

**AWARDEE NAME:**  
**University of Idaho, Moscow**

**PROJECT OR AWARDEE ACCOUNT NO.:**  
**DE-FG02-06ER64198**

**PROJECT TITLE:**  
**Coupled Biogeochemical Process Evaluation for Conceptualizing Trichloroethylene  
Cometabolism**

**Project Principal Investigator:**  
**Ronald L. Crawford, University of Idaho**

**Final Progress Report: 02-28-10**

**Subproject title:** Proteomic and Genomic Analyses of Test Area North (TAN) Subsurface Microbial Communities for Evidence of Methane Monooxygenase (MMO)

**Overall Project Background:** Our goal within the overall project is to demonstrate the presence and abundance of methane monooxygenases (MMOs) enzymes and their genes within the microbial community of the INL TAN site. MMOs are thought to be the primary catalysts of natural attenuation of trichloroethylene (TCE) in contaminated groundwater at this location. The actual presence of the proteins making up MMO complexes would provide direct evidence for its participation in TCE degradation. The quantitative estimation of MMO genes and their translation products (sMMO and pMMO proteins) and the knowledge about kinetics and substrate specificity of MMOs will be used to develop mathematical models of the natural attenuation process in the TAN aquifer. The model will be particularly useful in prediction of TCE degradation rate in TAN and possibly in the other DOE sites.

Bacteria known as methanotrophs produce a set of proteins that assemble to form methane monooxygenase complexes (MMOs), enzymes that oxidize methane as their natural substrate, thereby providing a carbon and energy source for the organisms. MMOs are also capable of co-metabolically transforming chlorinated solvents like TCE into nontoxic end products such as CO<sub>2</sub> and Cl<sup>-</sup>. There are two known forms of methane monooxygenase, 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). All methanotrophs will produce a methanol dehydrogenase to channel the product of methane oxidation (methanol) into the central metabolite formaldehyde.

University of Idaho (UI) efforts are currently focusing on proteomic analyses using mass spectrometry and genomic analyses using RT-PCR to characterize these enzyme systems. UI's specific objectives are to develop the proteomics and genomic tools to assess the presence of the methane monooxygenase (MMO) proteins in the aquifers under study and

relate this to the enumeration of methanotrophic microorganisms. We are targeting the identification of both sMMO and pMMO. We believe that the copper level in the TAN aquifer is most likely suppressing the expression of sMMO and mediates the higher levels of pMMO expression. Hence our investigations include the identification of both forms of MMOs, and we are expecting higher concentration of pMMO proteins in TAN samples. The amounts of these proteins present are to be correlated with numbers of methanotrophs determined by us and other members of the research team using PCR-based methods.

#### **STATEMENT OF ACCOMPLISHMENTS RELATIVE TO ORIGINAL GOALS**

We feel we have accomplished without exception all of our original goals for the project year.

#### **CHANGES TO ORIGINAL PROJECT PLAN AND OBJECTIVES: NONE**

#### **ACTUAL OR ANTICIPATED PROBLEMS OR DELAYS: NONE**

#### **CHANGES OF KEY PERSONNEL: NONE**

#### **PUBLICATIONS, SYMPOSIA AND PROCEEDINGS PRODUCED**

Delwiche, M. E., D. T. Newby, A. Wood, M. Bingham, R. L. Crawford, and F. S. Colwell. The effect of trichloroethylene on minimum energy requirement and gene expression in a nutrient limited methanotroph. Joint Intl. Symp. for Subsurface Microbiology and Environmental Biogeochemistry, Jackson Hole, WY; Aug. 14-19-2005.

Paidisetti, R. K., J. L. Strap, F. Colwell, R. L. Crawford, and A. Paszczynski. Identification of proteins from *Methylosinus trichosporium* OB3b for methane oxidation using proteomic tools. INRA Subsurface Biotechnology and Bioremediation Symposium, Bozeman, MT; June 22, 2006.

Paszczynski, A. J. and R. Crawford. Detection of methanotrophic activity in INL Test Area North (TAN) aquifer using ecoproteomics and ecogenomics approaches. INRA 2007 Environmental Sensing Symposium, Boise, ID, October 25<sup>th</sup>-26<sup>th</sup>, 2007.

Benardini, J., A. Paszczynski, and R. Crawford. Monitoring natural attenuation utilizing ecoproteomics. INRA 2007 Environmental Sensing Symposium, Boise, ID; October 25<sup>th</sup>-26<sup>th</sup>, 2007.

Paidisetti, R., A. Johnson, R. Crawford, and A. Paszczynski. Using proteomics to sense natural attenuation in the INL Test Area North (TAN) aquifer. INRA 2007 Environmental Sensing Symposium, Boise, ID; October 25-26, 2007.

Colwell, F., M. Conrad, A. Paszczynski, E. Brodie, M. Delwiche, C. Radtke, M. Lee, R. Paidisetti, R. Crawford, N. Benardini, A. Johnson, R. Starr, D. Swift, D. Newby, and J. Barnes. Biogeochemistry of methane-driven destruction of trichloroethylene in a basalt

aquifer. American Geophysical Union fall meeting, San Francisco, CA; December 15-19, 2008.

Radtke, C., M. Lee, M. Delwiche, D. Newby, A. Paszczynski, R. Paidisetti, R. Crawford, N. Bernardini, A. Johnson, M. Conrad, E. Brodie, R. Starr, D. Swift, and R. Colwell. Coupled biogeochemical process evaluation for conceptualizing trichloroethene co-metabolism. 7<sup>th</sup> Intl. Symp. for Subsurface Microbiology, Shizuoka, Japan; November 16-20, 2008.

Paszczynski, A., R. Paidisetti, A. Johnson, R. L. Crawford, F. S. Colwell, T. Green, M. Delwiche, H. Lee, D. Newby, E. L. Brodie and M. Conrad. 2010. Proteomic and Genomic Analyses of Subsurface Microbial Communities for Presence of Methane Monooxygenase. To be submitted.

### **INVENTIONS, PATENTS OR OTHER PRODUCTS: NONE**

### **SUMMARY OF ACCOMPLISHMENTS**

**SUMMARY OF OVERALL PROJECT PROGRESS** (Andrzej Paszczynski, Ravindra Paidisetti, Andrew Johnson, Tonia Green and Ronald L Crawford of the University of Idaho; Frederick S Colwell, Oregon State University; Mark Delwiche, Deborah Newby and Corey Radtke, Idaho National Laboratory; Mark Conrad and Eoin Brodie, Lawrence Berkeley National Laboratory; and Hope Lee, Dana Swift and Bob Star, Northwind, Inc.; Compiled by Corey Radtke, Idaho National Laboratory, Idaho Falls, ID 83415 for the 2008 Annual ERSP PI Meeting).

The focus of this project is to determine the diverse biogeochemical processes involved in the microbial transformation of trichloroethene (TCE) in the Test Area North (TAN) contaminated aquifer at the Idaho National Lab. Our primary goals are to define the microbial processes involved in TCE co-metabolism and to quantify the contribution of these processes to TCE remediation within the medial zone of the TAN plume. Ultimately the studies are designed to provide multiple lines of evidence to determine whether or not co-metabolism via the methane monooxygenase pathway is contributing to natural attenuation of TCE within this zone of the plume. Flow-through *in situ* reactors (FTISRs) containing crushed basalt were incubated *in situ* for 238 days at TAN-35. After removal of the FTISRs, basalt and residual water collected from the reactors were immediately sampled and distributed for specific analyses. Aliquots of basalt were subjected to a comprehensive suite of analyses, including community proteomics, gene expression, metabolic activity, microbial community structure, and kinetics of TCE degradation. In addition to FTISR experiments, stable carbon isotope measurements and PhyloChip analyses were done on a set of groundwater samples collected from the source area of the TCE plume to the leading edge of the defined medial zone. The results to date provide a comprehensive and multi-tiered analysis of co-metabolic TCE remediation, and support the contribution of this process to the attenuation of TCE at this site. Incubation experiments performed with <sup>13</sup>C-TCE added basalt

and groundwater from the FTISRs indicate that the microorganisms in the FTISRs are capable of co-metabolically degrading approximately 7.5 µg of TCE per liter of groundwater. A novel time-course cell lysis method was developed for proteomics allowing increased peptide coverage resulting in a greater number of total proteins being detected. This demonstrated the presence and activity of aerobic methanotrophs in the medial zone. Confirmation of methanotrophic activity was achieved through combined use of targeted enzyme biochemical probes and fluorescent *in situ* hybridization (FISH) which consistently identified methanotrophs and active sMMO enzyme both in the planktonic phase (groundwater) and biofilm (basalt) communities derived from the reactors. Real-time PCR identified ~3000 copies of mmoX per g of basalt; while RT-PCR confirmed that the mmoX subunit was being actively transcribed. Stable carbon isotope ratios of dissolved inorganic carbon (DIC) and dissolved methane indicated increased levels of methane oxidation with distance from the source area (-55% to -28% for methane; >8% to -13% for DIC) corresponding to increased dissolved oxygen concentrations. These data corresponded well with site geochemistry and microbial assessments for community composition (PhyloChip) and activity (FISH) that identified a gradient of methanogenic to methanotrophic populations within the contaminant plume. Currently, the FTISRs are being redesigned to allow *in situ* stable isotope tracer studies, intended to derive TCE degradation rates (and methane incorporation rates) as close to *in situ* conditions as possible. Re-installation of the FTISRs in the aquifer is scheduled for March 2008.

## **SUMMARY OF UNIVERSITY OF IDAHO (UI) PROJECT ACCOMPLISHMENTS** (Compiled by Ronald L. Crawford, University of Idaho, Moscow ID, 83844-1052)

### **Proteomic and Genomic Analyses of Subsurface Microbial Communities for Evidence of Methane Monooxygenases (MMOs) Presence** (Andrzej Paszczynski, Ravindra Paidiseti, Andrew Johnson, Tonia Green and Ronald L Crawford).

**Brief Summary of UI Progress:** Proteomics-based techniques for monitoring the contribution of biological processes to pollutant degradation in the environment are rapidly developing as reliable tools for improved management and understanding of environmental remediation. Recently, tandem mass spectrometry has been used to measure gene expression at the protein level to provide real-time information about interactions between pollutants and microbial communities in different environment niches. During the past year we applied environmental proteomics to monitor proteins that are involved in the co-metabolic degradation of trichloroethylene (TCE) in groundwater of the Test Area North (TAN) site on lands of the Idaho National Laboratory (INL) near Idaho Falls, ID. To acquire peptide sequences information we used an ultra performance chromatography (UPLC) system coupled with QToF Premiere nano-electrospray tandem quadrupole-time of flight mass spectrometer. Our goal was to identify signature peptides of methane monooxygenases (MMOs) within methanotrophic bacteria that are active in cometabolic degradation of TCE. We developed a new method for extracting total proteins from environmental planktonic and/or biofilm samples that involve a new time course cell lysis and protein extraction method in combination with chromatographic separation of peptide and tandem mass spectrometry sequencing. The techniques resulted in successful extraction and identification of MMO-based peptides from both pure cultures and TAN site samples.

## FULL TECHNICAL REPORT

### BACKGROUND

Proteomics is one of the fastest developing research areas contributing to the understanding of global protein distribution and, in particular, the dynamics of metabolic pathways both in pure bacterial cultures and complex biological systems. The latter contain numerous, different microbial cells with total cell numbers of given species ranging across multiple orders of magnitude. There are a number of new approaches to isolate and sequence proteins extracted directly from environmental samples<sup>15, 25</sup> without need for the intricate and time-consuming process of separation of the involved organisms or individual proteins produced by these organisms<sup>4</sup>. Particularly, the combination of mass spectrometry with electrospray ionization and ultra performance liquid chromatography has opened new analytical opportunities for the detection and characterization of biocatalysts involved in the degradation of environmental contaminants. The technological advancement of reaching an extremely low detection limit ( $10^{-15}$  M) in electrospray mass spectrometry (ES/MS-MS) and matrix assisted laser desorption mass spectrometry (MALDI), and the capability to sequence peptides using MS-MS data represent revolutionary breakthroughs in proteomics-based analyses of complex samples including natural microbial communities<sup>38</sup>.

Proteomics, unlike genomic techniques, provides direct and dynamic evidence of the incidence and distribution of a given protein within a natural community of microorganisms<sup>34</sup>, confirming the existence of given biological activity at the protein expression level. Although many genomic approaches including measuring levels of gene expression (mRNA) have been used successfully, recent studies show that proteomics information has more potential to reveal actual microbial involvement in chemical transformations in the environment<sup>37</sup>. Systems biology studies indicate that the differential expression of protein and formation of metabolites is directly dependent on cellular system conditions that are determined by physiological and environmental factors<sup>39</sup>. Moreover the characterization of native metaproteomes (the collective information about proteins from an environmental sample) may provide information that can be very useful in understanding, monitoring, and providing evidence that remediation has reached a bench mark level. Proteomics data together with geochemical information will help in the design of remediation strategies for a given pollutant at a given contamination site.<sup>8,39</sup>

Environmental pollutants in soil and water are a major problem worldwide because they cause many toxic, mutagenic, and carcinogenic threats to human and environment health<sup>39</sup>. One pollutant of such environmental concern is trichloroethylene (TCE), an anthropogenic compound that currently contaminates aquifers worldwide. TCE has been used as an additive in adhesives, paint removers, and typewriter correction fluids as well as being used in degreasing operations and radionuclide processing<sup>26</sup>. As results of these applications there are numerous TCE plumes at industrial and government facilities in the United States <http://www.osti.gov/energycitations/purl.cover.jsp?purl=/836439-DI67Yx/native/>.

Work discussed here focused on co-metabolic degradation of TCE by methanotrophic microorganisms in groundwater at the Test Area North (TAN) site on Idaho National Laboratory (INL) land near Idaho Falls, ID. The site contains large TCE plume (Figure 1). There has been growing interest in bioremediation of anthropogenic chemicals (including TCE) because it is cost effective and poses lower human exposure risk and causes minimal environmental disturbance<sup>15,26</sup>. Monitoring of the TCE plume at the TAN site indicates diminution of TCE through natural attenuation (NA) processes. However, confirmation that biodegradation of TCE contributes to NA at TAN requires proof of the presence of metabolically active methanotrophs. Methanotrophs are ubiquitous gram negative bacteria which produce a set of proteins called methane monooxygenases (MMOs). MMOs oxidize methane to methanol, thereby providing a carbon and energy source for the organisms<sup>30,40</sup>. MMOs are also capable of co-metabolically transforming chlorinated solvents like TCE into nontoxic end products such as CO<sub>2</sub> and Cl<sup>-</sup><sup>6</sup>.

Proteomic analysis requires knowledge of a targeted protein's component structure and amino acids sequence. The MMO amino acid sequences of several species have been determined and are in the Swissprot database. There are two known forms of methane monooxygenase, a membrane-bound particulate (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<sup>30</sup>. sMMO has been observed primarily in type II and type X methanotrophs<sup>24,24,30</sup>, with a few exceptions that include the identification of sMMO in the type I methanotroph *Methylomonas methanica*<sup>19</sup>. The expression of MMOs by methanotrophic bacteria is dependent upon the copper concentration in the environment. The expression of pMMO is promoted by a high copper-to-biomass ratio (~4 μM), whereas a low copper-to-biomass (0.8 μM) level results in the expression of sMMO<sup>5,24,32,40</sup>.

This report focuses on the detection of both particulate and soluble forms of MMO. Detection involved using tandem mass-spectrometric analyses of trypsinized whole-protein extract of both *M. trichosporium* OB3b (a pure laboratory culture) and field samples (planktonic and biofilm). Planktonic cells were concentrated from groundwater by ultrafiltration, while biofilms were grown on sterile basalt chips lowered into wells in the TCE-contaminated TAN aquifer. In order to obtain reliable data we had to develop a new cells lysis procedure. Application of our method resulted in an increased number of proteins identified in each sample and also in an increased percentage of peptide coverage of identified proteins. Using this method we have been able to extract and identify MMO signature peptides. We correlated the amounts of protein extracted with the numbers of methanotrophs present as determined using PCR-based methods. We enumerated methanotrophic cells in water samples collected from TAN wells using quantitative PCR (qPCR). We used DNA from known numbers of *Methylosinus trichosporium* OB3b cells to generate standard curves using the cycle threshold (CT) method. These curves were used to convert qPCR data into numbers of methanotroph cells in TAN samples.

An array of experimental approaches were utilized to understand the biogeochemical processes driving TCE co-metabolism at TAN including methanotroph enumeration by quantitative polymerase chain reaction (qPCR) and direct detection of MMO proteins using a shotgun proteomics approach which are both described in this study. Aerobic methanotrophs

were enumerated by qPCR using primers targeting conserved regions of the genes necessary for particulate (pMMO) and soluble (sMMO) MMO expression, *pmoA* and *mmoX*, respectively, as well as the gene for the downstream enzyme methanol dehydrogenase (*mxh*). Identification of proteins in extracts from planktonic and biofilm samples from TAN was determined using 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. The detection of MMO in samples from TAN provides direct evidence of the catalytic potential of the indigenous microbial community to transform TCE by co-metabolism. The complexity of environmental protein extracts used for the proteomics experiments was further reduced by fractionating proteins using a recently developed lysis protocol. Using this method, MMO proteins were found to be abundant in samples collected from wells within and adjacent to the TCE plume at TAN.

## MATERIALS AND METHODS

***Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath cultures:** Cultures were grown in nitrogen supplemented mineral salt media (NMS) at 30 °C with shaking at 200 rpm. NMS media contained 1g of MgSO<sub>4</sub> x 7H<sub>2</sub>O, 1 g of KNO<sub>3</sub>, 0.717 g of Na<sub>2</sub>HPO<sub>4</sub> x 12H<sub>2</sub>O, 0.272 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of CaCl<sub>2</sub> x 6H<sub>2</sub>O, 1 ml solution containing 2 mg of ferric ammonium citrate and 2 mg of disodium EDTA and 0.5 mL of trace element solution in a final volume of 1 liter of deionized water, pH 6.8. The trace element solution was prepared by dissolving 0.5 g of disodium EDTA, 0.2 g of FeSO<sub>4</sub> x 7H<sub>2</sub>O, 0.03 g of H<sub>3</sub>BO<sub>3</sub>, 0.02 g of CoCl<sub>2</sub> x 6H<sub>2</sub>O, 0.01 g of ZnSO<sub>4</sub> x 7H<sub>2</sub>O, 3 mg of MnCl<sub>2</sub> x 4H<sub>2</sub>O, 3 mg of Na<sub>2</sub>MoO<sub>4</sub> x 2H<sub>2</sub>O, 2 mg of NiCl<sub>2</sub> x 6H<sub>2</sub>O and 1 mg CaCl<sub>2</sub> x 2H<sub>2</sub>O in 1 L of deionized water<sup>2</sup>. The media were sterilized by autoclaving at 121° C for 30 min. Liquid cultures (50 mL) were grown in 150 mL serum bottles with a headspace filled with sterile methane and air 3:7 (V/V). Every two days the headspace was flushed with fresh gas mixture for 2 minutes. 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:** Water samples from 4 wells (TAN-29, TAN- 35, TAN-36 and TAN-58) located within different zones of the TAN (Figure 1) site were collected and cells were concentrated using a KrosFlo hollow fiber ultra filtration system (hollow fiber 0.05-µm pore size, Cole-Parmer, Vernon Hills, IL). The filtrate was further concentrated by centrifugation at 5,500 g for 30 min and the collected cells suspended in TAN water were stored at -80 °C prior to proteomic analysis. Water from TAN wells was sampled twice; the initial sampling was only from TAN-29 and TAN-35 and was performed during May 2006. The second sampling included water samples from TAN-29, TAN-35, TAN-36, and TAN-58 and was performed during June 2007. Typically for a proteomic experiment 500 to 600 L of TAN well water was concentrated into a final volume of 15-20 ml. Biomass contained in a volume of 1 to 0.1 ml was used for protein extraction and trypsin digestion. Each digest

produced 20  $\mu$ L of peptide solution; 2  $\mu$ L of this solution was used for a particular proteomic analysis. Each sample was analyzed at least three times.

**Basalt biofilms:** Basalt chips (basalt substrate) of size 4 mm to 8 mm were selected and washed thoroughly with deionized water at least ten times. The washed basalt was sterilized by autoclaving a minimum of 5 times with an interval of 24 h between cycles. The basalt substrate was placed into pre-sterilized, perforated polypropylene columns (1 ft in length, InterNet Inc., Anoka, MN) which were sealed at the ends. The substrate columns were placed into wells TAN-29, TAN-35 and TAN-58 and left for 6 to 8 months. Upon retrieval, contents of columns were emptied into sterile plastic boxes and stored at  $-80^{\circ}\text{C}$  until further genomic and proteomic analysis.

**Enumeration of bacteria:** 1 mL of an *M. trichosporium* OB3b cell culture or a TAN planktonic cell sample was collected and 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 hour on a rocking platform shaker prior to staining. Next, cells were washed with PBS and resuspended in 1mg/mL of acridine orange solution<sup>17</sup>. After incubation at room temperature for 3 min, cells were transferred to 0.22  $\mu$ m black polycarbonate filters (25mm in diameter, GE Water & Process Technologies, Minnetonka, MN) and fluorescing cells were counted using an Olympus DP 70 microscope (Figure 2); 1mL of *M. trichosporium* OB3b cell culture was also collected for optical density reading at 600nm using an Agilent (Santa Clara, CA) 8453 UV-VIS diode array spectrophotometer.

**Extraction of protein from planktonic samples (time-course lysis; Figure3):** Cells from planktonic or pure cultures suspended in 20% formic acid were incubated in a boiling water bath for 30 minutes. Every 3 minutes the sample was removed from water bath, centrifuged and supernatant transferred to a new tube. The pellet was resuspended in fresh 20% formic acid and incubated in the boiling water bath again for 3 min. The procedure was repeated until the overall lysis time reached 30 min. The supernatant from each time point was dried (DNA Speed Vac, Savant, Ramsey, MN). After solubilization in 100 mM ammonium bicarbonate buffer (pH 8), disulfide bridges (-S-S-) were reduced by incubating with 10 mM dithiothreitol (DTT) at  $50^{\circ}\text{C}$  for 15 min. Subsequently, the sulfhydryl groups (-SH) were alkylated by adding 10 mM iodoacetamide (IAM) and incubated in the dark at room temperature for 15 min. Finally, trypsin was added (trypsin: protein ratio 1:50 W/W) and the sample incubated at  $37^{\circ}\text{C}$  for 15 h. Digested protein samples were dried again in the Speed Vac. The dry samples were reconstituted in 30  $\mu$ L of  $\text{H}_2\text{O}$  containing 5% acetonitrile and 0.1% formic acid. Immediately prior to MS analysis, samples were clarified by centrifugation at 10,000 g for 10 min.

**Protein extraction from basalt biofilms:** Fifteen grams of basalt rock covered with biofilm were submerged in 10 mL of 1% triton-X100. The samples were sonicated for 10 min followed by incubation in a boiling water bath for another 30 min. The supernatants were collected by centrifugation at 1000 x 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, suspended in 20% formic acid, and the hydrolysis procedure was followed as described for the planktonic samples. Washing samples with acetone was necessary to remove triton detergent that is not compatible with ES/MS analyses.

**Analysis of peptides:** A Waters Nanoaquity ultra performance LC (UPLC) was used to separate peptides in sample hydrolysates prior to delivery to a QToF Premiere MS/MS. Trypsin-digested sample (2  $\mu\text{L}$ ) was loaded into a fused silica capillary column (Waters Atlantis Nanoease: dC18, 3  $\mu\text{m}$ ; 75  $\mu\text{m}$  x 100 mm) in tandem with a trap column (Waters Symmetry: C18, 5  $\mu\text{m}$ ; 180  $\mu\text{m}$  x 20 mm). Peptides were first trapped using 100% solution A (0.1% formic acid in  $\text{H}_2\text{O}$ ) at a flow rate of 5  $\mu\text{L min}^{-1}$  for 3 min. Trapped peptides were then eluted and separated on the dC18 column using the following steps at a flow rate of 0.25  $\mu\text{L min}^{-1}$ : (1) isocratic 97% A and 3% B (0.1% formic acid in acetonitrile) for 1 min. ; (2) gradient 60% of A and 40% B for 90 min. ; (3) gradient 10% A and 90% B for 10 min. ; (4) isocratic 10% A and 90% B for 10 min. ; and (5) 97% A and 3% B for 1 min. The column effluent was delivered directly to the QToF Premiere nanospray source. A potential of 3.75 kV was applied to the emitter capillary, 35 volts to the sampling cone, 4.5 volts to the extraction cone; 1.9 KV potential was used on the MCP detector. The MS and UPLC were controlled by MassLynx 4.1 software (Waters). The source temperature was 80  $^{\circ}\text{C}$  and desolvation temperature (time of flight (ToF) was 180  $^{\circ}\text{C}$ . Nanoflow gas pressure was 0.9 Bar and desolvation gas flow was 600 L/Hr. Mass spectra were collected in the positive ion mode. Survey scans used a 400-2000 Daltons mass range in continuum mode and up to three peptides with charges of +2, +3, and +4 were sequenced (MS/MS) in a given time window using a 50-2000 Dalton mass window. Low mass resolution was set at 5,000 and high mass resolution at 15,000.

**Mass spectrometric data analysis:** We followed the Molecular and Cellular Proteomics Editorial Board guidance for analysis and identification of peptides and proteins. The guidelines for the analysis and documentation of peptide and protein identification were obtained from the Molecular and Cellular Proteomics journal website (MCP web site <http://www.mcponline.org>). ProteinLynx Global Server 2.2.5 (PLGS) and Protein Expression Informatics System software version 2.2 were used for mass spectra analysis, peptide sequencing and protein identification. This software uses both probability scoring (protein identification by peptide mass fingerprinting; <http://www.waters.com>) and a cross-correlation algorithm for protein identification by comparison of MS/MS data with database protein sequences (<http://www.thermo.com>). The raw data were converted into peak lists (\*.pkl files) by PLGS using the following parameters: (a) smooth channels = 4, number of smooths = 2, smooth mode = Savitzky Golay, (b) percentage of peak height to calculate the centroid spectra = 80%, and (c) no baseline subtract was allowed. Identification of proteins and peptides was performed by searching the MS spectra against the Swissprot, Non redundant (nr) and MMO (downloaded from NCBI website <ftp://ftp.ncbi.nih.gov>) databases. To verify data obtained with PLGS, Mascot software (<http://www.matrixscience.com>) that uses probability scoring for mass fingerprinting and the Molecular Weight Search (MOWSE) for additional MS/MS database searches was used. We used MSDB or TREMBL protein amino acid sequences obtained from Matrix Science Inc., Boston, MA. Parameter settings for the Mascot searches were: (a) trypsin as the specific enzyme; (b) peptide window tolerance (error window on experimental peptide mass values)  $\pm 2$  Da; and (c) fragment mass tolerance

of 0.8 Da. During searches, “carbamidomethyl C” modification was the only amino acid modification allowed. If needed, Mass-Lynx’s 4.0 protein sequencing tool for manual confirmation of peptide sequences was used. To generate centroid singly charged MS/MS spectra suitable for manual amino acid sequence analysis we used the Max-Ent 3 algorithm with the following parameters: (i) background subtract polynomial order 2, below curve 40%; (ii) smooth, peak width 1.00, number of smooths = 2, smooth mode = Savitzky Golay; and (iii) percentage of peak height for calculation of centroid masses, 80.

#### **DNA extractions for qPCR:**

**a) Pure cultures:** 1 mL of OB3b cell culture was centrifuged and cells were resuspended in 1 mL TE buffer. Serial dilutions were made from  $10^0$  to  $10^{-5}$ , cells were centrifuged again, and pellets were resuspended in 50 uL of 1% triton-X 100. Cells were lysed by boiling for 10 min and cooled quickly on ice. Aliquots from these preparations were used in preparing standard curves for qPCR.

**b) Planktonic and TAN samples:** Concentrated planktonic samples were initially centrifuged at 1,000 rpm to remove debris. Cells were collected by centrifuging at 14,000 rpm for 5 minutes and resuspended in 0.5mL 1% triton-X 100. Cells were boiled for 20 minutes and quick-cooled on ice. DNA in the samples was further purified using phenol/chloroform extraction, and ethanol precipitated. Finally, samples were desalted using MicroSpin Sephadex G-25 columns (GE Healthcare, Buckinghamshire, UK).

**c) Basalt biofilms:** Fifteen grams of basalt rock from substrate columns was submerged in 25 ml of 1% triton-X 100 and boiled for 20 minutes. Cell debris from the samples was removed by centrifuging for 20 minutes at 5,500 x g. Basalt was rinsed once with an additional 5 mL of 1% triton-X 100 and centrifuged again as before. Supernatants were combined, frozen, 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. The samples were run through MicroSpin G-25 columns clean up step using TE buffer.

**Quantitative PCR (qPCR) assays:** A series of quantitative PCR assays were performed using genomic DNA extracted from TAN planktonic and basalt biofilm samples to enumerate the methanotrophic bacteria. The DNA extracted from samples was amplified with the primers specific to particulate and soluble methane monooxygenase genes. The following primers were used for the amplification of pMMO, 189f (5' - GGNGACTGGGACTTCTGG-3') and mb661r (5' -CCGGMGCAACGTCYTTACC-3')<sup>13,28</sup>. The mmoX portion of sMMO was amplified using primer pair 536f (5' -CGCTGTGGAAGGGCATGAAGCG-3') and 898r (5' - GCTCGACCTTGAACCTGGAGCC-3')<sup>28, 10</sup>. Amplification of methanol dehydrogenase was performed using the primer pair *mx*a f1103 (5' -GCGGCACCAACTGGGGCTGGT-3') and *mx*a r1561 (5' -GGGCAGCATGAAGGGCTCCC-3')<sup>31</sup>. For all PCR reactions the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA) was used. Data analysis was performed using StepOne Plus software. The cycle at which the fluorescence of a certain target molecule number exceeded the background fluorescence (threshold cycle -  $C_T$ ) was determined from the dilution series of target DNA of pure cultures containing defined amounts of target DNA sequences. The  $C_T$  values are reverse proportion to the logarithm of

the target molecule numbers. Each measurement was performed in triplicate. PCR amplifications were performed in total volumes of 25  $\mu\text{L}$  consisting of 12.5  $\mu\text{L}$  of Power SYBR green master mix (Applied Biosystems), 300 nM of each primer, and 1  $\mu\text{L}$  of DNA standard or sample. Following the initial denaturation step at 95 $^{\circ}\text{C}$  for 10 minutes, all PCR amplifications were performed using 40 cycles of 95 $^{\circ}\text{C}$  for 1 minute, 55-60 $^{\circ}\text{C}$  for 1 minute, and 72 $^{\circ}\text{C}$  for 1 minute. Fluorescence readings were taken during the annealing step at the 55-60 $^{\circ}\text{C}$  incubation. Melt curve analysis was performed following every run to confirm product specificity. qPCR data analysis was conducted as described elsewhere<sup>33,41</sup>.

## RESULTS

**Enumeration of bacteria:** The number of cells in each sample was enumerated using an acridine orange epifluorescence technique for counting bacteria<sup>9</sup>. Although 0.05  $\mu\text{M}$  filters were used for concentrating TAN water samples, we used 0.22  $\mu\text{M}$  filters for the enumeration due to the non availability of 0.05 micron filters compatible for fluorescence-based counting methods. The cell numbers (cells/mL) determined for concentrated samples were  $6 \times 10^8$ ,  $3.6 \times 10^7$ ,  $3.2 \times 10^5$ ,  $8.7 \times 10^5$  and  $6.5 \times 10^5$  for TAN-29\_2006, TAN-29\_2007, TAN-35, TAN-36 and TAN-58, respectively (Figure 2).

**Identification of proteins from environmental samples:** Initially the extractions of total proteins from pure cultures and planktonic samples were carried out by treating the samples with 20% formic acid for 30 min in a boiling water bath. The lysed cell supernatants were dried by evaporation and digested through an in-solution digestion method<sup>36</sup>. Samples were reduced by treatment with dithiothreitol (DTT) followed by alkylation using iodoacetamide (IAM). Digestion of protein samples was carried out by treating the alkylated samples with trypsin. Prior to mass spectrometric analyses, the peptide samples were chromatographically separated by UPLC. To obtain MS/MS amino acid sequences data, we used Mass Lynx in data dependent acquisition (DDA) mode. Protein sequence data obtained from mass spectral analysis was searched against the known protein sequences available in the MSDB and Swissprot sequence databases. Protein identification was achieved using probability match and the individual peptide scores obtained by MASCOT and PLGS search engines. Although peptide sequence-database search analysis of pure cultures identified peptides from subunits of the methane monooxygenase complex of proteins, the samples were found to be biased toward the fragments of the most abundant proteins known to be found in the cells. Analysis of the *M. trichosporium* OB3b grown without copper showed peptides from most of the subunits of sMMO, but the number of peptides obtained was low and the results were not consistent between runs (results not shown). Similarly, only peptides belonging to PmoB were observed from the *M. trichosporium* OB3b cultures grown with copper; peptides from PmoA and PmoC were not detected in these samples, due perhaps to the intrinsic association of these subunits (PmoA and PmoC) with the bacterial membrane<sup>7</sup>. In another experiment, a mixed culture of *M. trichosporium* OB3b and *E. coli* was hydrolyzed. The analysis of this lysate indicated that sequencing results were completely biased towards proteins belonging to *E. coli*, masking the majority of *M. trichosporium* OB3b proteins. Assuming that environmental samples will contain cells with a wide range of resistance to lysis, this problem was solved by implementing a time-course lysis procedure that allowed for the

separation of proteins from microorganisms having different susceptibility to lysis by formic acid.

**Time-course lysis:** The findings described above led to the development of a time-course lysis procedure to maximize extraction of proteins from environmental microbial communities. As shown in Figure 3, samples were hydrolyzed by adding formic acid and boiling for 3 min intervals at 100° C. Supernatants from each time point were used for trypsin digestion and ESI-MS/MS analysis. Table 1 lists all the peptides related to methane assimilation identified from the hydrolysis of *M. trichosporium* OB3b grown in media containing 12  $\mu$ M copper. The use of the time-course lysis method enabled the identification of peptide sequences from all the subunits of pMMO. Peptides corresponding to nearly 61% of amino acid sequence of the PmoB subunit were observed. Along with PmoB, peptides from subunits PmoA and PmoC were observed with sequence coverage at 10% and 8%, respectively. Similarly, peptide sequences from all the subunits of soluble methane monooxygenase were identified from the analysis of pure *M. trichosporium* OB3b cultures grown in the absence of copper. All identified peptides belonging to sMMO complex proteins are listed in Table 2. Approximately 60% of the  $\alpha$ -subunit of sMMO was detected in the peptide populations. Peptide sequences identified from other subunits of sMMO such as the  $\beta$ ,  $\gamma$ , regulatory proteins, and the reductase protein were also observed (Table 2). In addition to subunits of pMMO and sMMO complex proteins, Table 1 and Table 2 list the identified peptide sequences of methanol dehydrogenase and formaldehyde dehydrogenase, the enzymes involved in further methanol and formaldehyde oxidation. During our analysis, peptide masses ranged from 407.72 Da ( $\pm$  0.8 Da) to 1600.28 Da ( $\pm$  0.8 Da).

The application of time-course lysis to planktonic TAN samples identified peptide sequences corresponding to pMMO. Tables 3A and 3B list the peptide sequences detected in planktonic samples obtained from the TAN-29 well sampled during 2006 and 2007, respectively. Table 3A shows amino acid sequences of peptides related to the PmoA and PmoB subunits from various methanotrophs. 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 3B). Peptides associated with formaldehyde metabolism were identified during the TAN-29 sample collected during 2006. These proteins were not identified from the TAN-29\_2007 sample. No peptides from the subunits of sMMO complex were observed during the analysis of either of the TAN-29 samples suggesting that these organisms, if present, are below detection limit. Our proteomics results indicated that detection limits for MMOs peptides was  $10^3$  cells in pure culture and  $10^4$  in natural planktonic samples. During the analysis of environmentally-derived planktonic samples it was observed that the number of peptide sequences identified was smaller than observed with pure cultures; however, when compared to results from single time point hydrolysis, we observed more peptide sequences and also a greater number of proteins were represented. Our results indicated that only two pMMO peptides (RVSFLNAGEPGPVLVRT, and RLADLIYDPDSRF) were identified throughout the time-course. The peptide sequences of the methanotrophic bacteria that belong to methanotrophic proteins involved in other metabolic pathways are listed in Tables 3A and B. Peptides from other structural and functional proteins such as hypothetical proteins, chaperonins, and

transcriptional regulatory proteins, trans-membrane proteins, and DNA repair proteins were also observed.

Similar analysis of microbial planktonic samples from TAN-36 (Table 4) and TAN-58 (Table 5) showed peptides from methanotrophic proteins. Although peptides from only the PmoB subunit were identified in the TAN-36 (distal zone of the TCE plume), peptides from PmoB and PmoA subunits were identified in TAN-58 (outside the distal zone of the TCE plume). Similar to TAN-29 (medial zone of the TCE plume), proteins from non-methane metabolic pathways were identified in both TAN-36 and TAN-58 samples. These results indicate that methanotrophs are active across the whole TAN aquifer suggesting that methane is an important substrate for microbes inhabiting TAN groundwaters. Proteins that were identified as being from different microorganisms associated with biodegradation, drug resistance, geomicrobial and pathogenic processes observed in TAN-29, 35, 36 and 58 samples are listed in Tables 6, 7, 8 and 9. Most of the bacterial protein sources observed were common among all four well samples with exception of TAN-35. The large portion of identified proteins in TAN-35 well belong to known and uncultured methanogenic species supporting biogenic methane production in TAN water. Another process that could also be occurring is anaerobic oxidation of methane by a process which can be thought of as reverse methanogenesis coupled with sulfate and/or nitrate reduction. The protein implicated in the initiation of reverse methanogenesis, or anaerobic methanotrophy, is highly similar to the methylcoenzyme M reductase (McrA) protein involved in the final assembly step of methane<sup>21,35</sup>. This could be occurring in TAN-35 well because of low oxygen concentrations and the identification of sulfate reducing proteins (Tables 9 and 11). The majority of the bacterial markers in TAN samples resemble sequences previously described in the *proteobacteria* group. Peptides indicative of bacteria from different genera, including *Pseudomonas*, *Burkholderia*, *Nitrosococcus* and *Nitrospira* were frequently observed. Peptides from sulfate/metal reducing, thermophilic and alkaliphilic bacteria similar to those from genera such as *Geobacter*, *Alkaliphilus*, *Desulfotomaculum*, *Desulfovibrio*, *Desulfotalea*, *Shewanella*, *Alcaligenes*, *Geobacillus*, and *Thermus* were frequently observed in all three planktonic biofilm samples. The 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 known to be expressed in bacterial species such as *Streptomyces* and nitrogen-fixing bacteria such as *Corynebacterium* and *Frankia* were detected. Proteins from uncultured organisms that are thought to be capable of oxidation of pentachlorophenol (PCP) and sulfur reducing were also detected.

**Quantization of methanotrophs in environmental samples:** Quantitative PCR (qPCR) and proteomic methods were used to enumerate methanotrophs in the environmentally-derived planktonic and basalt biofilm samples. Quantitative PCR assays were developed to detect and enumerate the number of methanotrophic bacteria in different well samples from the TAN site. The assays employed primers for conserved sequence portions of pMMO and sMMO. For each sample, the experimental conditions including primer concentrations and annealing temperatures were optimized to reduce non-specific amplification. Optimized conditions were tested using genomic DNA extracted from pure cultures of *M. trichosporium* OB3b as a positive control and no DNA template as a negative control. A standard curve for qPCR was

developed from at least triplicate measurements of a dilution series ( $10^6$  to  $10^2$  cells) of positive-control DNA extracted from *M. trichosporium* OB3b. The Ct (cycle threshold) values from the TAN samples were compared to the Ct values of *M. trichosporium* OB3b standards to obtain the numbers of methanotrophic microorganisms. The samples with more gene copies amplifiable by the MMO primers used in the assay produced an amplicon detectable at earlier cycle, and hence, have small Ct values, and *vice versa*. Results from qPCR using pMMO specific primers showed the TAN-29\_2006 sample to have a higher number of methanotrophic bacteria. In contrast, a sample collected from the same well after one year (TAN-29\_2007) exhibited higher numbers for cycle Ct values suggesting wide fluctuation of methanotroph numbers in this well. The size of PCR products from these samples was confirmed by electrophoresis on 1% agarose gels (Figure 4). The qPCR assays estimated a minimum of  $5.25 \times 10^6$  cells/mL of methanotrophic bacteria out of total  $6 \times 10^8$  cells/mL of total bacteria (acridine orange direct counts, AODC) present in TAN-29\_2006 sample using pMMO specific primers (Figure 5A). Similarly, the total cell numbers (determined by AODC) from TAN-29\_2007 ( $3.6 \times 10^7$  cells/mL), TAN-36 ( $8.7 \times 10^5$  cells/mL) and TAN-58 ( $6.5 \times 10^6$  cells/mL) well were compared to methanotrophs numbers using qPCR giving  $3.2 \times 10^5$  methanotrophs/mL,  $1.1 \times 10^4$  methanotrophs/mL, and  $2.5 \times 10^4$  methanotrophs/mL, respectively. Methanotrophic bacterial numbers from basalt biofilms were also determined using pMMO specific primers in qPCR assays. TAN-35 basalt biofilm contained 15 cells of methanotrophic bacteria per 1g of basalt chips. Samples from UP1 contained of  $8.5 \times 10^3$  methanotrophs per 1 g of basalt chips. Quantitative PCR assays using soluble methane monooxygenase primers were performed in TAN-29\_2006 and TAN-35 only (Figure 5B). These assays showed as low as 55 cells/mL of methanotrophs in TAN-29\_2006 and nearly  $3 \times 10^3$  methanotrophs/g in the TAN-35 basalt biofilm sample.

In another approach, methanotrophic bacteria were quantified using the intensity of pMMO peptide sequences' MS signals observed in both pure and environmental samples during mass spectrometric analysis (Figure 6). Dilutions of *M. trichosporium* OB3b cells ( $10^2$  to  $10^5$ ) were prepared and time-course lysis performed. The mass spectral peak intensities of a peptide (observed throughout the time-course of both pure cultures and TAN samples) were added 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 plotted on a graph to estimate the number of methanotrophic bacteria. In the present study, peptide R.VSFLNAGEPGPVLVR.T (belonging to the PmoB subunit) was found throughout the entire time-course of *M. trichosporium* pure cultures and all three TAN planktonic samples. The cumulative intensity of this peptide was used to estimate the number of methanotrophic bacteria in each sample. This approach allowed us to estimate  $3.9 \times 10^4$  methanotrophs/mL (TAN-29\_2006),  $2.5 \times 10^4$  methanotrophs/mL (TAN-29\_2007),  $9.4 \times 10^3$  methanotrophs/mL (TAN-58) and  $3.7 \times 10^3$  methanotrophs/mL (TAN-36) in the various samples. These values were found to be smaller than the numbers estimated by qPCR using pMMO specific primers, reflecting lower detection limits for qPCR than our proteomics analyses (10 cells versus 1000 cells in pure culture) As the time-course lysis method is still being optimized for TAN basalt biofilm samples, this approach could not be used universally to estimate the number of methanotrophs.

## DISCUSSION

The focus of this study was the extraction, identification, and quantification of peptides derived from methane monooxygenase proteins from planktonic bacteria and basalt biofilm samples from a TCE-contaminated aquifer. A high-throughput protein identification method using mass spectrometry was used to categorize the proteins from these samples. The samples used in the study were collected from contaminated groundwater wells of the Test Area North (TAN) site of the Idaho National Laboratory near Idaho Falls, ID (USA) and from a pristine well outside the TAN site located at the University of Idaho campus in Idaho Falls, ID (USA).

The TCE plume at the TAN site is divided into 3 zones based on historical concentrations of TCE; hotspot (>20 mg/L); medial zone (1- 20 mg/L); and distal zone (<1 mg/L). Four wells were chosen to perform proteomic analyses and evaluate the metabolic state of the microorganisms present in zones of different concentrations of TCE and methane. Wells TAN-29 and 35 are located in the medial zone and TAN-36 and 58 are located in the distal zone and outside the distal zone, respectively (Figure 1). During the time of sampling the concentrations of TCE and methane in the TAN medial zone wells ranged from 263-407 µg/L and 126-2500 µg/L, respectively (Table 11). Results from previous investigations suggested that cost effective remediation of these sites can be achieved through natural attenuation. The USEPA has defined natural attenuation as a remediation approach involving physical, chemical, or biological processes, which under favorable conditions can without intervention of humans lead to the reduction of mass, toxicity, mobility, volume or concentration of contaminants in soil and/or groundwater. The reduction takes place as a result of processes such as biodegradation, dispersion, dilution, sorption, volatilization, radioactive decay, transformation, and chemical or biological stabilization (EPA, OSWER Directive 9200.4-17P). Investigators have reported degradation of chemical contaminants including chlorinated compounds through co-metabolic processes which are due to non-specific enzymatic activities of microorganisms<sup>12,22,29,46</sup>. However, the suggestion that co-metabolic degradation of TCE occurs at TAN requires experimental lines of supporting evidence.

Proteomic analysis of a pure culture of the methanotroph strain OB3b indicated that the largest number of peptide sequences identified were always associated with proteins of methane assimilation. This was not a surprise because methane catabolism is a major source of carbon and energy for this microbe. The tryptic digestion of whole proteins from *M. trichosporium* OB3b cultures grown in the presence of copper resulted in peptide sequences of the PmoB subunit of particulate methane monooxygenase being detectable after 3 min of lysis, and they continued to be detectable until the 30 min time point. Peptide sequences such as KTGTPVRDPEYGTR were detected at the 9 min time point and were not detected after the 21 min time point; whereas other peptide sequences such as RAPVGVPEQEVHKW were detected at the 12 min time point and then throughout the rest of the time-course lysis. Some peptides such as RQWLIEPERY appeared at only at a single time point during the whole course of lysis. The number of peptide sequences corresponding to an individual protein was found to increase during the time-course, thereby increasing peptide percentage of coverage. Although the PmoB subunit was detectable at early time points of hydrolysis, the other subunits of particulate methane monooxygenase, PmoA and PmoC, were detectable

only in later time points (6 and 18 min, respectively) presumably indicating their closer association with bacterial membranes. Peptide sequences belonging to subunits of sMMO also were detected after 6 min, indicating that the cell membranes of *M. trichosporium* OB3b were moderately resistant to acid hydrolysis.

In order to provide such evidence of co-metabolic degradation, an ecoproteomics study was undertaken. The planktonic TAN samples were obtained by filtering and concentrating biomass from the contaminated groundwaters taken from wells TAN-29, 35, 36 and 58 (Figure 1). Microscopic examination of samples from these wells found some microbial cells were less than 0.22 micron in size; hence, we used 0.05  $\mu$ M filters for concentrating the planktonic cell samples from TAN water. The biofilm samples were grown on pre-sterilized basalt chips contained in nylon mesh columns. Cell numbers within well water were estimated using acridine orange DNA staining and microscopic counts of the fluorescent cells (Table 10, Figure 2). The use of a 0.22 micron size filter instead of a 0.05 micron size filter (which was unavailable) during acridine orange direct counts (AODC) might have caused loss of some kinds of cells. Numbers of microorganisms fluctuated widely in TAN well water. Samples of TAN-35, 36 and 58 wells were found to have 2 to 3 orders of magnitude fewer cells as compared to TAN-29. Also cell numbers from original groundwater collected from TAN-29 during 2007 were 10 times lower than a year earlier.

Methanotrophic cell numbers from these samples were estimated using qPCR (Table 10, Figure 4). The results from these assays in which pMMO specific primers were used indicated that in all samples except TAN-58 approximately 1% (0.9 to 1.3 %) of the total bacteria were methanotrophs. In the TAN-58 sample, the number of methanotrophic bacteria was 3.8% of the total bacterial cell numbers. The number of methanotrophic bacteria calculated from TAN-29\_2007 sample was found to be equivalent to the sample (TAN-29\_2006) taken one year earlier from the same site (Table 10). Although numbers of methanotrophs were also estimated from basalt biofilm samples using qPCR assays, we were unable to estimate the percentage of methanotrophs among the total number of bacteria. This is because cell number determinations from basalt biofilm required removing all cells from the basalt chips, which is virtually impossible. Results from qPCR assays performed using soluble MMO specific primers indicated that the number of type II methanotrophic bacteria in TAN-29\_2006 was too low to count. We could not identify any sMMO peptides during proteomic analysis as well. qPCR results from amplification of DNA from the TAN-35 sample using sMMO specific primers showed a significant number of possible sMMO containing species; although no peptides from sMMO proteins were identified using proteomic tools. It appears the conditions for protein extraction from basalt biofilms must be further optimized to isolate these peptides if these proteins are indeed being expressed.

We also have taken a proteomics approach to estimate numbers of methanotrophs in our samples. We compared the intensity of a peptide (RVSFLNAGEPGPVLVRT) detected in the environmental samples to the intensity of the same peptide from serial dilutions of a pure *M. trichosporium* OB3b culture. This approach allowed us to quantify metabolically active *M. trichosporium* OB3b-like bacteria based on the intensity of the MMO peptide signal obtained during mass spectrometric analysis. Numbers of methanotrophic cells obtained through this method were found to be one to two orders of magnitude smaller as compared to numbers

obtained from qPCR assays. This discrepancy can be due to the differences in sensitivity of these methods (qPCR-10 cells, MS/MS-1,000 cells) as well as specificity of the primers used for qPCR, as the peptide-peak intensity method estimates the metabolically active methanotrophs only, whereas the qPCR can estimate every cell with a gene copy specific to the primers used.

The focus of the present study was identification of biomarkers (signature peptides from either sMMO or pMMO) of methanotrophs inhabiting the TAN aquifer. A method was developed for extraction and analysis of total proteins from planktonic and basalt biofilm samples. Cells from pure cultures of *M. trichosporium* OB3b provided the necessary standards to develop and validate the approach. *M. trichosporium* OB3b cultures were grown in the presence of 12  $\mu\text{M}$  of copper to stimulate expression of the pMMO. Although 4  $\mu\text{M}$  of copper can induce the expression of pMMO<sup>24</sup>, a concentration of 12  $\mu\text{M}$  was used to help over express this enzyme. Although enzymatic lysis has been traditionally used to hydrolyze pure cultures, the specific activity of lytic enzymes on either gram positive or gram negative bacteria<sup>11</sup> is a major limitation for their application to environmental samples. However, we found chemical-based lysis to be effective in lysing cells from environmental samples because of its efficiency in breaking cell walls of most bacteria. In this method, boiling cells in 20% (v/v) formic acid was sufficient for lysis. The amount of protein released by this method was found to be much higher when compared to other methods such as sonication (data not shown). Formic-acid-based cell lysis was previously applied by Swatkoski et al.<sup>42</sup> for rapid proteomic identification of *Bacillus* spores. Their method was based on the selective release and chemical digestion of small acid-soluble spore proteins<sup>42</sup>. Since this method proved effective in extracting proteins from bacterial spores, it was chosen to extract proteins from microbial communities in the environmental samples of this study. Another advantage of using formic acid is its compatibility with most LC mobile phases. In dilute concentrations, formic acid can partially hydrolyze proteins at aspartic acid residues<sup>23,42</sup>. Since formic acid present in samples would interfere with further enzymatic digestion and downstream applications, it was removed by evaporation (Figure 3). The application of formic acid to the basalt biofilms samples most likely resulted in the release of metal ions and other chemicals from the basalt substrate. These nonvolatile chemicals might interfere with successive reduction, alkylation and trypsin digestion of released proteins. Hence for these samples we replaced formic acid with 1% Triton X-100 to extract proteins from basalt biofilms. Although this method was able to extract some proteins, further optimization is required to increase the yield of proteins from the basalt surfaces and to replace Triton X-100 with another compound because of this detergent's incompatibility with ES-MS/MS analyses.

Cultures of *M. trichosporium* OB3b were grown with and without copper to express pMMO and sMMO, respectively. These cultures were used to optimize the conditions for the identification of methane monooxygenase (MMO) peptides in the planktonic groundwater samples. The application of a time-course lysis procedure to the *M. trichosporium* OB3b pure cultures was found to greatly increase the number of peptides identified for each subunit of the methane monooxygenase and increased the percentage of peptide coverage of the respective protein. This procedure also allowed for classification of a greater number of proteins from *M. trichosporium* OB3b. The results from single time point hydrolysis of equal

dilutions of *E. coli* and *M. trichosporium* OB3b (data not shown) indicated that the released proteins in this complex protein mixture can be dominated by members of the most easily hydrolysable microorganisms in the sample. This is an important consideration when dealing with environmentally derived samples. Our new time-course lysis method showed that such an effect can be reduced by collecting proteins from easily lysable organisms at early lysis time points thereby exposing the more resistant organisms in the sample to longer lysis times. The efficiency of the time-course lysis method in lysing all types of bacteria can be determined by visualization of non-lysed bacteria using microscopic examination.

Application of time-course lysis on TAN samples identified proteins from different metabolic pathways. For example, results from analysis of TAN-29\_2006 sample showed the presence of pMMO complex proteins. The identification of these proteins in the sample collected from the same well during 2007 indicated that these proteins were present over extended periods. Samples from TAN-36 showed the presence of peptides from subunit PmoB. Although the number of peptides detected was lower as compared to TAN-29, their presence in this sample provides evidence for intrinsic methane oxidation. The detection of MMO peptides from the TAN-58 sample was interesting as this well is located outside the distal contamination zone (approximately 8000 feet from the original TCE injection). The expression of MMOs by methanotrophs in this well might be due to the presence of trace amounts of methane transported by down gradient flow of groundwater. As no reports of methane, TCE, and other geochemical values at this site are available, these observations remain inconclusive.

During single time lysis only a few peptides were observed for a given protein; however, the number of peptides per protein was increased during the time-course lysis. This increase in the number of peptides might be related to the reduction of sample complexity by serial removal of proteins at each three-minute interval. The lower percentage of peptide coverage identified in the TAN samples when compared to the pure culture sample might be related to the complexity of the microbial communities in the groundwaters of TAN wells. The time-course lysis method was able to reduce the complexity of environmental samples by extracting proteins from easily lysable microbes at earlier time points and from more resistant microbes at later time points.

During our analyses we observed that peptides from subunit PmoC were only observed in TAN-29\_2007 sample. Perhaps this fully membrane integrated protein was not released from the membrane portion of cells during lysis or perhaps these peptides were lost during the chromatographic separation of lysates from environmental samples. In addition, we suggest that some proteins that were not digested properly during trypsination would release peptides of more than 20 amino acids in length. These peptides might have remained undetected during analyses due to the sensitivity of the ESI-MS/MS (<http://www.waters.com>). Further experimental analyses are required to understand the reason for the non-detection of these peptides. During analysis of all samples (both planktonic and basalt biofilm) peptides from pMMO subunits only were observed. The inability to identify peptides from sMMO was probably due to the presence of copper levels in TAN groundwaters that were adequate for suppressing the expression of sMMO in type II and type X methanotrophs and few sMMO-expressing type I methanotrophs<sup>19,24,30</sup>. Our results, however, allowed identification of

proteins from different microbes that should aid in the understanding of the current status of different metabolic activities taking place in the aquifer. The identification of methane catabolic proteins implied the occurrence of methane degradation which can simultaneously perform the co-metabolic degradation of TCE that is known to occur in this aquifer.

Along with the methanotrophs, the analysis on TAN samples provided preliminary data indicating the presence of different groups of microorganisms in the TAN groundwaters. The detection of proteins associated with non-methanotrophic bacterial species such as *Pseudomonas*, *Burkholderia*, *Nitrosococcus* and *Nitrospira* can also support the concept of co-metabolic degradation of TCE. Some bacterial species that belong to the genera *Pseudomonas* and *Burkholderia* have been reported to harbor toluene monooxygenase genes that are involved in non-specific co-metabolic oxidation of TCE<sup>3,14,29</sup>. The results also showed the presence of ammonia monooxygenase (Amo) complex proteins derived from *Nitrosococcus* and *Nitrospira* or closely related bacteria. The Amo proteins function in oxidation of ammonia, share certain sequence homology with pMMO proteins, and can participate in non-specific oxidation of TCE<sup>18,45</sup>. Although there was no previous investigation of the involvement of *Nitrospira* NpAV in the oxidation of chlorinated compounds, this species having Amo complex proteins might also be capable of non-specific oxidation of TCE<sup>20</sup>. Similarly, the oxygenase proteins identified from *Streptomyces rochei* species were found to be involved in the aerobic degradation of chlorophenols<sup>48</sup>. The presence of these proteins might indicate the capability to oxidize many other chemical contaminants such as chlorophenols simultaneously with the degradation of TCE in the TAN aquifer<sup>1, 29</sup>.

The observation of proteins related to those of sulfate/metal-reducing bacteria such as *Geobacter sulfurreducens*, *Alkaliphilus metalliredigenes* QYMF, *Desulfotomaculum reducens*, *Desulfovibrio desulfuricans*, *Geobacillus kaustophilus*, and *Desulfotalea psychrophila* could be linked to the presence of high amounts of metal ions in these groundwaters<sup>1,27,44</sup>. Metal-reducing bacteria have been found to play a major role in the reduction of heavy metals such as Co, Cr, Mn and U<sup>43,47</sup>. Identification of proteins indicative of the presence of these bacteria in all TAN planktonic samples is consistent with the presence of minerals such as nitrate, nitrite, sulphate, and ammonium (Table 11). Although mineral data is not available from other wells, we assume that waters from the other wells share similar compositions regarding these minerals. The presence of proteins associated with pathogenic and/or anaerobic bacteria, such as *Salmonella typhimurium*, *Rickettsia montana*, *Bordetella pertusis*, and *Porphyromonas gingivalis*, might be due to the historical sewage injection in to well TSF-05 which is located upstream to the wells sampled<sup>16,44</sup>. Well TSF-05 has received organic and radioactive waste during the period from 1953 to 1972<sup>16,44</sup>. The detection of proteins related to those of nitrogen-fixing bacteria *Corynebacterium efficiens* and *Frankia* sp indicates that the gaseous nitrogen in the aquifer might be converted to ammonia. These microorganisms undoubtedly play crucial roles in supplying nitrogen to other members of the TAN aquifer microbial community.

In conclusion, the identification of different proteins from all three TAN planktonic samples illustrates the current metabolic state in the aquifer. The confirmed expression of methane monooxygenase proteins in high concentrations in all planktonic samples indicates the

occurrence of methane oxidation which is likely to be linked to co-metabolic oxidation TCE. Proteins observed from bacteria other than methanotrophs also indicated the possibility for degradation for TCE through non-methanotrophic pathways. Our results provide direct evidence of the occurrence of TCE degradation through natural attenuation in the TAN aquifer. Although the time-course lysis method was efficient in identifying proteins from planktonic samples, the efficiency in peptide coverage of individual proteins can be improved by further optimization of cell lysis conditions. The optimization of formic acid concentration may further improve the time-course lysis method. Thus far, we have been able to identify only a few proteins from basalt biofilms through single time point hydrolysis. Further development of time-course lysis method is needed to extract and identify more proteins from basalt biofilm samples. With the further improvement, the developed time-course cell lysis method should find application to a variety of environmental samples to study the microbial response to ever-changing environmental conditions.

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**Table 1.** Peptides of particulate MMO complex proteins detected through time-course lysis of *Methylosinus trichosporium* OB3b cells.

<b>**Protein Name (Accession ID)</b>	<b>Sequence (Individual ion score* range)</b>	<b>Observed mass</b>	<b>Time points where peptide was observed (in minutes)***</b>
PmoA (Q50541,Q5S3R7)	K.SGGAIGPFHVSVAEAAGCVK.T (57-115)	605.63	6 to 30
	R.TSMPEYIR.M (47-65)	498.74	12 to 30
PmoB (Q9KX50)	K.SQQAFLR..M (29-46)	425.21	3 to 30
	R.TLNWYDVK.W (14-45)	519.75	3 to 30
	K.LGEYTAAGLR.F (40-75)	525.76	3 to 30
	K.VTNKTDEPLK.L (46-73)	572.79	3 to 30
	R.YATEIGGPVIPK.F (25-69)	622.82	3 to 30
	R.TAQFIGEQFAPR.S (54-80)	682.84	3 to 30
	R.GLSTDPPLAPGETK.T (49-89)	742.36	3 to 30
	K.SSFLNAGEPGPVLVR.T (43-100)	771.91	3 to 30
	K.VHFSAWPQAVANPK.S (68-93)	825.93	3 to 30
	R.SVSLEVGKDYAFSIDLK.A (58-137)	624.34	3 to 30
	R.FLNPDVFTTKPEFDYLLADR.G (89-123)	833.41	3 to 30
	R.LSDLAYDTSQIGLLMFFSPSGK.R (86-185)	1281.62	3 to 30
	R.TIPLQAGLQKPLTPIIEEGTAGVGPVVTAEK. (123-240)	845.21	3 to 30
	K.GGVYKVPGR.E (14-38)	466.75	6 to 30
	K.RYATEIGGPVIPK.F (11-60)	700.89	6 to 30
	K.TSLNVNESMVLSGK.V (45-96)	739.86	6 to 30
	R.WHVHAQINVEGGPIIGPGQWIEIK.G (37)	684.62	6
	K.DYAFSIDLK.A (18-25)	536.27	9 to 27
	K.TTVNVNEEMVLSGK.I (15)	761.37	9
	K.GDMADFKDPVTLTLDGTTVDLETYIDR (85-119)	986.46	12 to 30
K.TDEPLKLGELYTAAGLR.F (53-90)	578.64	18 to 30	
K.ISEKDEEQIGDDRR.V (23-111)	674.29	21 to 30	
PmoC (Q9KX37, Q9KX51)	R.DRNVDAVAPR.E (45-79)	556.79	18 to 30
	R.LIGKDCVAALVG.- (77)	608.33	30
Methanol dehydrogenase (Q8KM55, Q883WWO, MTBXXDH, Q9AQ48)	K.SGLPVRDPEYGTR.M (14-42)	723.85	3 to 30
	R.TDGTLVSADKIDTVNVFK.K (41-135)	1019.49	3 to 30
	R.NGVITYLDR.T (13-33)	526.26	3 to 27
	K.TGTPVRDPEYGTR (12-15)	723.87	9 to 21
	R.DSDTGDLLWK.F (8-54)	574.29	3 to 30
	K.GLGATWEGDAWK.I (24-72)	696.32	3 to 27
	R.AVACCDLVNR.G (33-78)	589.26	6 to 30
	K.LLTHPDR.N (10-25)	426.24	9 to 18
	K.ILWQDKPK.Q (15-19)	514.28	3 to 12
	R.QNAEGLGQIK.A (15-32)	529.26	3 to 9
	K.DVCPSAMGYHNQGLDSYDPTK.E (7-20)	785.67	12 to 18
	K.DGFTLLAVVAPNLCKPNTILFNK.V (47-102)	915.14	3 to 30
Formaldehyde activating enzyme (Q6QQL3, Q6QQN4)	K.QAVQMFGPAQAGVAK.A (46-78)	751.86	3 to 30
	R.GSAAETAFANLVNND.D (11-96)	789.36	3 to 30

\*Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores > 47 indicate identity or extensive homology ( $P < 0.05$ ). \*\* PmoA, PmoB and PmoC; Particulate methane monooxygenase subunits A, B and C, respectively. \*\*\* Range of cell lysis time points where the particular peptide was detected. For example 3-30 min represent that a particular peptide was observed at 3, 6, 9, 12, 15, 18, 24, 27 and 30 min time points.

**Table 2.** Peptides of soluble MMO complex proteins detected through time-course lysis of *Methylosinus trichosporium* OB3b cells.

Protein Name (Accession ID)	Sequence (Individual ion score* range)	Observed mass	Time points where peptide was observed (in minutes) **
<b>α-subunit</b>	K.FKVEPWVK.T (13-29)	516.8	12-30
	K.TLIAQPHTR.G (18-43)	518.79	6-30
	K.AATDALKVNR.A (76-78)	529.8	6-30
	R.QWLIEPER.Y (39)	535.79	6
	K.YGVESPASLR.D (21-67)	539.78	6-30
	K.HSLTDDWGER.Q (11-55)	608.27	6-30
	R.VSQVPFIPSLAK.G (14-44)	642.82	6-30
	R.AGCVFPLAKF.- (21-39)	661.33	6-30
	K.KHSLTDDWGER.Q (46-57)	672.32	6-15
	K.NGYLAQVLDEIR.H (65-90)	695.87	6-30
	R.ELSEVIAEGHGVRSDBGK.T (64-77)	698.36	6-30
	R.SDGKTLIAQPHTR.G (10)	475.26	6
	R.GDNLWTLEDIKR.A (39-72)	730.38	6-30
	R.YECHNVFEQYEGR.E (43-90)	577.58	6-21
	R.VSQVPFIPSLAKGTGSLR.V (96-100)	619.69	6,9
	K.SGFIPYQWLLANGHDVYIDR.V (40-96)	788.72	6-30
	R.APVGVPEPQEVHKWLQSFNDFK.E (40)	661.07	6
	R.VHEYNGIKHSLTDDWGER.M (14)	539.51	6
	R.LALPDEEDQAWFEANYPGWADHYGK.I (80-164)	908.49	6-30
	R.DAYWAHHDLALAAYAMWPLAFAR.L (19-76)	666.32	9-27
	R.QFGTLLDGLTR.L (41-54)	610.85	12-27
	R.APVGVPEPQEVHK.W (68)	645.34	12-27
	K.WLQSFNDFKFNK.T (42-90)	685.84	12-30
	K.YHMANETKEQFK.V (48)	509.24	12
	K.VNRAPVGVPEPQEVHK.W (39-43)	553.64	12-30
	K.YFTPVLGYLFEYGSK.F (77,97)	892.44	12-30
	R.HTHQCAFINHYSK.H (53-98)	602.61	12-18
	K.FLNTDLNNAFWTQQK.Y (76-83)	920.46	12-30
	R.HMANGYQTVVSIANDPASAK.F (67-102)	1037.51	12-18
	K.VISNFLEVGEYNAIAASAMLWDSATAAEQK.N (151-168)	1600.28	12-30
	-.GDDALKVNR.A (23)	494.77	12
	R.APVGVPEPQEVHK.W (54-68)	645.34	12-30
	K.IISNFLEVGEYNAIAASAMLWDSATAAEQK.N (20-29)	1072.52	18-30
K.YLNSDLNNAFWTQQK.Y (47)	920.45	21	
<b>Regulatory protein (Protein B)</b>	K.FTITSELMGLDR.A (33-88)	691.85	6-30
	-.SSAHNAYNAGIMQK.T (92-110)	746.35	6-30
	K.SDEIDAIIEDIVLKGK.A (45-120)	605.66	9-27
	K.NPSIVVEDK.A (41-47)	500.77	12-27
	K.AKNPSIVVEDK.A (28-67)	600.34	12-27

**Table 2.** Continued.....

<b>Protein Name (Accession ID)</b>	<b>Sequence (Individual ion score* range)</b>	<b>Observed mass</b>	<b>Time points where peptide was observed (in minutes) **</b>
<b>β-subunit</b>	R.TTDWYR.H (16-23)	421.19	6-18
	K.YHYFIQPR.W (21-59)	562.29	6-30
	M.SQPQSSQVTKR.G (37-61)	623.34	6-12
	R.KYHYFIQPR.W (44-61)	417.88	6-27
	R.FLAAYSSEGSIR.T (61-81)	650.84	6-30
	K.LVPGFDASTDVPK.K (41-67)	673.36	6-30
	R.AAIIAAVDPHALDTQR.K (96-135)	578.32	6-15
	K.FHGGRPSWGNESTELR.T (27-68)	610.62	6-30
	R.AAIIAAVDPHALDTQRK.Y (97-111)	621.02	6-30
	R.GLTDPERAAIIAAVDPHALDTQR.K (89,	834.44	9
	K.RGLTDPER.A (25)	472.25	12
	R.DCLSDTIR.Q (29-47)	490.23	12-30
	K.VDAVLAGYKN.-(25-49)	525.28	12-27
	R.RWHHPYVK.D (11-18)	561.81	12-15
	K.IWTTDPIYSGAR.A (45-57)	690.36	12-27
	R.TFLNAWTEHYLASSVAALK.D (108-137)	708.04	12-27
	K.YFGALLYSEYGLFNAHSSVGR.D (102-143)	784.38	12-27
	R.QTAVFAALDKVDNAQMIQMER.L (117-147)	793.74	12-18
	R.GAIDDLFVYCLANDSEFGAHR.T (136-185)	1242.57	12-21
	R.LATVYGDTLTPFFTAQSQTTFQTR.G (107-168)	953.15	12-30
	R.TFLNAWTEHYLASSVAALKDFVGLYAK.V (37-136)	754.64	12-18
	R.AGVSEALQR.V (63-66)	465.74	12-30
	R.LSEYEQLSCYAQPNDWIAGGLDWGDWTQK.F (124-141)	1176.54	12-21
	K.VDNAQMIQMER.L(74-87)	662.82	15-27
	R.TIDPYWRDEILNK.Y(53-65)	554.96	15-27
	R.REFFQR.L (20)	441.72	18-27
<b>Reductase protein (Protein C)</b>	K.TLAQSMPTLGVR.I (20-59)	637.36	12-24
	R.LVLQPLTADGAAR.I (32-59)	662.89	12-30
	R.VFVAGGTGLSPVLSMIR.Q (63-102)	852.48	12-30
	R.GPAGSFFLHDHGGR.S (54)	485.56	27
<b>Formaldehyde activating enzyme</b>	K.DGFTLLAVVAPNLPCKPNTILFNK.V (93-105)	915.16	15-21
	R.GSAAETAFANALVNNK.D (33-102)	789.42	15-27
	K.QAVQMFQPAQAVAK.A (27,53)	751.39	21-30

**Table 2.** Continued.....

<b>Protein Name (Accession ID)</b>	<b>Sequence (Individual ion score* range)</b>	<b>Observed mass</b>	<b>Time points where peptide was observed (in minutes)**</b>
<b>γ-subunit</b>	R.LAYTSPFRK.S (29-45)	541.8	6-30
	R.NTDYYGTPLEGLRK.E (50-69)	542.94	6-30
	K.SYDLVDVYQYIER.K (33-78)	839.88	6-30
	K.LTSVDQATKFIQDFR.L (39-101)	590.31	6-18
	K.IAKLTSVDQATK.F (50)	637.87	9,12
	R.IHIEFR.Q (31)	407.72	12-24
	K.LPVADLITK.A (41-52)	485.3	12-30
	K.TEKLVPADLITK.A (34-48)	664.4	12-30
	R.TDAALGTVLMEIR.N (105-108)	695.37	12-27
	K.ATTGEDAAAVEATWIAK.I (94-130)	852.94	12-30
	R.QLYKPPVLPVNVFLR.T (30-71)	595.02	12-27
	R.KSYDIDVDYQYIER.K (30-83)	602.96	12-21
	R.EPIHDNSIR.T (17-48)	540.78	12-21
	R.EPIHDNSIRTEWEAK.I (14)	608.97	12
	K.FIQDFR.V (26-31)	413.2	15-27
	<b>Methanol dehydrogenase</b>	R.AVACCDLVNR.G (23-81)	589.28
K.GLGTATWEGDAWK.I (65-83)		696.34	6-30
K.LLTHPDRNGIVYTLDR.T (55)		628.35	6
K.TPHDEWDYAGINFMLSEQK.D (36-57)		804.69	6-15
R.KLLTHPDR.N (16-35)		490.29	12-24
K.WTMTIWGR.D (23)		525.76	12
R.NGIVYTLDR.T (21-43)		525.78	12-30
K.SGLPVRDPEYGTR.M (22,15)		723.87	12-30
R.TDGTLSADKIDDTVNVFK.K (44-130)		680.02	12-30
R.DSDTGDLLWK.F (33-41)		574.79	12-30
K.TGTPVRDPEYGTR.M (16,26)		723.87	12-30
K.ILWQDKPK.Q (29,16)		514.3	12-24
-PDTNLVYYGSGNPAPWNETMRPGDNK.W (156)		965.12	15
R.AGQFFVVGATLNMYPGPK.G (40,16)		899.46	21-24

\*Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores  $> 47$  indicate identity or extensive homology ( $P < 0.05$ ). \*\* Range of cell lysis time points where the particular peptide was detected. For example 3-30 min represent that a particular peptide was observed at 3, 6, 9, 12, 15, 18, 24, 27 and 30 min time points.

**Table 3A.** Peptides of methanotrophic proteins detected in TAN-29\_2006 sample.

Accession	Protein	Peptide (Individual ion score* range)	Methanotrophic species
<b>Methane metabolic proteins</b>			
Q2FAX8	PmoA	R.TGTPEYIR.M (40)	<i>Methylococcaceae bacterium LC 2</i>
Q9KX36	PmoB	R.VSFLNAGEPGPVLVR.T (19-76)**	<i>Methylocystis sp.</i>
Q9KX36	PmoB	R.TLNWYDVQWSK.T (14)	<i>Methylocystis sp.</i>
Q9KX36	PmoB	R.FAAEIGGPVPIK.F (21)	<i>Methylocystis sp.</i>
Q9KX36	PmoB	R.LGEYTAAGLR.F (45)	<i>Methylocystis sp.</i>
Q9KX36	PmoB	R.TIPLQAGLQKPLPLVTDGTAGVGK.E (11)	<i>Methylocystis sp.</i>
Q9KX36	PmoB	R.FLNPDVFTTKPDFDYLLADR.G (54)	<i>Methylocystis sp.</i>
B57266	PmoB	K.VEDATYRVPGR.A (33)	<i>Methylococcus capsulatus</i>
Q25BV2	Methane monooxygenase protein B	R.AGSWIGGQLVPR.S (34)	<i>Methylomicrobium sp</i>
Q25BV2	Methane monooxygenase protein B	R.TIHWFDLNWSK.D (56)	<i>Methylomicrobium sp</i>
Q25BV2	Methane monooxygenase protein B	R.LADLIYDPDSR.F (33-75)	<i>Methylomicrobium sp</i>
Q1GY79	Formaldehyde-activating enzyme	R.QAVQMFQPAQR.G (54)	<i>Methylobacillus flagellatus</i>
<b>Other proteins from methanotrophic bacteria</b>			
T51709	Hypothetical protein	R.QLPEEFR.S (21)	<i>Methylomicrobium sp</i>
Q606K3	Prolyl-tRNA synthetase	R.IARGIEVGHIFQLGTK.Y (16)	<i>Methylococcus capsulatus</i>
Q605B0	Translation elongation factor Tu	K.VGEEIEIVGIRPTAK.T (39)	<i>Methylococcus capsulatus</i>
Q608I2	TPR domain protein	K.TDYVSIALELSR.D (20)	<i>Methylococcus capsulatus</i>
Q60BG0	DnaK suppressor protein	K.TLDEIRER.Q (34-44)	<i>Methylococcus capsulatus</i>
Q608X6	Hydrophobe/amphiphile Efflux-1 (HAE1) family protein	K.NAILVVEFAR.E (18)	<i>Methylococcus capsulatus</i>
Q608X6	Class II aldolase/adducin-like protein	K.LVIVNDPDPFNWDNR.E (18)	<i>Methylococcus capsulatus</i>
Q608X6	DnaA domain protein	R.DAAAVLLACR.E (16)	<i>Methylococcus capsulatus</i>
Q608X6	TidD protein	M.ATEIVAIAER.A (16)	<i>Methylococcus capsulatus</i>
Q608X6	Chaperonin GroEL	R.AAVEEGIVPGGGVALLR.A (18)	<i>Methylobacillus flagellatus</i>
Q608X6	Translation elongation factor Tu	K.TTLTAAITVLT.K (22)	<i>Methylobacillus flagellatus</i>
Q608X6	Translation elongation factor Tu	K.LLDQAGQAGDNVGVLLR.G (69)	<i>Methylobacillus flagellatus</i>
Q1H0Q7	Formylmethanofuran dehydrogenase	R.VPEVLAALR.A (16)	<i>Methylobacillus flagellatus</i>
Q1H4M1	Ribosomal protein L18	K.VLASASTLEVEVR.K (17-46)	<i>Methylobacillus flagellatus</i>
Q1H4M1	Ribosomal protein L18	K.NGGNIAAAALVGR.I (6)	<i>Methylobacillus flagellatus</i>
Q1H485	Two component transcriptional regulator	R.TNLKEAIPILMITNR.S (19)	<i>Methylobacillus flagellatus</i>
Q1H485	Two component transcriptional regulator	K.TTLTAAITVLT.K (19)	<i>Methylobacillus flagellatus</i>
Q1GXE4	ATPase	K.SLLAAFAANADQVTPR.H (18)	<i>Methylobacillus flagellatus</i>
Q1H1N7	DNA repair protein RadA	K.QVIDGLDVK.G (17)	<i>Methylobacillus flagellatus</i>
Q1H4W5	Transmembrane protein	R.GAQPCVVTHIR.L (21)	<i>Methylobacillus flagellatus</i>
Q1H3X7	Transcriptional regulator, AraC family	R.LMTSLVGGTR.Y (18)	<i>Methylobacillus flagellatus</i>
Q2VNL7	Hypothetical protein	R.VSRVDIGSAR.T (20)	<i>Methylocapsa acidiphila</i>

\* Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores > 47 indicate identity or extensive homology ( $P < 0.05$ ). \*\* R.VSFLNAGEPGPVLVR.T peptide was used for quantitative estimation of methanotrophic numbers from planktonic sample.

**Table 3B.** Peptides of methanotrophic proteins detected in TAN-29\_2007 sample.

Accession	Protein	Peptide (Individual ion score* range)	Methanotrophic species
<b>Methane metabolic proteins</b>			
Q25BV2	PmoB	R.AGSWIGGQLVPR.S (34-57)	<i>Methylobacterium</i> sp. NI
Q25BV2	PmoB	R.TIHWFDLNWSK.D (33-72)	<i>Methylobacterium</i> sp. NI
Q25BV2	PmoB	R.TVDVTASDAAWVEVYR.L (9)	<i>Methylobacterium</i> sp. NI
Q25BV2	PmoB	R.LADLIYDPDSR.F (28-59)	<i>Methylobacterium</i> sp. NI
Q25BV4	PmoC	K.ADAAEAPLLNQK.N (54)	<i>Methylobacterium</i> sp. NI
Q70EF2	PmoB	R.LGEYTAAGLR.F (17-46)	<i>Methylocystis</i> sp. SC2
Q70EF2	PmoB	R.FAAEIGGPVVPK.F (13)	<i>Methylocystis</i> sp. SC2
Q70EF2	PmoB	R.VSFLNAGEPGPVLVR.T (35-54)**	<i>Methylocystis</i> sp. SC2
Q70EF2	PmoB	R.FLNPDVFTTKPDFDYLLADR.G (16-81)	<i>Methylocystis</i> sp. SC2
B57266	PmoB	K.VEDATYR.V (42)	<i>Methylococcus capsulatus</i>
B57266	PmoB	K.VEDATYRVPGR.A (35)	<i>Methylococcus capsulatus</i>
Q6UDA8	PmoA	R.SATPEYLR.I (26)	uncultured bacterium
Q8KJ2	PmoA	R.TGTPEYLR.Q (26-39)	<i>Methylomonas</i> sp. LC
Q70EF2	PmoB	R.TIPLQAGLQKPLTPLVTDGTAGVGK.E (83)	<i>Methylocystis</i> sp. SC2
<b>Other proteins from methanotrophic bacteria</b>			
Q602F1	SPFH domain/Band 7 family	R.QAEILKAEGEK.Q (20-32)	<i>Methylococcus capsulatus</i>
Q602F1	SPFH domain/Band 7 family	K.RAAILEAEGHR.Q (19)	<i>Methylococcus capsulatus</i>
Q3V8A0	Putative cobalamin biosynthetic protein CobC	K.GWHAALR.K (21)	<i>Methylococcus capsulatus</i>
Q1H485	Two component transcriptional regulator	R.TNLKEAIPILMITNR.S (21)	<i>Methylobacillus flagellatus</i> KT
Q60AC8	CRISPR-associated helicase Cas3	R.DDETAELIRR.G (23)	<i>Methylococcus capsulatus</i>
Q1H511	Inner-membrane translocator	R.GIAGILDKQR.E (13)	<i>Methylobacillus flagellatus</i> KT
Q1GXJ1	TonB-dependent receptor	K.GNFTDWR.L (15)	<i>Methylobacillus flagellatus</i> KT
Q606G7	Glycyl-tRNA synthetase	K.AGLSYAGIEAYATPRR.L (12)	<i>Methylococcus capsulatus</i>
AAU92021	AE017282 NID Multidrug efflux system outer membrane subunit	R.VTEIIGCIRR.D (29)	<i>Methylococcus capsulatus</i> str. Bath
Q608A2		R.AVLVTLGGDVAR.A (21)	<i>Methylococcus capsulatus</i>
Q1H4F2	Chaperonin GroEL	K.VTLGPK.G (18)	<i>Methylobacillus flagellatus</i> KT
Q1H4F2	Chaperonin GroEL	R.AAVEEGIVPGGGVALLR.A (44)	<i>Methylobacillus flagellatus</i> KT
Q605R4	Sensor histidine kinase KdpD	K.ETEALLEGLEMLPVR.T (18)	<i>Methylococcus capsulatus</i>
Q604T9	Outer membrane protein, OMP85 family	K.GIGLAEGKVFDR.Q (25)	<i>Methylococcus capsulatus</i>
Q60BG0	DnaK suppressor protein	K.TLDEIRER.Q (33)	<i>Methylococcus capsulatus</i>

\* Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores > 47 indicate identity or extensive homology ( $P < 0.05$ ). \*\* R.VSFLNAGEPGPVLVR.T peptide was used for quantitative estimation of methanotrophic numbers from planktonic sample.

**Table 4.** Peptides of methanotrophic proteins detected in TAN-36 sample.

Accession	Protein	Peptide (Individual ion score* range)	Methanotrophic species
Q9KX50	PmoB	R.TAQFIGEQFAPR.S (36-42)	<i>Methylosinus trichosporium</i>
Q9KX50	PmoB	K.SSFLNAGEPGPVLVR.T (37)**	<i>Methylosinus trichosporium</i>
Q1GXE3	Type II and III secretion system protein	K.VLSSPK.L (18)	<i>Methylobacillus flagellatus KT</i>
AAU90503	AE017282 NID	R.LPRECALMLR.E (13)	<i>Methylococcus capsulatus str. Bath</i>
AAU92020	AE017282 NID	R.ANGLAPQELIMDISSR.L (5)	<i>Methylococcus capsulatus str. Bath</i>
Q602F7	ATP-binding subunit ClpB	K.GVLNDIAKQEGR.V (10)	<i>Methylococcus capsulatus</i>
Q606W4	Cysteine synthase B	K.DEARIPGIR.R (14)	<i>Methylococcus capsulatus</i>
Q1H4Y0	Methionyl-tRNA formyltransferase	R.IDELQLPGGK.R (13)	<i>Methylococcus capsulatus str. Bath</i>

\* Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores > 47 indicate identity or extensive homology ( $P < 0.05$ ). \*\* K.SSFLNAGEPGPVLVR.T peptide was used for quantitative estimation of methanotrophic numbers from planktonic sample.

**Table 5.** Peptides of methanotrophic proteins detected in TAN-58 sample.

Accession	Protein	Peptide (Individual ion score* range)	Methanotrophic species
Q9KX50	PmoB	R.TAQFIGEQFAPR.S (35-52)	<i>Methylosinus trichosporium</i>
Q9KX50	PmoB	K.SSFLNAGEPGPVLVR.T (39-53)**	<i>Methylosinus trichosporium</i>
Q4FS5	PmoA	R.IIEVGSLRTFGGHTWWISAFFSAFK.G (17)	uncultured bacterium
Q60BG0	DnaK suppressor protein Methionyl-tRNA	K.TLDEIRER.Q (23-38)	<i>Methylococcus capsulatus</i>
Q1H4Y0	formyltransferase	R.IDELQLPGGK.R (15)	<i>Methylobacillus flagellatus KT</i>

\*Ion score is  $-10 \cdot \log(P)$ , where P is probability that the observed match is a random event. Individual ion scores > 47 indicate identity or extensive homology ( $P < 0.05$ ). \*\* K.SSFLNAGEPGPVLVR.T peptide was used for quantitative estimation of methanotrophic numbers from planktonic sample.

**Table 6.** Peptides of proteins from non-methanotrophic microorganisms that could play a role in degradation of contaminants, detected in TAN-29 sample.

Accession	Protein	Sequence	Bacterial species
AAR35294	AE017180 NID	K.TAEDAKIAIR.N	<i>Geobacter sulfurreducens</i>
AAR33446	AE017180 NID	R.VVNAIGQPIDGK.G	<i>Geobacter sulfurreducens</i>
AAR33446	AE017180 NID	K.KEFTA.-	<i>Geobacter sulfurreducens</i>
Q74E71	Hypothetical protein	R.LASPLR.I	<i>Geobacter sulfurreducens</i>
Q749B8	Membrane protein	R.TTGGNGIELESR.A	<i>Geobacter sulfurreducens</i>
Q74CW4	Mechanosensitive ion channel family protein	MDTMGQYQQLVVTYATVIGTK.I D	<i>Geobacter sulfurreducens</i>
Q74GJ3	Sensory box histidine kinase	R.LQLSERAR.C	<i>Geobacter sulfurreducens</i>
Q8KSZ3	AmoA	R.SSHGNGCLPR.C	<i>Nitrosococcus oceani</i>
Q8KSZ3	AmoA	R.TGTPEYIR.M	<i>Nitrosococcus oceani</i>
O06935	Ammonia monooxygenase 3 subunit B	R.IGEFTTAGVR.F	<i>Nitrosospira</i> sp. NpAV
Q3C745	GAF:Metal-dependent phosphohydrolase, HD subdomain	R.QKNLLEAQR.N	<i>Alkaliphilus metalliredigenes</i>
Q3C745	GAF:Metal-dependent phosphohydrolase, HD subdomain	K.IKDFLDVDR.I	<i>Alkaliphilus metalliredigenes</i>
Q8FNC5	Putative phenol 2-monooxygenase	R.KEPTAEGAAR.R	<i>Corynebacterium efficiens</i>
Q8FNC5	Putative phenol 2-monooxygenase	R.AALDNK.D	<i>Corynebacterium efficiens</i>
Q83X40	Oxygenase	R.AVHAR.L	<i>Streptomyces rochei</i>
Q83X40	Oxygenase	R.AGEPGR.A	<i>Streptomyces rochei</i>
Q82DF2	Putative transcriptional regulator	R.ASIVSLPR.D	<i>Streptomyces avermitilis</i>
DEPSXA	3-methyl-2-oxobutanoate dehydrogenase (lipoamide),	R.QELGV.-	<i>Pseudomonas putida</i>
Q11A90	Hypothetical protein	K.VLDDLDLSAGR.C	<i>Pseudomonas entomophila</i>
Q4K6V4	Aminotransferase	K.LGEALSPR.T	<i>Pseudomonas fluorescens</i>
Q00620	NosA protein precursor	R.IMGGELGATYR.L	<i>Pseudomonas stutzeri</i>
Q4ZXY8	DTDP-glucose 4,6-dehydratase	R.LFAEHRPR.A	<i>Pseudomonas syringae</i>
Q3F1V1	Hypothetical protein	R.IDAIPPIRGLR.G	<i>Burkholderia ambifaria</i>
ABE35609	CP000271 NID	K.ENENESGRADLK.A	<i>Burkholderia xenovorans</i>
ABE31991	CP000271 NID	K.RLQADLDVAR.T	<i>Burkholderia xenovorans</i>
Q3JR37	Hypothetical protein	R.ARAFGDR.E	<i>Burkholderia pseudomallei</i>
Q8XYA8	Putative outer membrane multidrug resistance lipoprotein	R.KALAPR.L	<i>Ralstonia solanacearum</i>
B56274	sulfur-regulated protein SrpB	R.QRGGRPAGMR.T	<i>Synechococcus</i> sp
B56274	sulfur-regulated protein SrpB	R.SGRPR.V	<i>Synechococcus</i> sp
BYEBT	sulfate-binding protein	K.NVEVLDSGAR.G	<i>Salmonella typhimurium</i>
Q5L208	Multidrug ABC transporter (ATP-binding protein)	R.AEAKR.R	<i>Geobacillus kaustophilus</i>
Q5KVG5	Manganese catalase	K.LLPIPKLDNR.V	<i>Geobacillus kaustophilus</i>
Q5KVG5	Pentachlorophenol-4-monooxygenase-like protein	R.KETIRPR.W	uncultured bacterium
Q5KVG5	Multidrug transport protein	R.IFDHIGARR.L	<i>Lactobacillus plantarum</i>
Q5KVG5	Penicillin binding protein	K.NDLNVGTK.I	<i>Rickettsia montana</i>
Q5KVG5	Multidrug resistance efflux pump	MNTPADSSPPADR.Q	<i>Xanthomonas axonopodis</i>
Q5KVG5	Dissimilatory (Bi-)-sulfite reductase beta subunit	K.VAPIIGRLR.S	sulfate-reducing bacterium
Q7VXG4	Hypothetical protein	K.LVARVTEGR.D	<i>Bordetella pertussis</i>
A30196	probable alcohol dehydrogenase	R.LINVIESGR.V	<i>Alcaligenes eutrophus</i>
Q475J3	Pyruvate kinase	R.IGGDLSNKGINR.Q	<i>Alcaligenes eutrophus</i>
Q4CE35	Putative RNA methylase	K.GDISSIPR.A	<i>Clostridium thermocellum</i>
BAC15445	BA000028 NID	K.NGVFIAMKGAQAK.E	<i>Oceanobacillus iheyensis</i>
Q2JED5	Methyltransferase type 11	R.LGVLGEVTGR.D	<i>Frankia</i> sp
Q7MVB7	S4 domain protein	R.LAPSASNQGR.Q	<i>Porphyromonas gingivalis</i>
Q2CZM9	Phosphoglucosamine mutase	K.IVDCANGAAYAVAPR.I	<i>Desulfotomaculum reducens</i>
Q2DOAO	Nicotinate-nucleotide pyrophosphorylase	K.SLDISLDIGEIK.V	<i>Desulfotomaculum reducens</i>
Q72JV9	Hypothetical protein	R.LGYLSQARR.G	<i>Thermus thermophilus</i>
Q5SHY8	Sensor histidine kinase	R.ALEGALREAGR.M	<i>Thermus thermophilus</i>
Q2B2J8	DNA polymerase III subunit	R.IEDKLQYEK.E	<i>Bacillus</i> sp.
Q2B2J8	DNA polymerase III subunit	R.NLSLIETILSSIQR.K	<i>Bacillus</i> sp.

**Table 7.** Peptides of proteins from non-methanotrophic microorganisms that could play a role in degradation of contaminants, detected in TAN-36 sample.

Accession	Protein	Sequence	Bacterial species
Q74BG5	Cytochrome c family protein	R.IIASATLATGK.T	<i>Geobacter sulfurreducens</i>
Q5L025	Aldehyde dehydrogenase	K.DVPVPK.R	<i>Geobacillus kaustophilus</i>
Q2BJL4	Hypothetical protein	K.QSGVSIPR.T	<i>Bacillus</i> sp
Q1MC05	Hypothetical protein	K.LSSPASGSVLK.V	<i>Bacillus</i> sp
Q2B2J8	DNA polymerase III subunit alpha	R.IEDKLQYEK.E	<i>Bacillus</i> sp
Q2B2J8	DNA polymerase III subunit alpha	R.NLSLIETLSSIQR.K	<i>Bacillus</i> sp
Q9RHG6	Resolvase	K.GDTVVVYK.L	<i>Bacillus cereus</i>
Q9LCZ4	FtsH	K.KSLVLSPR.D	<i>Thermus thermophilus</i>
Q74LL3	Hypothetical protein	K.VIPVPK.A	<i>Lactobacillus johnsonii</i>
Q1U869	Hypothetical protein	K.VATHPK.L	<i>Lactobacillus reuteri</i>
Q5FHY1	Hypothetical protein	R.YEGEIQITKR.D	<i>Lactobacillus acidophilus</i> .
Q8GFE4	Surface layer protein SlpC	K.ATVLSVYK.N	<i>Lactobacillus brevis</i>
Q30W91	Methyl-accepting chemotaxis sensory transducer	M.VPVLPE.E	<i>Desulfovibrio desulfuricans</i>
Q2D0A0	Nicotinate-nucleotide pyrophosphorylase	K.SLDISLDIGEIK.V	<i>Desulfotomaculum reducens</i>
Q6AM10	two-component system sensor histidine kinase	R.SLDLLQIQYVR.E	<i>Desulfotalea psychrophila</i>
Q1K3R4	NADH dehydrogenase	K.ATGIEVPR.R	<i>Desulfuromonas acetoxidans</i>
Q88119	SCO1/SenC family protein/cytochrome c	R.QRDANWLV.R.W	<i>Pseudomonas putida</i>
Q88717	GGDEF domain protein	R.AAVESLPR.Y	<i>Pseudomonas syringae</i>
Q00620	NosA protein precursor	R.IMGGELGATYR.L	<i>Pseudomonas stutzeri</i>
C83420	probable two-component response regulator PA1799	K.VLLVEDDQK.L	<i>Pseudomonas aeruginosa</i>
Q3K3J6	Transcriptional Regulator, MarR family	R.VLIDLTSAR.G	<i>Pseudomonas fluorescens</i>
Q3K1J0	2OG-Fe(II) oxygenase	K.LRIDITQTR.H	<i>Pseudomonas fluorescens</i>
ABE31991	CP000270 NID	K.RLQADLDVAR.T	<i>Burkholderia xenovorans</i>
ABE36742	CP000272 NID	R.GRAGLMLDGLR.E	<i>Burkholderia xenovorans</i>
Q3JV68	Hypothetical protein	R.ATVLGEVR.A	<i>Burkholderia pseudomallei</i>
Q3JJ06	Hypothetical protein	R.VIDIERER.R	<i>Burkholderia pseudomallei</i>
Q63JC6	Putative LysR-family transcriptional regulator	MKPLDLDAVR.A	<i>Burkholderia pseudomallei</i>
Q7W341	Haloacid dehalogenase-like hydrolase	K.AAEIEGVR.M	<i>Bordetella parapertussis</i>
Q33U40	Indole-3-glycerol-phosphate synthase	K.DAELIKQAFTALR.E	<i>Shewanella</i> sp
Q3NK07	AAA ATPase, central region	K.VTDVEIAEVLK.A	<i>Shewanella frigidimarina</i>
Q3QKT8	Branched-chain amino acid transport system II carrier protein	K.KGVDAPADQTR.A	<i>Shewanella amazonensis</i>
Q3QHE5	Deoxyribodipyrimidine photolyase	R.TIDNQLTR.A	<i>Shewanella amazonensis</i>
Q3QDM0	Methylmalonate-semialdehyde dehydrogenase	K.VSSYVEAGVR.E	<i>Shewanella amazonensis</i>
Q82E10	Putative transcriptional regulator	R.SPLTALDGARR.F	<i>Streptomyces avermitilis</i>
Q82DF2	Putative transcriptional regulator	R.ASIVSLPR.D	<i>Streptomyces avermitilis</i>
Q1N495	Hypothetical protein	K.YEEIKPVSSK.V	<i>Oceanobacter</i> sp
Q475J2	Phosphoglycerate kinase	K.TIIDIMAKR.G	<i>Alcaligenes eutrophus</i>
Q3W6T5	Hypothetical protein	R.TDGYLDARR.D	<i>Frankia</i> sp
Q3WIN5	Amidohydrolase 2	R.GARWIDLNGR.K	<i>Frankia</i> sp

**Table 8.** Peptides of proteins from non-methanotrophic microorganisms that could play a role in degradation of contaminants, detected in TAN-58 sample.

Accession	Protein	Sequence	Bacterial species
Q3QKT8	Branched-chain amino acid transport system II	K.KGVDAPADQTR.A	<i>Shewanella amazonensis</i>
Q33U40	Indole-3-glycerol-phosphate synthase	K.DAELIKQAFTALR.E	<i>Shewanella</i> sp
Q2WXX4	Ribosomal protein L1	K.NGIIHTTIGKVDFTPVQLK.E	<i>Shewanella</i> sp
Q2X1A9	Chemotaxis sensory transducer	R.AEDIPEMDAALK.A	<i>Shewanella</i> sp
Q3NYZ5	NAD-glutamate dehydrogenase	K.QALDETLILLTK.S	<i>Shewanella denitrificans</i>
Q3NK07	AAA ATPase, central region:Clp, N terminal	K.VTDVEIAEVLK.A	<i>Shewanella frigidimarina</i>
Q3KCJ6	Transcriptional Regulator, MarR family	R.VLIDLTASAR.G	<i>Pseudomonas fluorescens</i>
Q4KBV3	Monoxygenase, putative	R.EWIDFSVLETVIGSTRVAV.-	<i>Pseudomonas fluorescens</i>
C83420	probable two-component response regulator PA1799	K.VLLVEDDQK.L	<i>Pseudomonas aeruginosa</i>
Q2X8S9	Cytochrome c', putative precursor	R.VDPNSPLGK.R	<i>Pseudomonas putida</i>
Q2X994	Hypothetical protein	K.KADPQVGR.L	<i>Pseudomonas putida</i>
Q4B784	Hypothetical protein	K.QRDDALAQLK.A	<i>Burkholderia vietnamiensis</i>
Q3JKX8	AMP-binding enzyme domain protein	K.AVPNARILVVR.E	<i>Burkholderia pseudomallei</i>
Q3JKX8	AMP-binding enzyme domain protein	R.ITGFAGVPLWIQLARAAPGEAR.A	<i>Burkholderia pseudomallei</i>
Q3JQU1	Hypothetical protein	R.RPRISPR.A	<i>Burkholderia pseudomallei</i>
ABE31991	CP000270 NID	K.RLQADLDVAR.T	<i>Burkholderia xenovorans</i>
Q39LR6	Short-chain dehydrogenase/reductase SDR	K.MTLDDFR.A	<i>Burkholderia</i> sp
Q2T4S0	Twin-arginine translocation pathway	K.RDGDIPAR.I	<i>Burkholderia thailandensis</i>
Q62J47	Multidrug efflux RND membrane fusion protein MexE	R.VDPTSGVVR.M	<i>Burkholderia mallei</i>
Q2D0A0	Nicotinate-nucleotide pyrophosphorylase	K.SLDISLDIGEIK.V	<i>Desulfotomaculum reducens</i>
Q5AM10	two-component system sensor histidine kinase	R.SLDLLQIQIYVR.E	<i>Desulfotalea psychrophila</i>
Q1K3R4	NADH dehydrogenase	K.ATGIEVPR.R	<i>Desulfuromonas acetoxidans</i>
Q1K2Y3	Hypothetical protein	K.ASLTVGPEK.G	<i>Desulfuromonas acetoxidans</i>
S27492	hypothetical protein B	K.EENLLDNAR.E	<i>Bacillus firmus</i>
Q2B7L4	Hypothetical protein	K.QSGVSIPR.T	<i>Bacillus</i> sp
Q1U677	ParB-like partition protein	K.KTQQINDAR.L	<i>Lactobacillus reuteri</i>
Q74GG3	Sensor histidine kinase	R.LLQEEKLAAIGR.M	<i>Geobacter sulfurreducens</i>
Q74BG5	Cytochrome c family protein	R.IIASATLATGK.T	<i>Geobacter sulfurreducens</i>
Q749B8	Membrane protein, putative	R.TTGGNGIELESR.A	<i>Geobacter sulfurreducens</i>
Q2DP72	Hypothetical protein	R.TIDEIEALK.A	<i>Geobacter uraniumreducens</i>
Q2W4B4	Delta 1-pyrroline-5-carboxylate dehydrogenase	R.TIAEALDNAR.A	<i>Magnetospirillum magneticum</i>
T47114	probable 3-carboxy-cis,cis-muconate cycloisomerase	K.DLDLSDLTDPAR.Y	<i>Streptomyces</i> sp
Q7W341	Haloacid dehalogenase-like hydrolase	K.AAEIEGVR.M	<i>Bordetella parapertussis</i>
Q3W351	Hypothetical protein	R.AAGAGGPAPR.S	<i>Frankia</i> sp
Q2J4T7	Hypothetical protein	R.AEDIEALAESVR.L	<i>Frankia</i> sp
Q46QD7	Pyruvate ferredoxin/ flavodoxin oxidoreductase	R.DELDTVQR.E	<i>Alcaligenes eutrophus</i>
Q46QD7	Pyruvate ferredoxin/ flavodoxin oxidoreductase	R.KQIESMFEQNVV.L	<i>Alcaligenes eutrophus</i>
Q475J2	Phosphoglycerate kinase	K.TIIDIMAKR.G	<i>Alcaligenes eutrophus</i>
Q1LL95	NADH:flavin oxidoreductase/NADH oxidase	R.IVDFVHANGDTK.I	<i>Alcaligenes eutrophus</i>

**Table 9.** Peptides of proteins from methanotrophic and methanogenic microorganisms that could play a role in methane anabolism and catabolism, detected in TAN-35 (08/2007)\*\* sample.

Accession	Protein	Peptide	Species
Q25BV2_9GAMM	pMMO B	R.AGSWIGGQLVPR.S (28-74)	<i>Methylochromium</i> sp. NI.
Q25BV2_9GAMM	pMMO B	R.TIHWFDLNWSK.D (22)	<i>Methylochromium</i> sp. NI.
Q25BV2_9GAMM	pMMO B	R.LADLIYDPDSR.F (7-44)	<i>Methylochromium</i> sp. NI.
Q9KX36_9RHIZ	pMMO B	R.VSFLNAGEPGPVLVR.T (69)	<i>Methylocystis</i> sp. M.
Q9C4C1_9EURY*	Methyl-coenzyme M reductase alpha subunit	R.GPNEPGGLSFGFLADIVQSHK.K (2-59)	Uncultured methanogen MRE-ME4
Q9C4C1_9EURY	Methyl-coenzyme M reductase alpha subunit	K.YPTTLEDHFHGGQR.A (31-86)	Uncultured methanogen MRE-ME5
Q2AAD9_9ARCH	Methyl-coenzyme M reductase alpha subunit	R.GPNEPGGLSFGHMADIVQTSR.K (8-150)	Uncultured Archaeon
Q75NC8_9ARCH	Methyl-coenzyme M reductase alpha subunit	R.GPNEPGGLSFGHMSDIVQTSR.K (62-99)	Uncultured Archaeon
MCRA_METBF	Methyl-coenzyme M reductase subunit alpha (EC 2.8.4.1)*	K.TSWQAIHIPTIVSR.T (16-25)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804)
MCRA_METBF	Methyl-coenzyme M reductase subunit alpha (EC 2.8.4.1)	R.GPNEPGGLSFGHLSDIVQTSR.V (14)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804)
MCRG_METBF	Methyl-coenzyme M reductase subunit gamma (EC 2.8.4.1)	R.GVDPGTLGSR.Q (26-36)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804).
MCRG_METBF	Methyl-coenzyme M reductase subunit gamma (EC 2.8.4.1)	K.RTTIYR.V (14)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804).
MCRG_METBF	Methyl-coenzyme M reductase subunit gamma (EC 2.8.4.1)	R.LQEDGVMFDMLDR.R (71)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804).
Q46FN1_METBF	Gas vesicle protein	R.VVVASVDTFLLHYAEEITK.I (8-90)	<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804)
Q3AQ85_CHLCH	Adenylylsulfate reductase, alpha subunit (EC 1.3.99.1)	R.MTTVEGLFTAGDGVGASGHK.F (65-90)	<i>Chlorobium chlorochromatii</i> (strain CaD3)
Q3AQ85_CHLCH	Adenylylsulfate reductase, alpha subunit (EC 1.3.99.1)	K.FSSGSHAEGR.I (13)	<i>Chlorobium chlorochromatii</i> (strain CaD3)
Q3AQ84_CHLCH	Adenylylsulfate reductase, beta subunit	K.ICPQQAIEVR.G (52)	<i>Chlorobium chlorochromatii</i> (strain CaD3)
Q3N5Z0_9DELT	AMP-dependent synthetase and ligase	R.ITVLTGLPTAYR.A (22-56)	<i>Syntrophobacter fumaroxidans</i> MPOB
Q3MYV1_9DELT	Formate--tetrahydrofolate ligase (EC 6.3.4.3)	K.YIEVTAITPTLGEK.S (66-70)	<i>Syntrophobacter fumaroxidans</i> MPOB
Q3N5E5_9DELT	PAS precursor	K.VSEALQGIIR.D (45-48)	<i>Syntrophobacter fumaroxidans</i> MPOB
Q3MZZ7_9DELT	Hypothetical protein	R.ETSALVQALR.T (45-48)	<i>Syntrophobacter fumaroxidans</i> MPOB
Q2LR05_SYNAS	ATP synthase beta chain (EC 3.6.3.14)	K.VIDLLVPFPR.G (39-46)	<i>Syntrophus aciditrophicus</i> (strain SB)
Q2LR05_SYNAS	ATP synthase beta chain (EC 3.6.3.14)	R.FTQAGSEVSALLGR.M (75)	<i>Syntrophus aciditrophicus</i> (strain SB)
Q1XEC6_9BACT	Sulfite reductase, dissimilatory-type alpha subunit (EC 1.8.99.3)	K.GWDLGGSGSAMR.T (16)	uncultured sulfate-reducing bacterium
Q1XEC6_9BACT	Sulfite reductase, dissimilatory-type alpha subunit (EC 1.8.99.3)	K.AYAGGEIKPAGGGTIYTK.L (36)	uncultured sulfate-reducing bacterium

**Table 10.** Methanotrophic cell numbers calculated for concentrated TAN water samples using qPCR assays and direct microscopic counts.

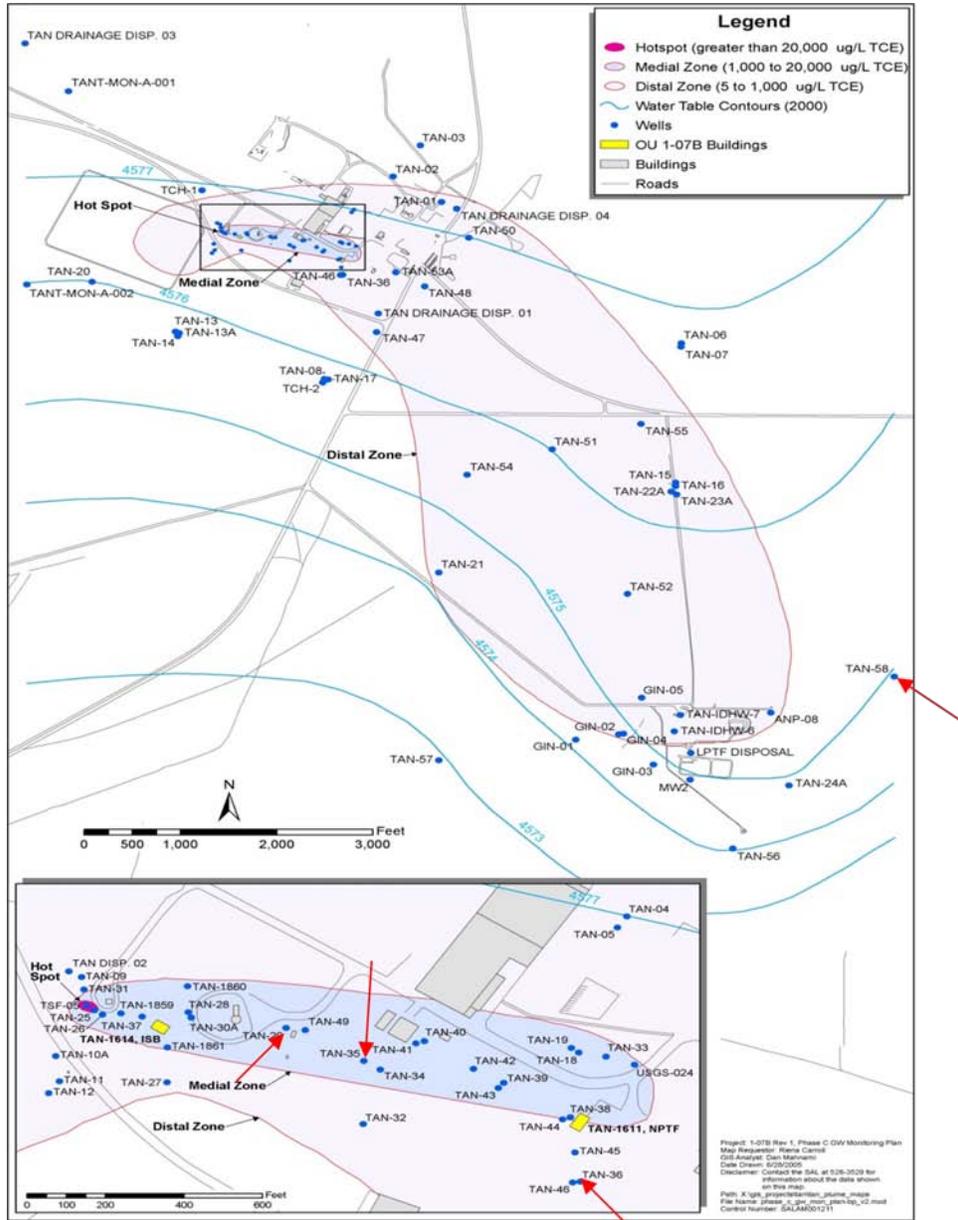
Primers	Sample analyzed	Number of Methanotrophs estimated (methanotrophs/mL)	Total cell counts (cells/mL)	% Methanotrophs
189f (5'-GGNGACTGGGACTTCTG-3') and mb661r (5'-CCGGMGCAACGTCYTTACC-3')	TAN-29_2006	5.25 x 10 <sup>6</sup>	6 x 10 <sup>8</sup>	0.875
	TAN-29_2007	1.42 x 10 <sup>6</sup>	3.6 x 10 <sup>7</sup>	3.94
	TAN-35-1	undetected	N/A	
	TAN-35-2	undetected	N/A	
	TAN-35-3	undetected	N/A	
	TAN-35-4	undetected	N/A	
	TAN-35-5	2.01 x 10 <sup>4</sup> methanotrophs/ g	N/A	
	TAN-35-6	undetected	N/A	
	TAN-36	4.57 x 10 <sup>4</sup>	8.7 x 10 <sup>5</sup>	5.25
	TAN-58	4.40 x 10 <sup>4</sup>	6.5 x 10 <sup>5</sup>	6.77
UP 1 (basalt)	8.5 x 10 <sup>3</sup> methanotrophs/ g	N/A		
536f (5'-CGCTGTGGAAGGGCATGAAGCG-3') and 898r (5'-GCTCGACCTTGAAGCTGGAGCC-3')	TAN-29_2007	5.44 x 10 <sup>3</sup>	3.6 x 10 <sup>7</sup>	0.015
	TAN-35-1	undetected	N/A	
	TAN-35-2	5.29 methanotrophs/g	N/A	
	TAN-35-3	8.20 methanotrophs/g	N/A	
	TAN-35-4	2.43 x 10 <sup>1</sup> methanotrophs/g	N/A	
	TAN-35-5	7.54 methanotrophs/g	N/A	
	TAN-35-6	4.17 methanotrophs/g	N/A	
	TAN-35 planktonic	4.02 x 10 <sup>3</sup>	3.2 x 10 <sup>5</sup>	1.26
	TAN-36	6.58 x 10 <sup>2</sup>	8.7 x 10 <sup>5</sup>	0.076
	TAN-58	1.43 x 10 <sup>3</sup>	6.5 x 10 <sup>5</sup>	0.22
<i>mx</i> a f1103 (5'-GCGGCACCAACTGGGGCTGGT-3') and <i>mx</i> a r1561 (5'-GGGCAGCATGAAGGGCTCCC-3')	TAN-29_2007	1.08 x 10 <sup>4</sup>	3.6 x 10 <sup>7</sup>	0.59
	TAN-35-1	undetected	N/A	
	TAN-35-2	3.73 <i>mx</i> a copies/g	N/A	
	TAN-35-3	undetected	N/A	
	TAN-35-4	undetected	N/A	
TAN-35-5	99.81 <i>mx</i> a copies/g	N/A		

TAN-35-6	undetected	N/A	
TAN-36	$9.48 \times 10^2$	$8.7 \times 10^5$	0.11
TAN-58	$1.30 \times 10^3$	$6.5 \times 10^5$	0.2

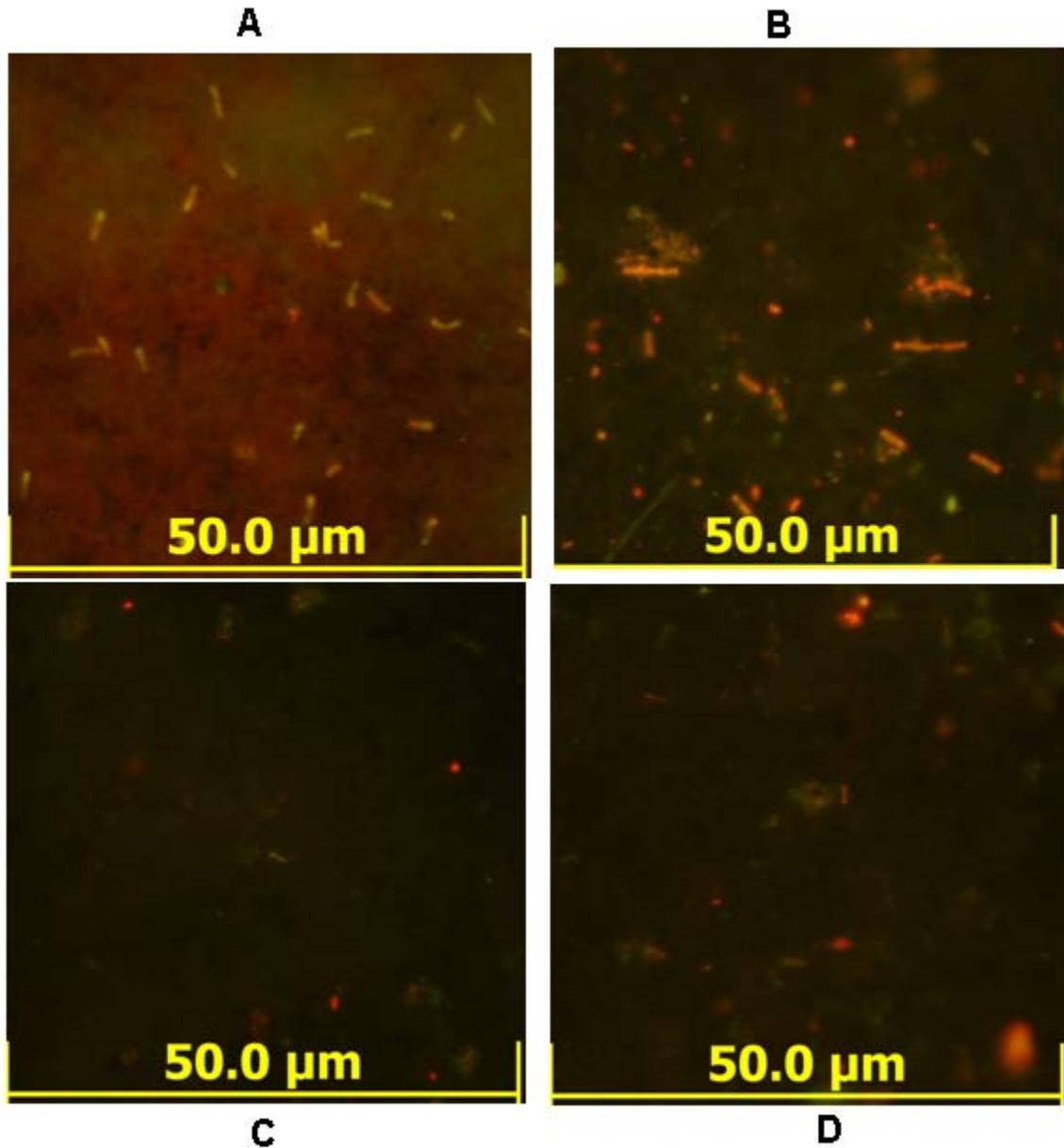
**Table 11.** Geochemical parameters at selected TAN wells

TAN well	TCE (µg/L)	Volume (liters)	Methane (µg/L)	DO (µg/L)	Chloride (mg/L)	Nitrite (mg/L)	Sulfate (mg/L)	Nitrate (mg/L)	Ammonium (mg/L)
TAN-29_2006	407	784	1725	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*	1935	1-7*	N/A	N/A	N/A	N/A	N/A	N/A
TAN-36	120	3197	125	>1000	65.16	1.46	21.67	1.35	2.0

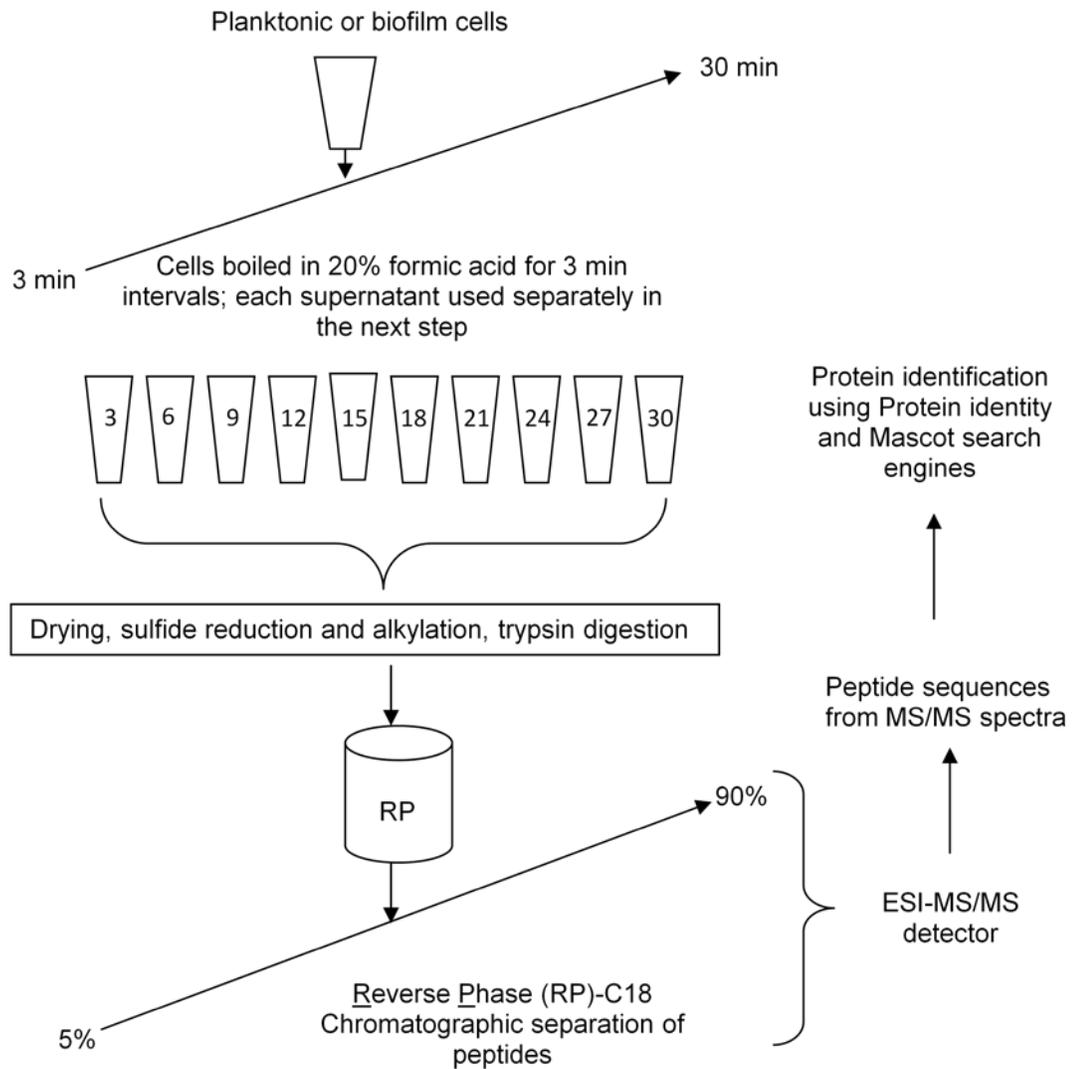
**Figure 1.** TCE plume in the Snake River Plain Aquifer and the wells used to monitor groundwater conditions. TCE and other liquid wastes were injected into well TSF-05 (near the Hotspot) from 1952 to 1972. The contamination has spread south-east with groundwater flow. Arrows point towards the wells from which planktonic and basalt biofilm 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 was collected from TAN-35.



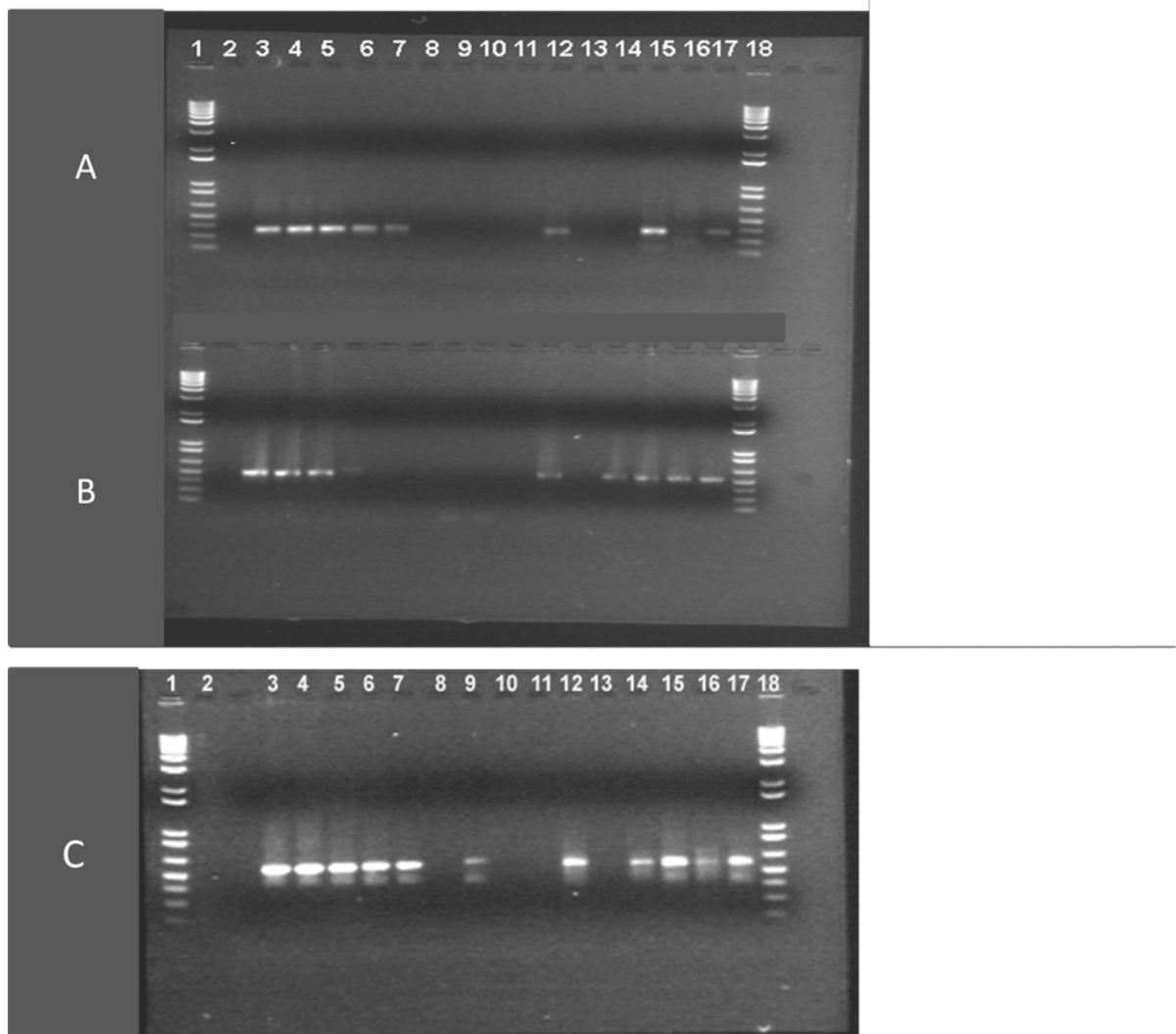
**Figure 2.** Enumeration of planktonic bacteria using acridine orange direct counts. Acridine orange stained cells from a pure culture of *M. trichosporium* OB3b (A), a TAN-29 planktonic sample (B), TAN-36 sample (C) and a TAN-58 sample (D). TAN-35 data are not shown. TAN planktonic samples show numerous cell clusters



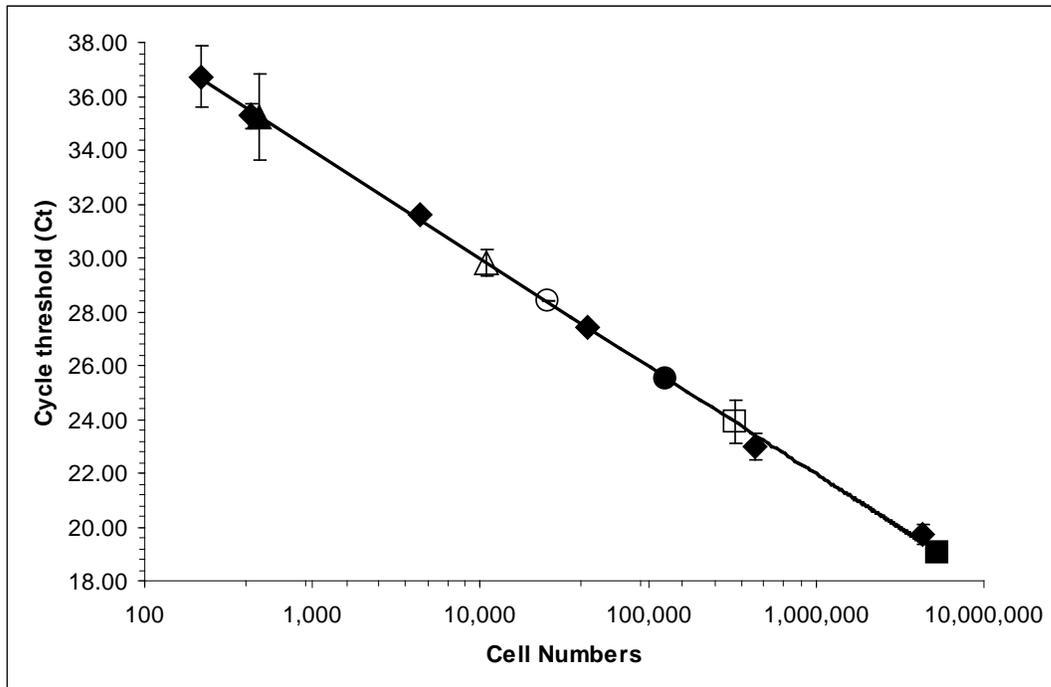
**Figure 3.** Extraction and identification of proteins. Pure culture, planktonic, and basalt biofilm samples were incubated in a boiling water bath and the supernatant from each time point was analyzed separately. The method included the following steps. **1)** Cells boiled in 20% formic acid for 3 min intervals and each supernatant used in the next step. **2)** Vacuum dry to evaporate formic acid. **3)** Resuspend the cell pellets in 100 mM ammonium bicarbonate (pH 8). **4)** Add 10 mM dithiothreitol (DTT) and incubate at 50°C for 15 min. **5)** Add 20 mM iodoacetamide (IAM) and incubate in dark at room temperature for 15 min. **6)** Trypsin (100 ng/μl) digestion (1:100 ratio). **7)** Analysis using UPLC ESI MS/MS. The mass spectral data generated from ESI-MS/MS was analyzed using Protein identity and Mascot software. Proteins were identified by BLAST searching peptides sequences against the Swissprot, MSDB or TREMBL databases.



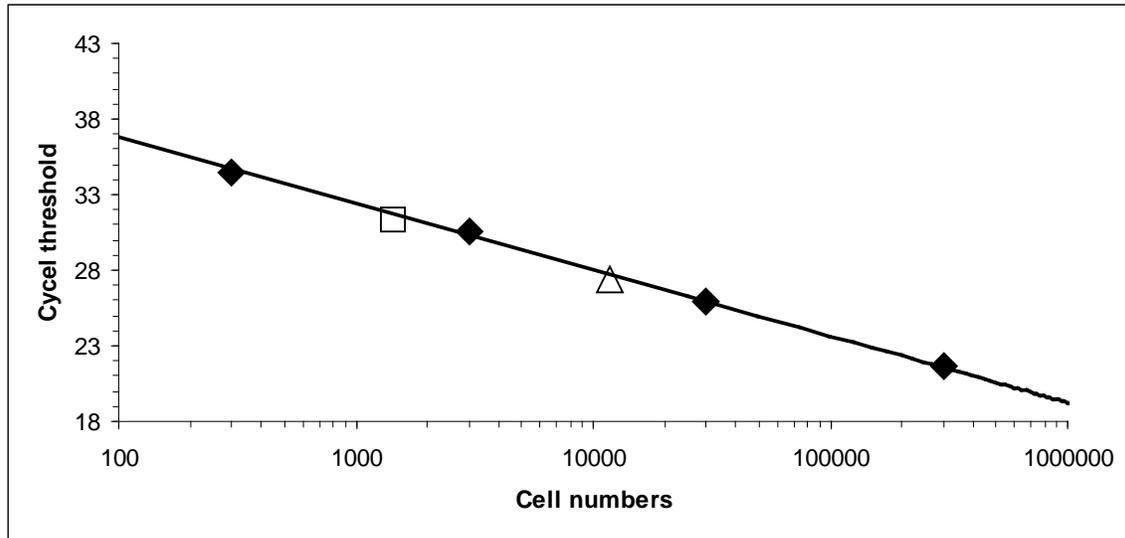
**Figure 4.** Ethidium bromide stained agarose gel showing qPCR products of *Methylosinus trichosporium* OB3b culture and samples from TAN. A) 369bp qPCR products using sMMO primers (536f/898r) B) 472bp qPCR products using pMMO primers (189f/mb661r) C) 557bp qPCR products using methanol dehydrogenase primers (mxaf1103/mxar1561). Lane 1: 1 KB+ ladder Lane 2: NTC (no template control) Lane 3-7:  $1.64E+05$  to  $1.64E+01$  OB3b cells Lane 8-13: TAN 35 basalt #1-6 Lane 14: TAN 35 basalt (1-9) Lane 15: TAN29 planktonic Lane 16: TAN36 planktonic Lane 17: TAN58 planktonic Lane 18: 1KB+ ladder.



**Figure 5 A.** Estimation of methanotrophic bacteria in TAN samples using qPCR with pMMO specific primers. (♦) Represents qPCR standards of *M. trichosporium* OB3b dilutions; (■) - TAN 29\_2006, Ct 19.07,  $5.25 \times 10^6$  methanotrophs/mL of sample; (□) - TAN 29\_2007 Ct 23.90,  $3.36 \times 10^5$  methanotrophs/mL sample; (▲) - TAN 35, Ct 35.24, 32.11 methanotrophs/ g of basalt sample; (Δ) TAN 36, Ct 29.81,  $1.1 \times 10^4$  methanotrophs/ mL of sample; (○) - TAN 58, Ct 28.37,  $2.5 \times 10^4$  methanotrophs/mL; (●) - UP 1, Ct 25.53,  $8.5 \times 10^3$  methanotrophs/g of basalt sample.



**Figure 5B.** Estimation of methanotrophic bacteria in TAN samples using qPCR with sMMO specific primers. (◆) Represents qPCR standards of OB3b dilutions; (□) - TAN 29, Ct 31.33, 55.07 methanotrophs/mL sample; (Δ) - TAN35, Ct 24.20  $3.1 \times 10^3$  methanotrophs/g of basalt; Significance value of the graph  $R^2 = 0.991$ .



**Figure 6.** Quantification of methanotrophic cells using specific peptide MS signal intensity. The cumulative intensity of the peptide “VSFLNAGEPGPVLVR” (●) obtained from the time-course hydrolysis of serial dilutions of *M. trichosporium* OB3b cells ( $10^2$  to  $10^5$ ). 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. (Δ) represents peptide intensity of TAN-29\_2006 ( $3.9 \times 10^4$  methanotrophs/mL); (○) represents peptide intensity TAN-29\_2007 sample ( $2.4 \times 10^4$  methanotrophs/mL); (□) represents peptide intensity from TAN-58 sample ( $9.4 \times 10^3$  methanotrophs/mL); (■) represents peptide intensity from TAN-36 sample ( $3.7 \times 10^3$  methanotrophs/mL)

