Poster number: C13B-0611

Identifying active CH_4-oxidizers in thawed Arctic permafrost by proteomics

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The rate of CH_4 release from thawing permafrost in the Arctic has been regarded as one of the determining factors on future global climate. It is uncertain how indigenous microorganisms would interact with such changing environmental conditions and hence their impact on the fate of carbon compounds that are sequestered in the cryosol. Multitudinous studies of native surface cryosol (top 5 cm) and microcosm experiments have provided growing evidence of effective methanotrophy (Fig. 2A&B).

Cryosol samples corresponding to the active layer were sampled from a sparsely vegetated, ice-wedge polygon at the McGill Arctic Research Station at Axel Heiberg Island, Nunavut, Canada (N79°24, W90°45) before the onset of annual thaw (Fig. 1). Pyrosequencing of 16S rRNA gene indicated the occurrence of methanotroph-containing bacterial families as minor components (~5%) in native cryosol including *Bradyrhizobiaceae*, *Methylobacteriaceae* and *Methylocystaceae* belonging to the alpha-Proteobacteria, and *Methylacidiphilaceae* within the Verrucomicrobia (Fig. 3). The potential of methanotrophy is supported by preliminary analysis of metagenome data, which detected the presence of putative methane monooxygenase gene (MMO) sequences related to *Bradyrhizobium* sp. and *Pseudonocardia* sp. (Fig. 4). Proteome profiling of native cryosols in general yielded minute traces of proteins, which likely hints at the dormant nature of the cryosol microbial consortia. The lack of a specific database for permafrost posed an additional challenge to protein identification. Microcosms exhibited a net CH_4 consumption of ~65 ng C-CH_4 per gram (fresh weight) of cryosol over 16 days of aerobic incubation at room temperature. Protein extraction and characterization identified 350 proteins from acetate-amended microcosms, whereas only 33 proteins could be identified in the control set. Most of the identified proteins are involved in energy metabolism or post-translational modification of proteins. Although the activity of *Shewanella* sp. was suppressed by the higher acetate concentration, other bacteria were activated. This was shown by at least a 10-fold increase in the number of identified proteins, which were primarily players in cellular energy metabolism. Among them, proteins belonging to the anaerobic Fe^{3+} reducer, *Geobacter* sp. and to methane-oxidizers, *Bradyrhizobium* sp., *Methylosinus* sp. and *Methylocystis* sp. appear dominant. This result indicates incubation experiment enhances microbial activities and causes significant shift in compositions of active community. (Fig. 6).

In order to advance the database for better biodiversity and functional identification, we are currently using two extraction protocols (Fig. 5) and consolidating metagenome data obtained from the same cryosol samples. A depth profile (from active to permafrost layer) for methanotrophs is being determined by examining native cores, thawed cryosols as well as enrichment cultures. The proteome information from these samples will be presented, which will be complemented by molecular studies.

Field site

McGill Arctic Research Station (79°24.918'N, 90°45.424'W)
Axel Heiberg Island Canadian High Arctic

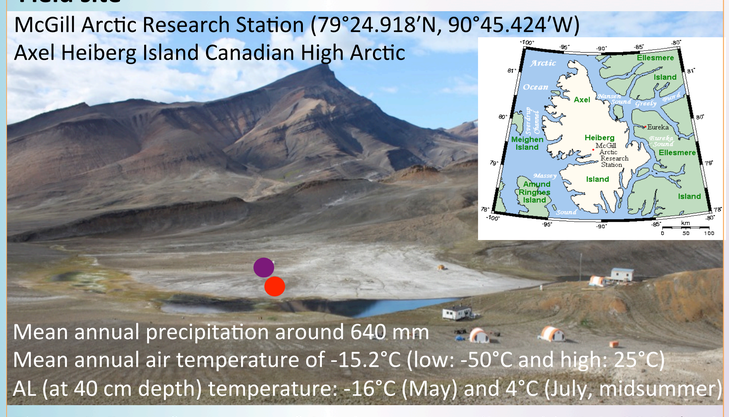


Figure 1. Permafrost cores of 1-m long were collected in July 2010 (red circle) and May 2011 (purple circle) for microcosm, enrichment cultivation and full-core thawing experiments.

Canadian Arctic cryosol is a sink for ambient CH_4

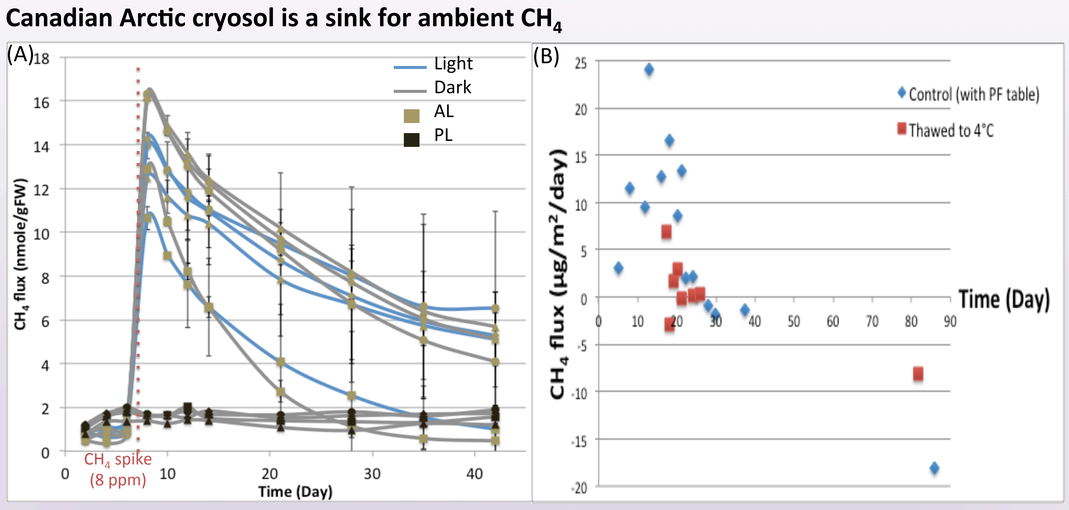
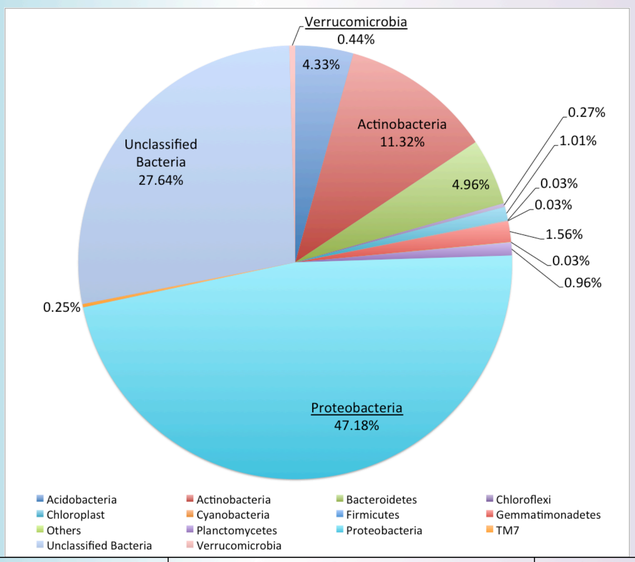


Figure 2. Net CH_4 flux rate in (A) microcosm and (B) full-core thawing experiment. (A) Five grams of active layer, AL, and permafrost layer, PL, were incubated aerobically and anaerobically respectively at 4°C in 24 hour light or darkness with or without amendments. All AL microcosms showed CH_4 consumption whereas all PL microcosms produced CH_4 at a very low concentrations. CH_4 consumption by AL was estimated to be at least 4 times faster than CH_4 production by PL under the experiment conditions. (B) A core was completely thawed and incubated at 4°C whereas the control was thawed just down to the PL table. An initial burst of CH_4 (1,000 µg/m²/day) was recorded in the first 15 days from the fully thawed core (data points omitted for visualization purpose). Rate of CH_4 release into the headspace decreased with time, and eventually the overall system switched to CH_4 uptake. Both experimental setups indicated that the whole system (AL+PL) is a sink for ambient CH_4 even when the top few tens cm of PL is thawed.

DNA-derived methanotrophic communities



Phylum	Genus	Relative Abund.
Verrucomicrobia	*Methylacidiphilum*	0.03%
Alpha-Proteobacteria	*Methylobacterium*	0.06%
	Methylocystis and others	1.59%
	Bradyrhizobium (*B. elkanii* and *B. japonicum*)	3.47%
	Total	5.07%

Figure 3. Bacterial composition of total community DNA in native AL cryosol. Using MOTHUR, phylogenetic affiliation of 3650 16S rRNA gene pyrosequences (V3 region) were identified using RDP classification scheme, based on a confidence threshold of 80%. Underlined phyla contain known methanotrophs, with detailed breakdown in the table.

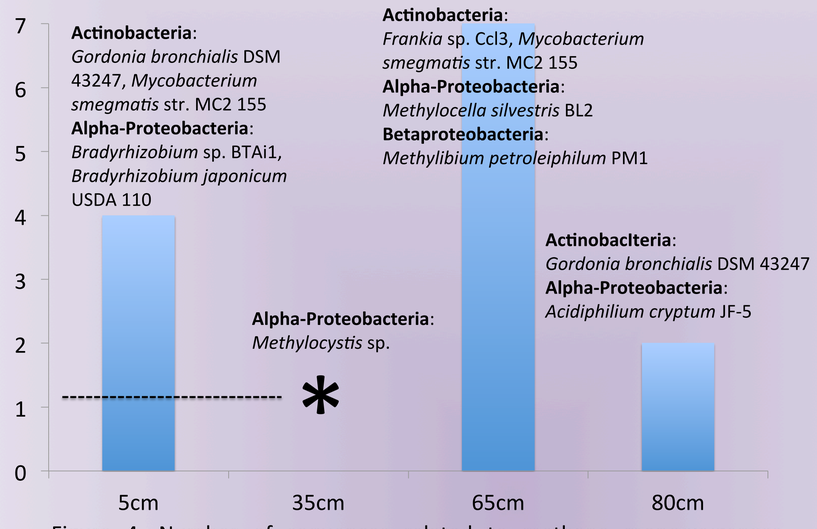


Figure 4. Number of sequences related to methane monooxygenase (MMO) retrieved in metagenome analysis of native cryosol at different depths. Functional identification was performed by comparing the metagenome data to various databases available on MG-RAST. Under the same criteria (max. e-value = 10^{-5}, min. identify = 50 %, and min. align. length = 50 aa), the search against SEED returned with the greatest number of identifiable proteins. * indicates the only result available for sample from 35cm, which was obtained via the search against GenBank. Caution has to be taken when interpreting this result because the presence of MMO genes does not indicate their expression in the environment and, hence, that the organisms hosting these genes are oxidizing CH_4 *in-situ*.

Proteome profiling strategy

Proteins were extracted from the cryosol samples using either the SDS –TCA or phenol/chloroform protocol (as shown in the flowchart). Protein extracts were analyzed via mass spectrometry using LTQ Orbitrap Velos. Peptides were chromatographically separated on biphasic resin column using a 24h MuDPIT setup. The Raw spectra were matched to the various databases (as described in the grey box) using either DBDigger or SEQUEST.

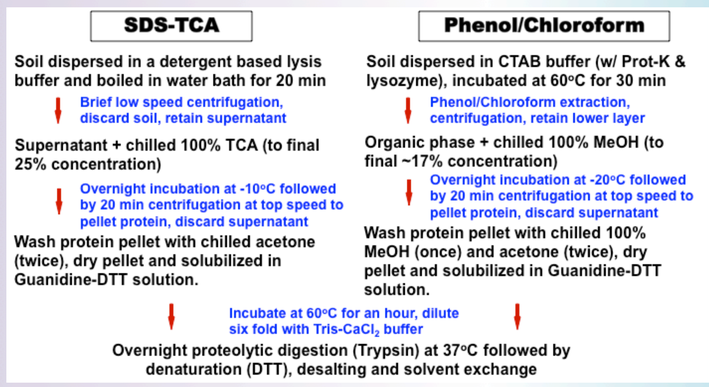


Figure 5. Cryosol protein extraction methodology

Two types of databases (DB) were constructed for protein identification

1) Assorted DBs assembled from completed and annotated genomes
   - Psychrophiles_DB (32 genomes of known psychrophiles downloaded from JGI)
   - Amplicon_DB (340 genomes of species related to indigenous microbial community of this study site as revealed by pyrosequencing)
   - AMO_DB (35 genomes of known anaerobic methane-oxidizers downloaded from JGI)
2) Empirical DBs were employed or constructed from metagenome data
   - AlaskanPF_DB (Cryosol metagenomes generated from Bonanza Creek, Alaska)
   - CanadianArcticPF_DB (Cryosol metagenomes of this study site)

Low protein yield

Table 1. Protein yield from different cryosol samples.

Cryosol samples	Extraction method	Protein Yield (µg / g of cryosol (FW))
Native cryosol	SDS-TCA	30^
AL microcosms	SDS-TCA	10 – 50
Fully-thawed core	Phenol/Chloroform	25
Enrichment cultures	SDS-TCA	500 – 4000

^ Spectra matched to databases with low % coverage and counts

Proteome profile of microcosms

Microcosm experiment was set up to investigate the effect of acetate amendment on microbial community and protein expression. 2.5 g of AL cryosol was amended with acetate (1 mM). Sterilized water of same volume was added to the control account for the enhanced water content. Microcosms, in duplicates, were incubated with filtered air (~1.8 ppmv of CH_4) at 21°C for 16 days without shaking.

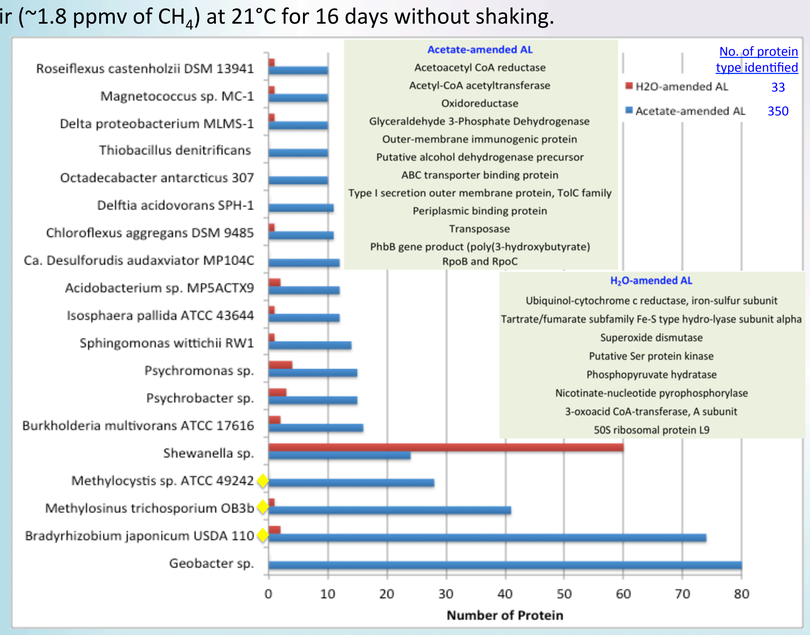


Figure 6. Distribution of protein abundance across different microbial species. Proteins were compared to Psychrophiles_DB and AMO_DB. Proteins that are common to both sets of microcosm are for protein-folding (Hsp 60, Hsp 70), transcription (Elongation Factor-Tu, DNA-dependent RNA polymerase), ATP synthesis (ATP synthase) and alkene reduction (2-alkenal reductase). A list of proteins that are unique to each type of amendment was overlain on the chart. ◆ marks taxa that are known CH_4-oxidizers.

Proteome profile of thawed permafrost core

A 1-m long core was completely thawed to 4°C (in cold room) and 5 g of samples were collected after 7-month incubation in order to characterize the variation in the microbial composition and protein expression across depth after prolonged thawing.

Table 2. Number of proteins identified by different databases

Sample	Amplicon_DB	AlaskanPF_DB	AMO_DB
5 cm	23	31	5
35 cm	21	24	13
65 cm	16	12	7

Results from Amplicon_DB :
- DNA polymerase and Flif M-ring protein were found at all three depths
- A few proteins, such as Precorrin-6x reductase, Hsp70, and Glucokinase, were identified in the 5 and 35 cm samples only.

Results from AlaskanPF_DB:
- DNA polymerase, Phosophoribulokinase, Malate dehydrogenase, Elongation Factor-Tu, Galactokinase, and Alphaglucosidase were found at all three depths.
- Upper cryosol layers had many protein sequences uniquely similar to Alaskan permafrost.

Results from AOM_DB:
- DNA polymerase and alpha-amylase (from *Acidobacterium* spp) were found at all depths.
- At 35 cm depth, many other proteins were also identified such as peptidases, biotin-acetyl CoA carboxylase, Methylmalonyl-CoA carboxylase.

All layers showed evidence of cellular motility and cellular growth. Many enzymes that are involved in glucose-utilizing metabolic pathways were identified.

Microbial taxanomy identified by the different databases were not consistent.

Proteome profile of enrichment cultures

1 g of cryosol was incubated with different medium and incubated at 10°C in order to selectively cultivate microbial consortia. Proteins extracted from high cell density cultures were analyzed and data-processing is in progress.

Conclusions:
- Alpha-proteobacteria, belonging to Type II methanotrophs, play an important role in atm. CH_4 oxidation in Canadian Arctic cryosols.
- Native cryosol layers yielded protein identifications of no confidence, implying dormancy of indigenous microorganisms as well as the insufficiency of species coverage in the databases.
- Microorganisms become metabolically active upon thawing although the activity level remained low.
- Incubation at higher temperature and with additional carbon sources selectively revived the microorganisms and significantly increased the number of identifiable proteins.
- Proteins involved in CH_4-cycling and other biogeochemical catabolic pathways have yet to be identified despite this increased biomass.
- The large percentage of unidentified proteins pointed out the uniqueness of proteins in Canadian Arctic cryosol and underscores the need for in-depth sequencing of the cryosol from this region and the development of a customized database with informative gene annotation if the impact of global warming of microbial processes is to be delineated.

Future work:
- Qualitative and quantitative biodiversity assessments of cryosol samples in various incubation experiments to complement the protein data.
- Expansion of databases to include a wider diversity of organisms, especially fungi and algae.
- Construct databases for specific functional groups, for example, methanogens and methanotrophs.
- Apply proteome profiling strategy to more samples.

Presentation of related research @ AGU:
- Gas fluxes in long-term thawing experiment (Poster #: Monday, C13B-0613)
- Microbial biodiversity (Poster #: Monday, C13B-0620)
- Isotopic analysis of lipids (Talk: B14D, Monday 4:00-4:15pm)
- Modeling of CH_4 flux (Poster #: Tuesday, B21D-0405)
- In-situ gas fluxes (Talk: B42A, Thursday, 10:50-11:05)

Acknowledgement

Research on carbon cycling in Arctic Permafrost was supported by U.S. Department of Energy's Office of Biological and Environmental Research, Genome Sciences Program's Biological Systems Research on the Role of Microbial Communities in Carbon Cycling managed by Dr. Joe Graber, grant number DE-SC0004902 to T.C. Onstott.

Oak Ridge National Laboratory is managed and operated by the University of Tennessee-Battelle, LLC., for U.S. Department of Energy.

AGU Fall Meeting, San Francisco, 3-7 December 2012