the groundwater was also tested. OB3b (2×10^8 cells/mL) in aquifer conditions degraded 43% TCE in one week and 50% in two weeks. Acetylene (0.03%) added to the headspace of the ampules successfully inhibited the TCE co-metabolism by OB3b. It is estimated that at least $1x10^7$ methanotrophs/mL will need to be concentrated from TAN groundwater in order to see a change of similar magnitude in TCE concentration within two weeks.

sMMO gene expression: One of our objectives is to quantify expression of the sMMO gene which codes for the primary methanotrophic enzyme implicated in TCE co-metabolism. Forward and reverse primer and probe concentrations have been optimized for this assay to target both *mmoX* and *mmoC* genes which code for corresponding subunits of the sMMO complex isolated from OB3b. The *mmoX* assay detected DNA concentrations of 0.0189 ng/uL whereas the *mmoC* assay detected DNA concentrations two orders of magnitude lower. Higher reaction efficiency and a more consistent standard curve were observed with genomic DNA than with cDNA for both primer sets.

Proteomics: Another goal is to demonstrate the presence and abundance of MMO and the genes encoding this enzyme within the microbial community of the INL TAN site.

The actual presence of the proteins making up MMO would provide direct evidence for its participation in TCE degradation and be useful in developing mathematical models of the process. MMO exists in two forms, a membrane-bound particulate form (pMMO) and a cytoplasmic soluble form (sMMO). pMMO consists of two components, pMMOH (a hydroxylase comprised of 47, 27, and 24-kDa subunits) and pMMOR (a reductase comprised of 63 and 8-kDa subunits). sMMO consists of three components: a hydroxylase (protein A-250 kDa), a dimer of three subunits ($\alpha 2\beta 2\gamma 2$), a regulatory protein (protein B-15.8 kDa), and a reductase (protein C-38.6 kDa). Our specific objectives are to use proteomics to assess the presence of both sMMO and pMMO proteins in the aquifers and relate this to the numbers of methanotrophs and MMO genes determined by us and other members of the research team using PCR-based methods. Although sMMO is more active in the co-metabolic degradation of TCE than is pMMO, high copper levels can suppress the expression of sMMO and mediate the expression of high levels of pMMO.

Our initial efforts involved the development of standard curves for MMO using pure cultures of OB3b, followed by identification and quantification of MMO in TAN site samples. We are doing so using tandem mass-spectrometric analyses of trypsinized whole-protein preparations of both OB3b (internal control) and field samples of both planktonic and biofilm biomass from the TAN aquifer (collected as described above). We have optimized the protein extraction and processing procedures needed to detect these signature peptides and thus far have a detection limit of about 10-50 picograms of sMMO peptide, representing 10^4-10^5 methanotroph cells. We hope to lower this detection limit further prior to analyzing environmental samples. Briefly, the method includes extraction of total proteins from pure and/or environmental samples by adding 1% triton-X100 and boiling the samples at 100º C for 20 min. The extraction is followed by purification of proteins by standard acetone precipitation. The precipitated proteins are then solubilized and digested with trypsin for ESI-MS/MS analysis. MMO has been detected in planktonic and biofilm samples from the TAN aquifer (Table 1). We estimate that methanotrophs comprise about 0.01% of the total biomass of planktonic cells filtered from TAN-29 water, and 21% of the total biomass collected as a biofilm grown on basalt chips submerged for ~8 months in TAN-35.

Table 1. Samples found to contain detectable levels of methanotroph proteins.

Sample origin	Sample condition or medium	Cell numbers*	Proteins detected
OB3b	NMS medium without Cu	4×10^5	sMMO-component A alpha chain; sMMO-component A beta chain; sMMO-component A gamma chain; sMMO-regulatory protein B; methanol dehydrogenase subunit precursor
OB3b	NMS medium with 12 µM Cu	4×10^5	pmoB; large subunit of methanol dehydrogenase
TAN-29	Planktonic cells	1.2×10^9	Q9KX50-pmo-B, *Methylosinus trichosporium;* Q9KX36-pmo-B, *Methylocystis* sp.; Q8RMH5-methanol dehydrogenase, *Methylobacillus* sp.; JQ0706, alcohol dehydrogenase, *Methylobacterium*; Q605M5, hypothetical protein, *Methylococcus capsulatus*
TAN-35	Basalt	4.65×10^6	Q60BG0, DnaK suppressor protein, *Methylococcus capsulatus*

	biofilms		Bath; Q604I5, hypothetical protein, *Methylococcus capsulatus* Bath; Q608P6-Hypothetical protein, *Methylococcus capsulatus* Bath; Q607J2, heavy metal efflux pump, CzcA family-*Methylococcus capsulatus* Bath; Q608C3-ATP-dependent RNA helicase, DEAD/DEAH box family, *Methylococcus capsulatus* Bath

* cell numbers determined by acridine orange direct counts

Co-metabolic enzyme activities: We have also evaluated the activity of enzymes responsible for TCE co-metabolism in the medial zone of the TAN plume. Previous studies have shown that co-metabolism by methanotrophs is an active TCE degradation mechanism in this aerobic aquifer. Oxygenase enzymes in general and sMMO in particular are thought to be responsible for the co-metabolism. An enzyme activity probe (coumarin) detected sMMO activity in eight medial zone wells. Three enzyme activity probes (3 hydroxy-phenylacetylene, phenylacetylene, and trans-cinnamonitrile) were used to assay for general oxygenase activity throughout the medial zone. Approximately 5 - 25% of the cells harvested from all wells sampled yielded a positive response for these enzymes suggesting substantial aerobic TCE co-metabolic activity. Microbial DNA and sMMO abundance were assayed using polymerase chain reaction (PCR) on biomass harvested from 6 medial-zone wells. Samples from all six wells studied showed evidence for the presence of sMMO genes. These results from the TAN medial zone show that the microbial community has the genetic potential to produce sMMO, and that both sMMO and other oxygenases are active throughout the medial zone indicating the potential for co-metabolic activity throughout the medial zone. In portions of the medial zone with elevated methane (> 100 µg/L) and low dissolved oxygen (< 1 mg/L), the sMMO pathway appears to be the dominant biological TCE degradation mechanism. In portions of the medial zone and farther down-gradient where methane concentrations are lower and dissolved oxygen concentrations are higher, other oxygenase pathways appear to be predominant.

Microcosm experiments were performed to simultaneously measure TCE co-metabolism and enzyme activity using coumarin, total cells (DAPI), and FISH for concentrated TAN aquifer water collected at TAN-29, as well as for attached bacteria at UP-1. Results of these microcosms produced degradation rates for planktonic communities. Presently, a microcosm experiment is ongoing for basalt chips colonized in TAN-35 and concentrated water collected from TAN-35. Initial results have shown 35 to 52% TCE degradation within 7 days. Enzyme activity using coumarin will also be analyzed for the TAN-35 microcosm experiment.

The studies described above were preformed using biomass harvested by filtering medial zone groundwater, and hence are biased toward planktonic organisms. Future phases of the project will focus on attached biomass. Accordingly, methods for assaying attached biomass were developed using basalt chips inoculated at well UP-1. The methods for quantifying sMMO activity, for measuring microbial activity using enzyme probes, FISH, and TCE co-metabolism in an attached community were developed.

Microbial community structure: The primary focus of this work is to identify the organisms involved in the production and consumption of methane in the TAN aquifer in

order to determine the importance of Type II methane-oxidizing bacteria (the organisms capable of co-metabolism of TCE) in the methane cycle. Phylochip analyses of water samples from three wells in the medial zone of the TCE plume identified several groups of methane-oxidizing bacteria (both Type I and Type II organisms). Although we did not quantify the relative levels of activity of methane-oxidizing organisms (possible by direct 16S rRNA hybridization), we did identify a general increase in the populations (by analysis of 16S rDNA) of the different groups of methane-oxidizing bacteria towards the down-gradient portion of the plume, i.e., away from the injection well. We were also able to identify several groups of methanogenic organisms in the samples despite the relatively high dissolved O_2 in the water. In general, methanogenic populations decreased with increasing distance from the injection well. The key implication of these findings is that the rate of methane cycling in the aquifer may be significantly higher than originally postulated based on the limited concentrations of dissolved methane in the water. This could mean that there is also a significantly increased potential for co-metabolism of TCE in the aquifer. Analyses of the structure of microbial communities attached on the basalt chips suspended in TAN-35 are underway.

Isotopic signatures of methanotrophic activity: In order to develop a technique for quantifying the levels of activity of the different groups of methane oxidizers and methanogens in the aquifer, we are conducting experiments with key organisms to determine the magnitude of shifts in the carbon isotope ratios of the different organisms. These efforts are currently focused on measuring the isotopic compositions of lipids extracted from the cells. In parallel, we are extracting and analyzing lipids from groundwater and basalt chip samples. The progress on this work has been slow to date, but preliminary results indicate that different groups of methane-oxidizing organisms produce large negative shifts in the carbon isotope ratios of the methane substrate. When coupled with the low carbon isotope ratios of the available methane, this should produce very distinctive carbon isotope ratios for the lipids from those organisms.

Groundwater model: Microbial studies performed in this project will lead to a better understanding of the factors that control TCE degradation kinetics. These experimentally derived parameters will be incorporated into a groundwater flow and transport model and used to predict evolution of a plume of TCE-contaminated groundwater. To accomplish this, a kinetic model that describes competitive-inhibition between methane and TCE for an enzyme (e.g., sMMO) and inhibition at low dissolved oxygen conditions has been developed. The reactive transport simulation code RT3D is being modified to include a reaction module based on this kinetic model. A simulation study will be performed to evaluate the ability of this kinetic model to realistically predict TCE transport and degradation, and to estimate the improvement in predictive ability of this more complex model relative to simpler models that require less data to parameterize.

Planned Activities

- Placement of FTISR in TAN-35 to test how methanotroph presence/activity is linked to hydraulic flow rate within the aquifer (Nov 06 - May 07)
- Development of TAN natural attenuation, groundwater flow, and transport model to include direct determinations of TCE co-metabolism rates. Improved predictions of evolution of TCE plume evolution. (June 07)

- Complete multiple analyses related methane-oxidizing and methanogenic microorganisms collected using the FTISR in the TAN aquifer. Analyses will include microcosm-derived co-metabolism rates, sMMO expression, Phylochip diversity analysis, proteomics, carbon isotope fractionation experiments under relevant nutrient conditions (analyzing carbon isotope ratios of CH_4, CO_2, bulk biomass, phospholipid fatty acids and nucleic acids), monitoring of carbon isotope compositions of dissolved methane, dissolved inorganic carbon and biomass from the FTISR (Aug 07)
- Linkage of parameters: relationship of methanotroph biomass and activity parameters to co-metabolic rates (Sept 07)
- Complete investigations at TAN and extend to other DOE sites (e.g., Paducah) (Sept 07)

Information Access

There are no manuscripts related to this research that have been submitted.

Respective contributions made by collaborating institutions

From the previous sections that summarize progress:

- Idaho National Laboratory – Oregon State University researchers are responsible for aquifer sampling using the groundwater filtration system and FTISR, design, construction and testing of the FTISR, TCE co-metabolism studies with microcosms, and sMMO gene expression studies.
- Lawrence Berkeley National Laboratory researchers are responsible for Phylochip investigations of microbial community diversity, methanotroph lipid analysis, and stable isotope analysis of groundwater constituents and microbial communities.
- North Wind Inc. researchers are responsible for assisting the collection of samples from the TAN aquifer, for enzyme activity and FISH assays, for microcosm experiments, for modeling of natural attenuation, and for exploring future opportunities at other DOE sites where natural attenuation may be occurring.
- University of Idaho researchers are responsible for methanotroph proteomic investigations and sMMO gene detection studies.