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ORIGINAL PAPER

Proteomic and targeted qPCR analyses of subsurface microbial communities for presence of methane monooxygenase

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Abstract The Test Area North (TAN) site at the Idaho National Laboratory near Idaho Falls, ID, USA, sits over a trichloroethylene (TCE) contaminant plume in the Snake River Plain fractured basalt aquifer. Past observations have provided evidence that TCE at TAN is being transformed by biological natural attenuation that may be primarily due to co-metabolism in aerobic portions of the plume by methanotrophs. TCE co-metabolism by methanotrophs is the result of the broad substrate specificity of microbial methane monooxygenase which permits non-specific oxidation of TCE in addition to the primary substrate, methane. Arrays of experimental approaches have been utilized to understand the biogeochemical processes driving intrinsic TCE co-metabolism at TAN. In this study, aerobic methanotrophs were enumerated by qPCR using primers targeting conserved regions of the genes *pmoA* and *mmoX* encoding subunits of the particulate MMO (pMMO) and soluble MMO (sMMO) enzymes, respectively, as well as the gene *mxa* encoding the downstream enzyme methanol dehydrogenase. Identification of proteins in planktonic and biofilm samples from TAN was determined using reverse phase ultra-performance liquid chromatography (UPLC) coupled with a quadrupole-time-of-flight (QToF) mass spectrometer to separate and sequence peptides from trypsin digests of the protein extracts. Detection of MMO in unenriched water samples from TAN provides direct evidence of intrinsic methane oxidation and TCE co-metabolic potential of the indigenous microbial population. Mass spectrometry is also well suited for distinguishing which form of MMO is expressed in situ either soluble or particulate. Using this method, pMMO proteins were found to be abundant in samples collected from wells within and adjacent to the TCE plume at TAN.

Keywords Proteomics · Methanotrophs · Co-metabolism · Methane monooxygenase · Trichloroethylene

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Introduction

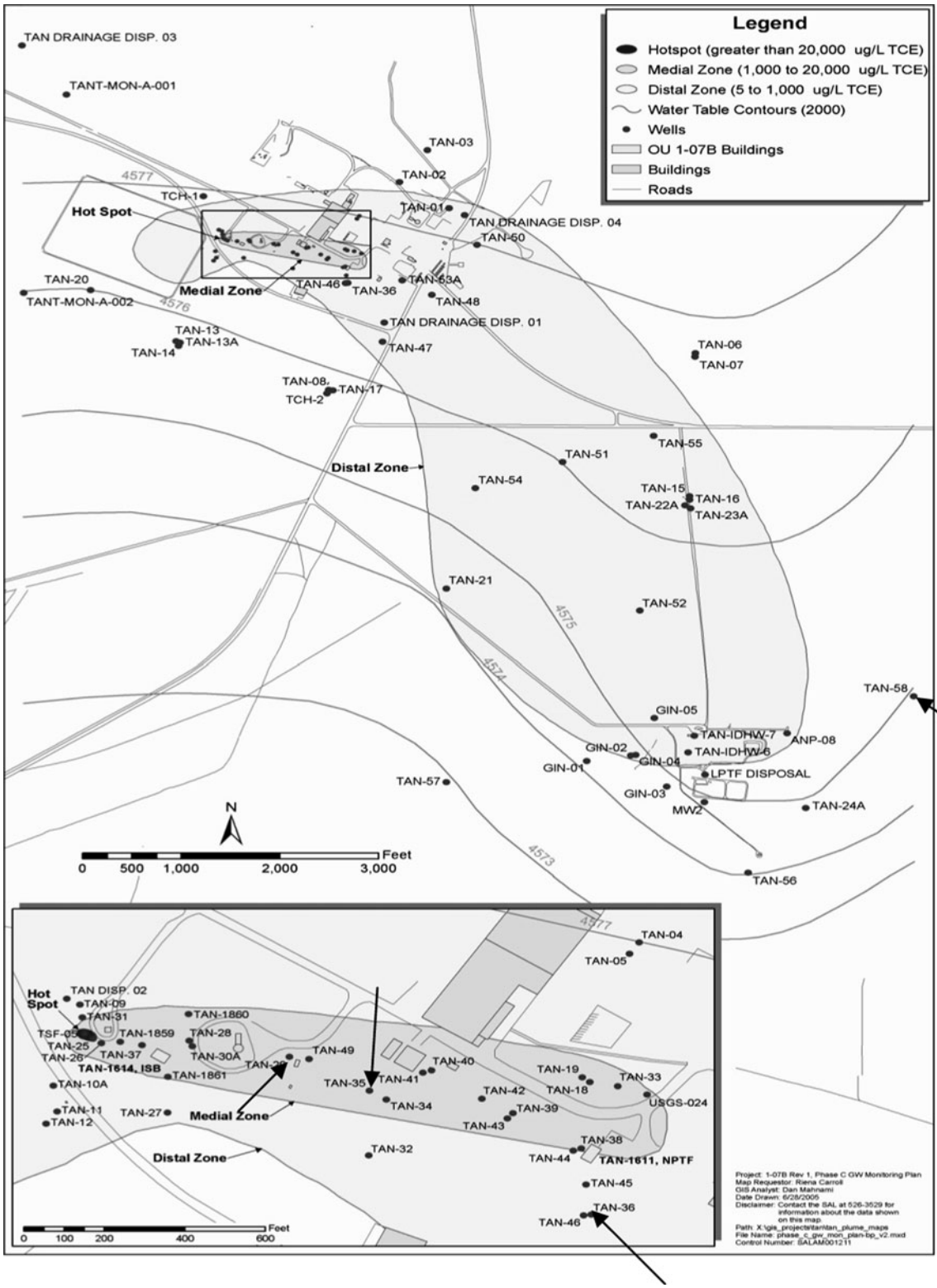
Environmental pollutants in soil and water are a major problem worldwide because they threaten the health of humans and the environment through

toxicity, mutagenicity, and carcinogenicity (Singh 2006). One pollutant of environmental concern is trichloroethylene (TCE), an anthropogenic compound that contaminates aquifers worldwide. TCE has been used as an additive in adhesives, paint removers, and typewriter correction fluids as well in degreasing and dry-cleaning operations and in large quantities in radionuclide processing (Lontoh and Semrau 1998). As a result of these applications, numerous TCE plumes exist at industrial and government facilities in the United States (Sorenson 2008). Aerobic degradation of TCE by co-metabolism has been well established in the laboratory and as a remediation strategy in the field (Arp et al. 2001; Mattes et al. 2010; Semprini et al. 1990). Co-metabolism involves the fortuitous transformation of a non-growth substrate by an enzyme actively engaged in the transformation of primary growth substrate. Until recently direct evidence for TCE attenuation by intrinsic co-metabolism was limited.

Aerobic methanotrophs are ubiquitous Gram-negative bacteria that use methane monooxygenases (MMOs) to oxidize methane to methanol, thereby providing a carbon and energy source for the organisms' growth (Stafford et al. 2003; McDonald et al. 1995). There are two known forms of methane monooxygenase, a membrane-bound particulate form (pMMO) and a cytoplasmic soluble form (sMMO). The particulate MMO has been observed in all types (type I, type II, and type X) of methanotrophs (McDonald et al. 1995). Soluble MMO has been observed primarily in type II and type X methanotrophs (McDonald et al. 1995; Koh et al. 1993), with a few exceptions that include the identification of sMMO in the type I methanotroph *Methylomonas methanica* (Koh et al. 1993). The expression of pMMO is promoted by a high copper-to-biomass ratio, whereas expression of sMMO is promoted by a low copper-to-biomass level (Stafford et al. 2003; Miller et al. 2002; Lieberman and Rosenzweig 2004; Basu et al. 2003). Due to the broad substrate specificity of MMO, TCE, among other potential substrates, can also be oxidized forming an unstable and reactive TCE epoxide which is toxic to the cell. The TCE epoxide intermediate is short-lived due to its reactivity and becomes completely mineralized to non-toxic end products CO_2 and Cl^- (Chu and varez-Cohen 1998; Arp et al. 2001). The short-lived nature of the intermediates of TCE co-metabolism makes detection in the environment difficult compared to anaerobic reductive dechlorination which results in the formation of many stable intermediates that are detectable in the environment (Mattes et al. 2010). For this reason alternative approaches must be taken to provide evidence for intrinsic aerobic TCE co-metabolism by indigenous microorganisms.

Fig. 1 TCE plume in the Snake River Plain Aquifer and the wells used to monitor ground water conditions. TCE and other liquid wastes were injected into well TSF-05 (Hotspot) from 1952 to 1972. The contamination spread southeast with groundwater flow. *Arrows* point to the wells from which planktonic and basalt biofilm samples were collected. Planktonic samples were collected from TAN-29 and TAN-35 (medial zone), TAN-36 (distal zone) and TAN-58 (outside the distal zone). Basalt biofilm samples were collected from FTISR reactors that were incubated in the groundwater in TAN-35 ▶

The Test Area North site (TAN) at the Idaho National Laboratory (INL) near Idaho Falls, ID, USA has a 3-km long TCE contamination plume in the Snake River Plain Aquifer (SRPA). The source of the contamination is the TSF-05 well which received chlorinated solvents, including TCE and tetrachloroethylene (PCE), radionuclides and sanitary sewage waste from the mid-1950s to 1972 (Sorenson et al. 2000). The plume is divided into three zones based upon historical TCE concentrations (Fig. 1). Sorenson et al. (2000) established that TCE in the area immediately surrounding the residual source was being degraded by anaerobic reductive dechlorination and had a half-life of 8 years. The majority of the plume downgradient of the source was aerobic and TCE concentration was monitored in relation to internal tracers, the recalcitrant co-contaminants PCE and tritium. Using the tracer corrected method it was determined that aerobic degradation was primarily responsible for the observed TCE attenuation in the aerobic portion of the plume as opposed to dispersion or sorption and a half-life of 13–21 years for TCE was established. The mechanism for the aerobic degradation of TCE was unknown at the time but co-metabolism was suspected. Continued investigation by Newby et al. (2004) and Erwin et al. (2005) found that groundwater samples taken from areas of the SRPA outside of the contaminant plume contained microorganisms that carried genes for methane monooxygenase and ammonia monooxygenase and it was predicted that these microorganisms would also be present within the contaminant plume.


Legend
Hotspot (greater than 20,000 ug/L TCE)
Medial Zone (1,000 to 20,000 ug/L TCE)
Distal Zone (5 to 1,000 ug/L TCE)
Water Table Contours (2000)
Wells
OU 1-07B Buildings
Buildings
Roads
TAN DRAINAGE DISP. 03
TANT-MON-A-001
TAN-03
TAN-02
TAN-01
TAN DRAINAGE DISP. 04
TAN-50
TCH-1
Hot Spot
Medial Zone
TAN-20
TANT-MON-A-002
TAN-46
TAN-36
TAN-53A
TAN-48
TAN DRAINAGE DISP. 01
TAN-47
TAN-13
TAN-13A
TAN-14
TAN-08
TCH-2
TAN-17
TAN-06
TAN-07
TAN-55
TAN-51
Distal Zone
TAN-54
TAN-15
TAN-22A
TAN-16
TAN-23A
TAN-21
TAN-52
TAN-58
GIN-05
TAN-IDHW-7
ANP-08
GIN-02
TAN-IDHW-6
GIN-04
GIN-01
LPTF DISPOSAL
TAN-57
GIN-03
MW2
TAN-24A
TAN-56
4577
4576
4575
4574
4573
N
0 500 1,000 2,000 3,000 Feet
Hot Spot
TAN DISP. 02
TAN-09
TAN-31
TAN-1860
TSF-05
TAN-1859
TAN-28
TAN-25
TAN-37
TAN-26
TAN-30A
TAN-1614, ISB
TAN-1861
TAN-10A
TAN-11
TAN-12
TAN-27
Medial Zone
Distal Zone
TAN-49
TAN-40
TAN-35
TAN-41
TAN-34
TAN-42
TAN-39
TAN-43
TAN-32
TAN-19
TAN-33
TAN-18
USGS-024
TAN-38
TAN-44
TAN-1611, NPTF
TAN-45
TAN-36
TAN-46
TAN-04
TAN-05
0 100 200 400 600 Feet
Project: 1-07B Rev 1, Phase C GW Monitoring Plan
Map Requestor: Riena Carroll
GIS Analyst: Dan Mahnami
Date Drawn: 6/28/2005
Disclaimer: Contact the SAL at 526-3529 for information about the data shown on this map.
Path: X:\gis_projects\tan\tan_plume_maps
File Name: phase_c_gw_mon_plan-bp_v2.mxd
Control Number: SALAM001211

Recently, Wymore et al. (2007) reevaluated the TCE degradation rates at TAN using the tracer corrected method and confirmed the likelihood of aerobic degradation of TCE with a half-life of 13 years. Additionally, the same study used enzyme activity probes for MMO enzyme and found sMMO activity, using phenylacetylene as a differential inhibitor of sMMO, from samples collected from wells in the distal zone of TAN providing additional evidence of intrinsic TCE co-metabolism at TAN. However, phenylacetylene is also capable of inhibiting pMMO at high concentrations (Lontoh et al. 2000). In addition, there has been recent evidence that pMMO can be inhibited at low phenylacetylene concentrations putting into question whether phenylacetylene can be used as a selective inhibitor of sMMO (Lee et al. 2009). Therefore additional evidence is required to determine whether pMMO or sMMO is being expressed in situ as this information is necessary in the evaluation of long term co-metabolic activity and kinetics. In in vitro experiments where methanotrophs were grown in the presence of a mixture of chlorinated solvents, cells expressing sMMO, despite having a broader substrate range and faster kinetics, exhibit slower growth due to competition with methane for binding to MMO and rapid buildup of toxic co-metabolites and are subsequently less capable of transformation of chlorinated solvents. However, cells expressing pMMO, which has a higher specificity for methane with slower kinetics, are capable of sustained high growth and as a result greater transformation of chlorinated solvents and also exhibit greater methane uptake at lower temperatures than cells expressing sMMO (Lee et al. 2006; Yoon and Semrau 2008).

Extremely low detection limits (10^{-15} mol) afforded by electrospray ionization mass spectrometry (ESMS) and peptide sequencing capabilities using MS–MS data represent a revolutionary breakthrough in proteomics-based analyses of complex microbial communities (Shevchenko et al. 1996; Aebersold and Mann 2003). This is an especially valuable tool since mRNA expression levels do not always correlate to the amount of protein produced (Ram et al. 2005; Richard et al. 2006). Proteomics in combination with metagenomics has been used to identify and catalog >2,000 proteins expressed by the few microbial species that make up biofilms at an acid mine drainage site (Ram et al. 2005). However, in most cases metagenomic information is not available for a given sample and the samples contain many different microbial species with cell numbers of different strains ranging across multiple orders of magnitude. In addition, proteomic analysis is limited by interfering species present in environmental sample matrices. These difficulties are exemplified in the study by Benndorf et al. (2007) where only 26 proteins could be identified from soil samples.

Here we describe an alternative method and approach to obtain evidence of intrinsic TCE co-metabolic potential by detection of MMO proteins directly from unenriched planktonic and biofilm environmental samples from TAN using mass spectrometry and enumeration of methanotrophs by quantitative PCR (qPCR). Biofilm, consisting of microbes indigenous to the aquifer, was established on pre-sterilized basalt chips inside of a flow-through in situ reactor (FTISR) placed in well TAN-35 for 8 months. A new procedure developed by our group (Bansal et al. 2009, 2011) for time-course formic acid lysis and prefractionation of proteins followed by peptide separation and sequencing by UPLC-ESI-QToF was used to reduce the sample complexity in order to detect signature peptides of the characteristic methanotrophic and methanogenic proteins MMO and methyl-coenzyme M reductase (Mcr). This mass spectrometric approach also enables us to determine whether sMMO of pMMO is being expressed in environmental samples. In addition, the MS peak intensity from a signature MMO peptide found in the pure culture *M. trichosporium* OB3b was used to establish the detection limit for the method and was used to estimate pMMO expression levels in TAN-29 relative to pure culture.

Materials and methods

Pure bacterial strains

Methylosinus trichosporium OB3b and *Methylococcus capsulatus* (Bath) cultures were grown in nitrogen-supplemented mineral salt media (NMS) pH 6.8 at 30°C with shaking at 200 rpm (Atlas 1993). Liquid cultures (50 ml) were grown in 150-ml serum bottles with a headspace filled with sterile methane and air 3:7 (v/v). Once every 2 d, the headspace was flushed with a fresh gas mixture for 2 min. To stimulate

expression of sMMO, cultures were grown in copper-free NMS media. Copper (12 μM) was added to the media to induce expression of pMMO in the *M. trichosporium* OB3b culture. The cells were harvested at mid-log phase by centrifugation at 5,500×*g* for 10 min. The pellets were suspended in NMS media with 5% dimethyl sulfoxide (DMSO) and stored in 1 ml aliquots at −80°C.

Planktonic cell samples

The TCE plume at the TAN site is divided into three zones based on historical concentrations of TCE: (1) hotspot (>20 mg l^{-1}); (2) medial zone (1–20 mg l^{-1}); and (3) distal zone (<1 mg l^{-1}). Four wells were sampled for evaluation of the metabolic state of the microorganisms present in the three different aquifer zones. Wells TAN-29 and TAN-35 are located in the medial zone, TAN-36 in the distal zone, and TAN-58 outside the distal zone (Fig. 1). Water samples from four wells (TAN-29, TAN-35, TAN-36, and TAN-58) were collected and cells concentrated using a KrosFlo Plus hollow-fiber ultra filtration system (0.05-μm pore size, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The filtrate was further concentrated by centrifugation at 5,500×*g* for 30 min; the collected cells suspended in TAN water were stored at −80°C prior to proteomic analysis. A sample was collected from TAN-29 in May 2006. All four wells were sampled during June 2007. For each proteomics experiment, the cells present in 500–600 l of TAN well water were concentrated to a final volume of 15–20 ml. Biomass contained in 0.1–1 ml of the concentrate was used for protein extraction and trypsin digestion. Each digest produced 20 μl of peptide solution; 2 μl of this solution was used for proteomic analysis. Every sample was analyzed at least three times.

Basalt biofilms

Flow-through in situ reactors (FTISR) were installed in TAN-35 in November 2006 and operated while submerged in the groundwater to maintain ambient temperatures (ca. 12°C from Wymore et al. (2007)). The FTISR consisted of six 1 m × 2.5 cm stainless steel cylindrical reactors filled with crushed basalt (Conrad et al. 2010a; Lehman 2007). Groundwater was pumped through the reactors at a rate of either 0.1 or 1 m/day to colonize the basalt chips with indigenous microbes. The reactors were retrieved in July 2007 and the basalt from each reactor was mixed separately. The samples were stored at −80°C until further use.

Enumeration of total cells

One ml of a *M. trichosporium* OB3b cell culture or a TAN water sample was centrifuged for 2 min at 17,000×*g*. Each cell pellet was washed with 1 ml of sterile phosphate-buffered saline (PBS) pH 7.3, centrifuged as before, resuspended in 1 ml of 70% ethanol, and fixed for 1 h on a rocking platform shaker prior to staining. Cells were then washed with PBS and resuspended in 1 mg ml^{-1} of acridine orange solution (Kepner and Pratt 1994; Suzuki et al. 1993; Francisco et al. 1973). After incubation at room temperature for 3 min, cells were collected on 0.22-μm black polycarbonate filters (25 mm in diameter, GE Water and Process Technologies, Minnetonka, MN), and fluorescing cells were counted using an Olympus DP 70 microscope (Olympus, Center Valley, PA). Cell counts were obtained from 10 random microscope fields of view.

DNA extractions for qPCR

For pure cultures, 1 ml of *M. trichosporium* OB3b cell culture was centrifuged, and cells were resuspended in 1 ml of TE buffer. Serial dilutions of the cell suspension were made from 10^0 to 10^{-5}, cells were centrifuged again, and pellets were resuspended in 50 μl of 1% Triton X-100. Cells were lysed by boiling for 10 min and then cooled quickly on ice (Wang et al. 1996). Aliquots from these preparations were used in preparing standard curves for qPCR.

For planktonic samples, concentrated solutions were initially centrifuged at 100×*g* to remove large debris. Cells then were collected by centrifuging at 20,000×*g* for 10 min and resuspended in 0.5 ml 1% Triton X-100. Cells were boiled for 20 min and quickly cooled on ice. DNA in the samples was further purified using phenol/chloroform extraction and then ethanol precipitation. Finally, samples were purified using MicroSpin Sephadex G-25 columns (GE Healthcare, Buckinghamshire, UK).

For basalt biofilm extractions, 15 g of basalt rock from FTISR reactors was submerged in 25 ml of 1%

Triton X-100 and boiled for 20 min. Cell debris from the samples was removed by centrifuging for 20 min at 5,500×*g*. Basalt was rinsed once with an additional 5 ml of 1% Triton X-100 and centrifuged again as before. Supernatants were combined, and lyophilized. The lyophilized pellet was resuspended in sterile water and DNA in the sample was further purified using phenol/chloroform extraction and ethanol precipitation. For clean-up, samples were run through MicroSpin G-25 columns using TE buffer.

Quantitative PCR (qPCR) assays

A series of qPCR assays were performed using genomic DNA extracted from TAN planktonic and basalt biofilm samples to enumerate methanotrophic bacteria. Standard curves for qPCR were generated using genomic DNA extracted from serial dilution (10^0–10^{-5}) of *M. trichosporium* OB3b. Each standard was measured in triplicate. The following primers were used for the amplification of pMMO (standard curve $r^2 = 0.999$), 189f (5′-GGNGACTGGGACTTC TGG-3′) and mb661r (5′-CCGGMGCAACGTCY TTACC-3′) (Lyew and Guiot 2003; Holmes et al. 1995). The *mmoX* portion of sMMO (standard curve $r^2 = 0.999$) was amplified using primer pair 536f (5′-CGCTGTGGAAGGGCATGAAGCG-3′) and 898r (5′-GCTCGACCTTGAACTTGGAGCC-3′) (Fuse et al. 1998; Lyew and Guiot 2003). Amplification of methanol dehydrogenase genes (standard curve $r^2 = 0.994$) was performed using the primer pair *mxa* f1103 (5′-GCGGCACCAACTGGGGCTGGT-3′) and *mxa* r1561 (5′-GGGCAGCATGAAGGGC TCCC-3′) (McDonald and Murrell 1997). For all PCR reactions, a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) was used. Data analysis was performed using StepOne Plus software. Cycle threshold values were obtained from a dilution series of pure culture target DNA. PCR amplifications were performed in triplicate using total volumes of 25 µl consisting of 12.5 µl of Power SYBR green master mix (Applied Biosystems), 300 nM of each primer, and 1 µl of DNA standard or sample. Following an initial denaturation step at 95°C for 10 min, all PCR amplifications were performed using 40 cycles of 95°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min. Fluorescence readings were taken during the 55–60°C incubation. Melt curve analysis was performed following every run to confirm product specificity. qPCR data analysis was conducted as previously described (Raeymaekers 2000; Suzuki et al. 2000).

Formic acid extraction of protein from planktonic samples (time-course lysis)

Formic acid time-course lysis was used to extract protein from pure cultures of *M. trichosporium* OB3b and planktonic samples for mass spectrometry. Full details of the formic acid lysis method, protein digestion and mass spectrometry conditions have been reported by our group previously (Bansal et al. 2009). Briefly, samples were boiled with 20% formic acid at 3 min intervals in a boiling water bath for a total duration of 30 min. Cells and cell debris were pelleted by centrifugation at the end of each interval and the supernatant collected into a new tube. Ten protein fractions were obtained for each sample using the 30 min time-course lysis procedure. The length of time-course formic acid lysis was determined by visualization of non-lysed cells using light microscopy. After 30 min, only cell debris was visible (data not shown). Each fraction was dried (DNA Speed Vac, Savant, Ramsey, MN) and the proteins in each fraction were reduced, alkylated and subjected to trypsin digestion. The resulting peptide fragments were separated and sequenced by reversed phase UPLC-ESI-QToF.

Protein extraction from basalt biofilms

Fifteen g of basalt rock were submerged in 10 ml of 1% Triton X-100. The samples were sonicated with a Virsonic 100 with sonicating probe (Virtis, Gardiner, NY) for 10 min followed by incubation in a boiling water bath for another 30 min. Supernatants were collected by centrifugation at 1,000×*g* for 5 min. Total proteins and unlysed cells were precipitated with acetone (4:1 acetone to supernatant ratio, 80% acetone final concentration) followed by centrifugation at 14,000×*g* for 15 min. The pellets were collected, washed twice with 80% acetone in order to remove Triton X-100, and suspended in 20% formic acid. A hydrolysis procedure identical to that described above for the planktonic samples was used.

Table 1 Enumeration of methanotrophic and total cell numbers in concentrated TAN water samples using qPCR assays and acridine orange direct microscopic counts

Well	AODC	qPCR enumeration[a]		
		pmoA	*mmoX*	*mxa*
	Cells ml^{-1}	Cells ml^{-1}	Cells ml^{-1}	Cells ml^{-1}
TAN 29_2006[b]	6.0×10^8	5.25×10^6		
TAN 29_2007[b]	3.6×10^7	3.36×10^5	5.44×10^3	1.08×10^4
TAN 35_2007 (Basalt)[c]		$<32\ g^{-1}$	$<24\ g^{-1}$	$<100\ g^{-1}$
TAN 36_2007[b]	8.7×10^5	1.1×10^4	6.58×10^2	9.48×10^2
TAN 58_2007[b]	6.5×10^5	2.5×10^4	1.43×10^3	1.30×10^3

[a] Conserved regions of the genes encoding pMMO (*pmoA*), sMMO (*mmoX*), and methanol dehydrogenase (*mxa*) were amplified using primers reported in the literature (see "Materials and methods" section)

[b] Planktonic samples were concentrated approximately ×500 by ultrafiltration. Cells numbers in unconcentrated groundwater would be expected to be 5×10^2-fold lower

[c] The qPCR results for TAN-35 basalt are close to detection limit. We were also not able to visualize the PCR product on a gel. Note that basalt samples were not concentrated as noted for the well water samples

Mass spectrometric data analysis

ProteinLynx Global Server 2.2.5 (PLGS, Waters) and Protein Expression Informatics System software version 2.2 were used for mass spectra analyses, peptide sequencing, and protein identification. The raw data were converted into peak lists (*.pkl files) by PLGS using the following parameters: (1) smooth channels = 4, number of smooths = 2, smooth mode = Savitzky Golay; (2) percentage of peak height to calculate the centroid spectra = 80%; and (3) no baseline subtract was allowed. Identification of proteins and peptides was performed by searching the processed MS spectra against the Swiss-Prot, Non-redundant (nr) (downloaded from NCBI website ftp://ftp.ncbi.nih.gov) and MMO (created in house from all known MMO sequences deposited) databases. The pkl file for each fraction of a sample was merged using Mascot Daemon and were search using Mascot Server version 2.2 (htpp://www.matrixscience.com) against MSDB or TrEMBL protein amino acid sequence databases (Matrix Science Inc., Boston, MA). Parameter settings for the Mascot searches were: (1) trypsin as the specific enzyme; (2) peptide window tolerance (error window on experimental peptide mass values) ± 0.8 Da; and (3) fragment mass tolerance of 0.8 Da. During searches, "carbamidomethyl C" modification was the only amino acid modification allowed. Individual ion scores > 47 indicate identity or extensive homology ($P < 0.05$). The ion score is $-10 * \log(P)$, where P is the probability that the observed match is a random event. Protein scores are non-probability based and are calculated from the peptide(s) score, number of identified peptides, and the number of queries.

Methanotroph enumeration by mass spectrometry

Methanotrophic bacteria numbers were also estimated using the intensity of MS signals from pMMO peptide sequences observed in both pure and environmental samples. We decided to measure the MMO proteins because they are responsible for assimilation of methane and are one of the constitutive proteins for these microbes. Dilutions of mid-log phase *M. trichosporium* OB3b cells (10^2–10^5) were prepared and time-course lysis performed. The mass spectral peak intensities of a peptide (observed throughout the time course for both pure cultures and TAN samples) were summed, and the cumulative peptide peak intensity was plotted against its corresponding OB3b cell dilution. The cumulative intensity of that particular peptide from TAN samples was added and compared to a standard curve to estimate the number of methanotrophic bacteria. Peptide RVSFLNAGEPGPVLVR.T (belonging to the PmoB subunit) was found throughout the entire time course of *M. trichosporium* pure cultures and in some planktonic samples.

Results

Enumeration of total planktonic bacteria by acridine orange direct counts

Planktonic cells were obtained by filtering and concentrating biomass from groundwater taken from wells TAN-29 and 35 (medial zone), TAN-36 (distal zone), and TAN-58 (outside distal zone) (Fig. 1). Although 0.05-μm filters were used for concentrating cells from TAN groundwater, 0.22-μm filters were used for enumeration because 0.05-μm filters compatible with fluorescence-based counting methods were not available (Suzuki et al. 1993). Cell numbers (ml^{-1}) obtained by AODC for concentrated planktonic samples are reported in Table 1. On average 500–600 l of groundwater was concentrated for each well to a final volume of ~1 l Therefore, the actual cell count for unconcentrated well water samples would be about 5×10^2-fold lower. To have sufficient biomass for proteomics experiments the samples were further concentrated by centrifugation to ~10 ml. Biofilm was established on pre-sterilized basalt chips inside of a FTISR placed in TAN-35 for 8 months. The crushed basalt was colonized with indigenous bacteria by pumping groundwater through the six reactors that made up the FTISR at 0.1 m/day for three reactors and 1 m/day for the other three reactors. Cell counts for basalt biofilms were not obtained as quantitative recovery of intact cells was not feasible. A planktonic sample from TAN-35 collected after the FTISR was removed contained 3.2×10^5 cells ml^{-1}. Cell numbers were higher in TAN-29 and TAN-35 located in the medial zone than from TAN-36 in the distal zone and TAN-58 which is outside the distal zone.

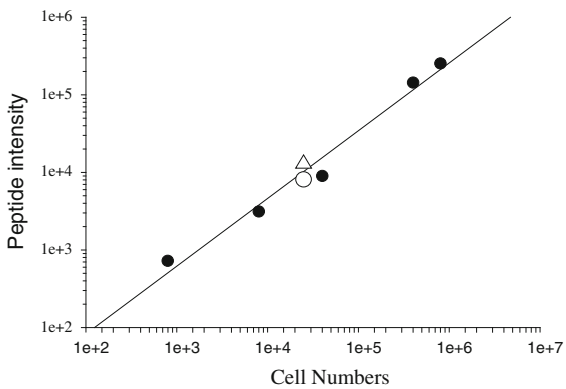


Fig. 2 Quantification of methanotrophic cells using specific peptide MS signal intensity. The cumulative intensity of the peptide "VSFLNAGEPGPVLVR" (*filled circles*) obtained from the time-course hydrolysis of serial dilutions of *M. trichosporium* OB3b cells (10^2–10^5). The cumulative intensity of the same peptide obtained during time-course hydrolysis of TAN samples was added and plotted to estimate the number of methanotrophic bacteria. *Open triangle* represents peptide intensity of a TAN-29 sample collected in 2006 (3.9×10^4 methanotrophs/ml); *open circle* represents peptide intensity of a TAN-29 sample collected in 2007 (2.4×10^4 methanotrophs/ml). Cells numbers are for concentrated groundwater

Quantification of methanotrophs by qPCR

Methanotrophs in planktonic and basalt biofilm samples were enumerated using qPCR by comparison to a standard curve generated using genomic DNA from a dilution series of *M. trichosporium* OB3b (10^6–10^2 cells) measured in triplicate. Primers targeting conserved sequence regions of the genes *PmoA*, *mmoX* and *mxa* which encode portions of the proteins pMMO, sMMO and methanol dehydrogenase respectively, were used in qPCR experiments to estimate the number of methanotrophic bacteria present in planktonic samples (Table 1). Results from these assays in which pMMO specific primers were used indicated that in all samples, except TAN-58, methanotrophs make up 0.9–1.3% of the total microbial population. In the TAN-58 sample, the number of methanotrophic bacteria was 3.8% of the total bacterial cell numbers (Table 1). The numbers of total methanotrophic bacteria decrease with distance from the source of the contaminant plume following the trend of the AODC cell counts. Numbers of methanotrophic bacteria from basalt biofilms were also determined using qPCR. Only one basalt biofilm sample from the TAN-35 well had measurable numbers of methanotrophs and contained 32 methanotrophs g^{-1} of basalt chips using primers targeting pMMO. Results from qPCR assays performed using sMMO-specific primers indicated that the number of type II methanotrophic bacteria in TAN-29 during the 2006 sampling event was below the detection limit.

Detection of MMO protein in pure culture and environmental samples

Formic acid time-course lysis was used to extract proteins from pure cultures of *M. trichosporium*

Table 2 Methanotrophic and methanogenic proteins identified in concentrated planktonic samples collected from Test Area North wells as noted

Well	pMMO		sMMO	Mcr	
	Accession [*Score**]	*Peptide*(*s*) [*Ion Score**]		*Accession* [*Score**]	*Peptide*(*s*) [*Ion Score**]
TAN 29_2006 (medial)	PmoB		ND	McrA	
	Q70EF2 [215]	R.VSFLNAGEPGPVLVR.T [76]***		Q9C4C1 [83]	K.YPTTLEDHFGGSQR.A [54]
		R.TIPLQAGLQKPLTPLVTDGT AGVGK.E [76]		*Q9C4C2***	R.GPNEPGGLSFGFLADIV QSHK.K [29]
		R.FLNPDVFTTKPDFPDYLLADR.G [54]			
		R.FAAEIGGPVIPK.F [42]			
		R.TLNWYDVQWSK.T [14]			
		R.LGEYTAAGLR.F [45]			
	Q25BV2 [138]	R.TIHWFDLNWSK.D [56]			
		R.LADLIYDPDSR.F [75]			
		R.AGSWIGGQLVPR.S [34]			
	Q9KX50 [55]	R.LGEYTAAGLR.F [42]			
		R.SVSLEVGKDYAFSIDLK.A [9]			
		R.FLNPDVFTTKPEFPDYLLADR.G [4]			
	PmoC			McrG	
	Q25BV4 [65]	K.ADAAEAPLLNQK.N [24]		MCRG [92]	R.LQEDGVMFDMLDR.R [64]
		R.KVMSITPR.E [20]		*Q8PXH7***	R.LQEDGVMFDMLDRR.R [28]
		K.VMSITPR.E [20]		*Q8THHO***	
				*Q9HHA9***	
				*D29525***	
TAN 29_2007 (medial)	PmoB		ND	ND	
	Q25BV2 [190]	R.TIHWFDLNWSK.D [71]			
		R.LADLIYDPDSR.F [56]			
		R.AGSWIGGQLVPR.S [56]			
		R.TVDVTASDAAWEVYR.L [9]			
	Q70EF2 [156]	R.VSFLNAGEPGPVLVR.T [53]***			
		R.FLNPDVFTTKPDFPDYLLADR.G [48]			
		R.LGEYTAAGLR.F [46]			
		R.FAAEIGGPVIPK.F [9]			
	PmoC				
	Q25BV4 [53]	K.ADAAEAPLLNQK.N [53]			

Table 2 continued

Well	pMMO		sMMO	Mcr	
TAN 35_2007 (medial)	PmoB		ND	McrA	
	Q25BV2 [229]	R.AGSWIGGQLVPR.S [73]		Q2AAD9 [1655]	R.GPNEPGGLSFGHMADIV QTSR.K [150]
		R.LADLIYDPDSR.F [44]		*Q2AAEO***	
		R.TIHWFDLNWSK.D [21]		*Q2AAE5***	
				*Q2ABR0***	
				*Q15BI6***	
	Q9KX36 [65]	R.VSFLNAGEPGPVLVR.T [56]		*Q75NC9***	
	*Q70EF2***			Q9C4C1 [535]	K.YPTTLEDHFGGSQR.A [85]
				*Q9C4C2***	K.FGSAK.A [6]
				*Q7ZA86***	
				*Q7ZAFO***	
				McrG	
				MCRG [66]	R.LQEDGVMFDMLDR.R [66]
				Q8PXH7**	R.GVDPGTLSGR.Q [40]
				Q8THHO**	K.RTTIYR.V [14]
				Q9HHA9**	
				D29525**	
TAN 36_2007 (distal)	PmoB		ND	ND	
	Q9KX50 [79]	R.TAQFIGEQFAPR.S [42]			
		K.SSFLNAGEPGPVLVR.T [37]			
TAN 58_2007 (outside plume)	PmoB		ND	ND	
	Q9KX50 [104]	R.TAQFIGEQFAPR.S [52]			
		K.SSFLNAGEPGPVLVR.T [52]			

Mcr methyl-coenzyme M reductase, *pMMO/sMMO* particulate and soluble methane monooxygenase, *ND* not detected

* Protein score is non-probability based and is calculated from the peptide(s) score, number of identified peptides, and the number of queries. Peptide ion score is $-10 * \log(P)$, where P the is probability that the observed match is a random event. Individual ion scores >47 indicate identity or extensive homology ($P < 0.05$). ** Protein homologues containing the same peptide(s). *** The tryptic peptide R.VSFLNAGEPGPVLVR.T was used for semi-quantitative estimation of methanotrophic numbers from planktonic samples

Table 3 Geochemical parameters at selected TAN wells

TAN well	TCE ($\mu g\ l^{-1}$)	Volume (l)	Methane ($\mu g\ l^{-1}$)	DO ($\mu g\ l^{-1}$)	Chloride ($mg\ l^{-1}$)	Nitrite ($mg\ l^{-1}$)	Sulfate ($mg\ l^{-1}$)	Nitrate ($mg\ l^{-1}$)	Ammonium ($mg\ l^{-1}$)
TAN-29 2006	407	784	1,725	215	N/A	N/A	N/A	N/A	N/A
TAN-29 2007	384	784	387	N/A	78.75	2.28	24.80	2.90	1.0
TAN-35	350–500[a]	1,935	1–7*	N/A	N/A	N/A	N/A	N/A	N/A
TAN-36	120	3,197	125	>1,000	65.15	1.46	21.67	1.35	2.0

TCE trichloroethylene, *DO* dissolved oxygen, *N/A* not available

[a] Historic range

OB3b which are then identified by separation and sequencing of (tryptic-digested) peptides with reversed phase UPLC-ESI-QToF (Bansal et al. 2009). Numerous peptide sequences belonging to proteins involved in methane assimilation were identified from hydrolysis products of *M. trichosporium* OB3b grown in media containing 12-μM copper. The particulate form of MMO also contains three different subunits PmoA, PmoB, and PmoC. Peptides corresponding to nearly 61% sequence coverage of the PmoB subunit were observed. Along with PmoB, peptides from subunits PmoA and PmoC were observed with sequence coverage of 10 and 8%, respectively. Similarly, peptide sequences from all subunits of sMMO were identified from the analysis of pure *M. trichosporium* OB3b cultures grown in the absence of copper (data not shown). Soluble MMO consists of three subunits MMOH, MMOR, and MMOB. The peptide RVSFLNAGEPGPVLVR.T (belonging to the PmoB subunit) was found throughout the entire time course of *M. trichosporium* OB3b pure cultures. The cumulative LCMS intensity of this particular peptide from *M. trichosporium* OB3b cell dilutions was used to generate and standard curve in order to establish the detection limit of the method. Proteomics results indicated that detection limits for an active culture of *M. trichosporium* OB3b expressing pMMO was 10^3 cells (Fig. 2).

Application of time-course lysis to planktonic TAN samples identified peptide sequences corresponding to PmoB subunit of pMMO in all planktonic samples and from various methanotrophs (Table 2). Peptides from the PmoB subunit were identified in medial zone wells of the TCE plume TAN-29 (in both 2006 and 2007 samples) and TAN-35, distal zone well TAN-36, and in TAN-58 which lies outside the distal zone of the TCE plume. Although we did not identify peptide sequences corresponding to the PmoC subunit from the TAN-29 2006 sample, peptides from the PmoC subunit were identified in the TAN-29 sample collected in 2007 (Table 2). Peptides associated with formaldehyde metabolism were identified in the 2006 sample. Peptides from the PmoA subunit were observed in the TAN-29 and TAN-58 samples but were below the scoring threshold. No proteins that met the Mascot scoring threshold were identified in TAN-35 basalt samples. However, peptides from the PmoB subunit of pMMO were identified in the planktonic sample collected from TAN-35 after the FTISR was removed. No peptides from sMMO complex subunits were observed during the analysis of TAN samples, suggesting that methanotrophs expressing sMMO, if present, were below detection limits.

As the RVSFLNAGEPGPVLVR.T peptide was also found in TAN-29 samples this approach allowed us to estimate the number of methanotrophs in those samples to compare to qPCR results. Using the cumulative peak intensity of the RVSFLNAGEPGPVLVR.T peptide the TAN-29 2006 and 2007 samples were found to bear the equivalent of 3.9×10^4 and 2.5×10^4 cells ml^{-1} of *M. trichosporium* OB3b grown under laboratory conditions, respectively. This result is one to two orders of magnitude lower than obtained from qPCR. The RVSFLNAGEPGPVLVR.T peptide was not found in TAN-36 and TAN-58 samples.

A large portion of identified proteins in TAN-29 2006 and TAN-35 planktonic samples belong to both known and uncultured methanogenic species supporting the premise that biogenic methane can be produced in TAN groundwater. Peptides from the McrA and McrG subunits of methyl-coenzyme M reductase were identified in both TAN-35 and

TAN-29 2006. Mcr protein consists of three different subunits McrA, McrB, and McrG. Geochemical data for TAN-35, however, indicated that there was little methane present in TAN-35 (Table 3). In addition, high scoring peptides (exceeding Mascot scoring thresholds) from proteins involved in sulfate reduction, including peptides from the alpha and beta subunit of adenylylsulfate reductase, were identified in TAN-35. Other proteins from non-methane metabolic pathways were identified from different microorganisms that are associated with biodegradation, drug resistance, geomicrobial, and pathogenic processes in all TAN well samples but the majority of identified proteins were below the Mascot scoring threshold (data not shown).

Discussion

The numbers of microorganisms that were detected in TAN well water fluctuated widely. Samples from TAN-35, 36, and 58 wells were found to have two to three orders of magnitude fewer cells when compared to TAN-29, but total cell numbers in these three wells were higher than generally observed in the Snake River Plain aquifer (Newby et al. 2004). Also, cell numbers from TAN-29 groundwater collected during 2007 were 10 times lower than those measured a year earlier. Because qPCR results presented here were generated using genomic DNA from *M. trichosporium* OB3b cells, our results account for the known two-copy number of *pmo* in the cells of methanotrophs enumerated in the samples (Gilbert et al. 2000). The results presented may overestimate the number of methanotrophs because the genomes of some species (e.g., *M. capsulatus* Bath) may contain more than two copies of *pmo* genes (McDonald et al. 2008). The qPCR results are consistent with previous findings that methanotrophs are present in the Snake River Plain aquifer (Newby et al. 2004), and supports previous findings that these cells are responsible for some of the observed natural attenuation of TCE that occurs in the aquifer (Wymore et al. 2007).

All planktonic samples from TAN showed the presence of peptides from subunit PmoB indicating active intrinsic methane oxidation, and TCE co-metabolic potential, throughout the medial and distal zones of the TCE plume as well as outside the plume. Basalt samples from TAN-35 were capable of degrading ^{13}C labeled TCE in microcosm experiments (Conrad et al. 2010). In addition, peptides from Mcr protein, which is a biomarker for methanogenesis, were identified in TAN-29 2006 and TAN-35 planktonic samples which suggest a biogenic source of methane in the medial zone. Stable carbon isotope analyses of methane and DIC have shown a transition from methanogenesis to methane oxidation with distance from the source of the TCE contaminant plume (Conrad et al. 2010). PhyloChip analysis from the same study also showed a shift in the structure of the microbial community with distance from the source well from bacteria involved in methanogenesis and reductive dechlorination to methanotrophic bacteria. The co-existence of aerobic methanotrophs and anaerobic methanogens in TAN-29 and TAN-35 planktonic samples is not unusual as methanogens can survive under aerobic conditions by inhabiting biofilms (Lyew and Guiot 2003). Likewise methanotrophs are capable of going dormant under anoxic conditions (Roslev and King 1995). And, certainly aquifer solids may allow the presence of microniches which would permit cells possessing these distinctive physiologies to occur in relatively close proximity.

Although the number of peptides detected in TAN-36 and TAN-58 samples was lower when compared to TAN-29 samples, their presence in these samples offers evidence that intrinsic methane oxidation is occurring in the distal zone and outside the TCE plume. The detection of MMO peptides in the TAN-58 sample was interesting, as this well is located outside the distal contamination zone (approximately 8,000 feet from the point of original TCE injection). The expression of MMOs by methanotrophs in this well may be due to the presence of trace amounts of methane transported by down-gradient groundwater flows or by methane that is naturally present in the aquifer away from the TAN site. Pristine wells in the aquifer contain dissolved methane concentrations as high as 1 μM and carbon stable isotope fractionation measured in aquifer methane and inorganic carbon suggest that both methanogens and methanotrophs are present in the groundwater and that one-carbon metabolism is actively occurring (Newby et al. 2004). Proteomic analysis of basalt biofilm samples from TAN-35 resulted in no protein identifications above the Mascot scoring threshold. This is most likely due to low methanotroph biomass on the basalt chips as indicated by qPCR results as well as release of

interfering species, such as metals, from the basalt chips during sample processing.

During analysis of planktonic samples, peptides from pMMO subunits, but not from sMMO subunits, were observed while qPCR data indicated the presence of methanotrophs targeted by sMMO specific primers in TAN 29 (from 2006), TAN-35, TAN-36 and TAN-58 samples. The inability to identify peptides from sMMO may be due to the presence of copper levels in TAN groundwater that were high enough to suppress the expression of sMMO in type II and type X methanotrophs and sMMO-expressing type I methanotrophs (Lieberman and Rosenzweig 2004; Koh et al. 1993; McDonald et al. 1995). Evidence of MMO expression by RT-PCR from different environments suggests that pMMO may be expressed predominately (Kolb et al. 2005; Chen et al. 2007, 2008; Angel and Conrad 2009). Cells expressing pMMO grown at 20°C also have a higher methane uptake than sMMO-expressing cells (Yoon and Semrau 2008).

Most of the bacterial peptides in TAN samples were below the Mascot scoring threshold and resemble sequences previously described in the *Proteobacteria* group. Peptides indicative of bacteria from different genera, including *Pseudomonas*, *Burkholderia*, *Nitrosococcus*, and *Nitrospira* were observed. Peptides from sulfate/metal reducing, thermophilic, and alkaliphilic bacterial groups similar to those from genera such as *Geobacter*, *Alkaliphilus*, *Desulfotomaculum*, *Desulfovibrio*, *Desulfotalea*, *Shewanella*, *Alcaligenes*, *Geobacillus*, and *Thermus* were frequently observed in all three planktonic samples. Proteomic analyses also revealed proteins previously observed in different species of anaerobic and/or pathogenic bacteria belonging to genera such as *Ralstonia*, *Rickettsia*, *Porphyromonas*, and *Bacillus*. In addition to proteins from these bacteria, peptides from multi-drug-resistance proteins that are expressed in bacterial species such as *Streptomyces* and nitrogen-fixing bacteria such as *Corynebacterium* and *Frankia* were detected.

In conclusion, the direct detection and identification of peptides from pMMO proteins by mass spectrometry provides evidence of active MMO expression by methanotrophs within the TCE plume and outside of the TCE plume. This evidence supports previous findings using enzyme activity probes for MMO and microcosm experiments that intrinsic methane oxidation and TCE co-metabolism is actively occurring in the medial and distal zones of the TAN site (Sorenson et al. 2000; Wymore et al. 2007; Conrad et al. 2010). Our detection of enzymes that are definitive for a specific degradation process is critical for accepting natural attenuation as a long-term strategy for remediating an aquifer (Madsen 1991). The detection of pMMO and not sMMO is important for future studies involving enzyme kinetics and modeling of TCE co-metabolism at TAN and other sites. Detection of peptides from Mcr protein subunits in TAN-35 and TAN-29 provides evidence of a source of biogenic methane supporting previous stable carbon isotope analyses and PhyloChip community profile experiments (Conrad et al. 2010).

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