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Characterization of Methane Degradation and Methane-Degrading Microbes in Alaska Coastal Waters

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Current State of Knowledge

Methane hydrates and other natural methane deposits in the Arctic potentially could contribute substantially to global atmospheric methane as the planet warms due to climate change. The Arctic is well known to harbor large reservoirs of methane on the continental shelf and in organic-rich permafrost on land. This methane-rich region, which has already experienced higher relative temperatures than any other part of the planet last year, is expected to warm even more in the coming decades (Corell 2006; Steele et al. 2008). Although most methane hydrates are probably beyond the reach of anthropogenic warming, release of methane from this huge source could come to rival that of the terrestrial biosphere (Archer 2007).

This release of methane is potentially ameliorated in part by the oxidation of methane by biotic and abiotic processes. Compilations of methane fluxes, however, often list only "soils" as a sink for atmospheric methane, along with atmospheric chemical oxidation (Canfield et al. 2005) (IPCC 2007), but all natural environments that release methane potentially harbor microbes capable of methane oxidation. A recent study demonstrated that methane oxidation reduced atmospheric release of methane by 85% (Guerin and Abril 2007), and microbial oxidation minimizes methane release from methane bubbles in the Black Sea (Schubert et al. 2006).

In spite of the importance of methane oxidation being widely appreciated, there are very few rate estimates especially for the Arctic (see below). We also know relatively little about the mechanism of methane oxidation and about the microbes that carry out this process. This project seeks to address these huge gaps in our understanding.

Why the coastal Arctic Ocean? - We need to look at the coastal Arctic Ocean because of its large reservoirs of methane and methane hydrates and because of its sensitivity to climate change. In addition to direct effects of temperature on methane hydrate stability, increasing temperature has contributed to decreasing sea ice in the Arctic over the last decade (Serreze et al. 2007), which could lead to even higher methane fluxes to the atmosphere in the near future. Methane concentrations have been observed to be higher in the winter than in summer in the Beaufort Sea perhaps due to ice capping the water column and preventing exchange with the atmosphere (Kvenvolden et al. 1993). An alternative hypothesis is that high methane oxidation in the summer reduces methane concentrations and minimizes release to the atmosphere. The two explanations cannot be tested without the estimates of methane oxidation for the Arctic to be made by this project.

It is now difficult to predict how methane fluxes to the atmosphere will change with climate change in the Arctic. This project will fill in critical gaps in our understanding of methane oxidation and improve models for this important system.

Microbes, Enzymes and Genes Involved in Aerobic Methane Oxidation - Aerobic methanotrophic bacteria can be classified into two broad groups that appear to vary with environmental conditions, including methane concentrations. In the Barents Sea, aerobic methanotrophs made up about 50% of all microbes in areas of intense methane seeps (Losekann et al. 2007). FISH assays revealed a correlation between the abundance of Type I and II aerobic methanotrophs and methane concentrations in permafrost soils of

Siberia (Liebner and Wagner 2007). Methanotroph abundance data have only rarely been coupled with data on methanotroph diversity and activity (Carini et al. 2005), such as what will be done by this project.

The initial step of methane degradation consists of the oxidation of methane to methanol. This step is catalyzed by the enzyme methane monooxygenase, which is found in both bacterial groups (Hakemian and Rosenzweig 2007). We need more data on the types of microbes and enzymes involved in methane oxidation.

Limitations of Existing Knowledge or Technology

There are very few estimates of methane degradation and methanotrophy in high latitude environments. We were able to find only four published studies that estimated methane degradation directly for the Arctic and only one, in the Chukchi Sea (Savvichev et al. 2007), somewhat close to the study region in the Beaufort Sea. The paucity of rate estimates makes it difficult to determine whether methane cycling in the Arctic differs substantially from that in low latitude environments.

Most of the work examining uncultured aerobic methanotrophs in natural environments has focused on a gene encoding a subunit of the particulate methane monooxygenase (*pmoA*) (Mcdonald et al. 2008). It appears that the phylogeny of this gene follows 16S rRNA phylogeny and of the entire organism (Mcdonald et al. 2008), which implies that diversity, abundance, and expression (mRNA) of *pmoA* can be used to deduce the diversity, abundance, and relative activity (at the mRNA level) of aerobic methanotrophy.

The diversity of *pmoA* in the Arctic has been examined only rarely (Jugnia et al. 2006; Pacheco-Oliver et al. 2002), one reason why this work is so essential. We are especially interested in comparing *pmoA* diversity in the Arctic with other high methane flux environments at lower latitudes. A study in the Gulf of Mexico found several *pmoA* types apparently unique to gas hydrates (Yan et al. 2006). Other studies described variation in *pmoA* diversity depending on methane sources, such as cold seeps (Inagaki et al. 2004). The work will determine if Arctic methanotrophs (*pmoA* genes) are similar to their low latitude counterparts.

Although relationships between *pmoA* diversity and methanotrophy have been found, the presence of *pmoA* genes does not necessarily mean that methanotrophs with those genes are actively carrying out methane degradation. The expression (mRNA synthesis) of that gene is closer to actual metabolic activity. Expression of *pmoA* has been examined in a few environments (Mcdonald et al. 2008). In forest soils, for example, some *pmoA* types were found to be present (detection of DNA) but apparently not active (no detectable mRNA) (Kolb et al. 2005). The paucity of data on *pmoA* expression is another argument for this project.

Development Strategies

We will examine methane degradation and methanotrophic microbes in Arctic coastal sediments and the water column on an expedition planned for August 2009, organized by NRL colleagues. This region of the Alaskan coast was selected because of high methane hydrate loadings found in the Mallik Wells on the tundra near the

Mackenzie Delta and the development of an Arctic tundra hydrate energy exploration established by BP Amoco in Alaska.

Summary of work - The overall goal of the project is to obtain a better understanding of methane degradation in order to improve predictive models of methane fluxes and how these fluxes may change with climate change.

1. *How does methane degradation depend on methane concentrations and methane hydrates?* **Hypothesis:** Methane degradation will correlate with methane concentrations, but not necessarily with the presence or extent of methane hydrates.

Methane concentrations and degradation rates will be measured in areas with and without methane hydrates located by multi-channel seismic profiling (to be done by NRL). Other geochemical data, including sulfate and sulfide concentrations and stable isotopes, will give further insights into the controls of methane degradation

2. What is the time-dependent response of methanotrophic microbes and methane degradation to changes in methane fluxes? **Hypothesis:** The abundance of methanotrophs and of key methane degradation genes (*pmoA* and *mcrA*) correlate only loosely with methane concentrations and will not change substantially on the hour to day time scale while methane degradation and expression of *pmoA* and *mcrA* genes are tightly coupled to methane concentrations.

Cell abundance of both aerobic and anaerobic methanotrophs will be estimated by microautoradiographic detection of cells incorporating ^{14}C-methane and by FISH. The abundance and transcript levels of *pmoA* and *mcrA* will be measured with Q-PCR. Samples from these assays will be taken from regions with and without methane hydrates with varying levels of methane concentrations.

3. How do methane degradation rates and key methane degradation genes (pmoA and mcrA) in the Arctic and low latitudes compare? **Hypothesis:** The methane degradation genes will be similar in the Arctic and low latitude environments and will vary more because of oxygen and methane concentrations than latitude.

The methane degradation rates from this project will be compared to published estimates in low latitude environments. Similarly, sequences of the methane degradation genes will also be compared.

Methane degradation - Microbial methane degradation in the water column will be determined using the approach that we have used previously in the Arctic. Seawater collected using Niskin bottles will be dispensed into serum vials. The vials will be sealed with rubber septa and radiolabeled methane will be injected through the septa using a gas-tight syringe. Killed controls will be poisoned with 2% formaldehyde. Incubations will be conducted at in situ temperature in the dark for 24 h. Uptake of ^{14}C-methane into particulate material will be determined by filtering the sample through a 0.45 µm pore

size nitrocellulose filter. The filtrate will be collected in an Erlenmeyer flask fitted with a cup holding a phenethylamine-saturated filter and sealed with a rubber stopper. Respiration of ^{14}C methane will be determined from the amount of radioactivity collected in the base trap after 48 h. The recovery efficiency of $^{14}CO_2$ using this approach is typically 96%. Radioactivity on the filters processed for uptake and respiration is determined by liquid scintillation counting.

Methane degradation rates in sediments will be determined using a modification of the procedure used for the water column. Anaerobic sediments will be handled in an anaerobic glove bag to minimize exposure to oxygen. Sediments collected by gravity coring will be sub-sampled using 10ml syringes that have had the tips cut away, exposing the full diameter of the syringe barrel. Methane concentrations will be determined by GC analysis of sub-samples taken from the equilibrated headspace. ^{14}C-methane will be added through the septum for the methane degradation assays. The vials will be incubated in the dark at in situ temperature for 24-h in parallel with formaldehyde killed controls. After the incubation the sediment slurry will be transferred to an Erlenmeyer flask fitted with a base trap and stopper. The sample will be acidified to capture the respired CO_2 in the base trap. The respiration of methane will be determined using liquid scintillation counting to measuring $^{14}CO_2$ in the base trap.

Methane degradation gene abundance and expression - DNA and RNA will be extracted using the CTAB method for extracting DNA and RNA. To determine diversity of methane degradation genes, known primers will be used in PCR experiments, and the amplified PCR product will then be cloned. The clone libraries will provide general information about the community structure, i.e. what types of genes are present in a particular location and season. One or more of the clones will also be useful as a positive control for other experiments.

Quantitative real-time PCR (qPCR) will be used to obtain the gene abundance and expression data for testing hypotheses about the time-dependent response of methanotrophic microbes to changes in methane fluxes. The qPCR assay will be used to determine the abundance of key methanotrophy genes in DNA extracted from sediment samples incubated with different levels of methane additions. In addition, expression levels of these genes will be quantified using RNA extracted from the same samples. In the latter case, the RNA will first be reverse transcribed back into DNA (cDNA) using a general primer. Gene specific primers will then be used to target the genes of interest in the DNA or cDNA.

Primers specific to the qPCR process for methane-oxidizing archaea have been developed by other groups (Kolb et al. 2003). These previously designed primers will be tested *in silico* using the clone library. If they do not successfully amplify specific genes in the library, information from the library will then be used to design new primers. Assuming that representatives from the *Archaea* and proteobacteria are found, specific primers designed specifically for each group will be used to quantify the abundance of the methane oxidizers in each group.

Single-cell analysis of methanotrophs - We will identify methane-assimilating microbes using incubations with ^{14}C methane and fluorescence in situ hybridization (FISH). Using this approach we are able to identify cells actively assimilating ^{14}C-methane as those cells

depositing silver grains in autoradiographic emulsion. The same cells are probed with fluorescent FISH probes targeting 16S rRNA to determine their phylogenetic identity.

Future

Our first goal of this project is to estimate methane degradation as a function of methane concentrations and other key biogeochemical properties, such as sulfate and oxygen concentrations. The main challenge here will be to obtain enough sediment samples that vary naturally in the key biogeochemical properties of interest. Our work will stand the best chance of meeting this challenge if the field sampling provides an adequate number of sediment cores collected within the study area. We anticipate that variation in biogeochemical properties with depth within sediment cores will also deliver much of the needed natural variation.

The second goal is to determine the time-dependent response of methane degradation and of the capacity for methane degradation (the methanotrophic community structure) to varying methane fluxes. The main challenge to attaining the second objective is identifying regions within the sampling area that differ in methane fluxes. Sampling over a wide geographic range within the study area will offer the best options for attaining this objective.

The third objective is to compare and contrast methane degradation rates and methane-degrading microbes in the Arctic with low-latitude environments. The main challenge to overcome in achieving this objective is obtaining a comprehensive identification of methane-degrading microbes. Attaining this goal is dependent on obtaining high quality nucleic acid samples from sediments for molecular biological analysis. We will apply the most effective methods for eliminating the inhibitory compounds in sediments that can inhibit such molecular analyses.

Potential impact of the research

The results of this work should help improve models for predicting methane fluxes in a globally changing environment. This project will add substantially to the data set on methane degradation rate estimates and molecular characterization of methanotrophic microbes. Both types of data are not extensive in any environment, but the Arctic is especially under sampled even though the presence of large methane reserves is well known.

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