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

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Abstract

The net flux of methane from methane hydrates and other sources to the atmosphere depends on methane degradation as well as methane production and release from geological sources. The goal of this project was to examine methane-degrading archaea and organic carbon oxidizing bacteria in methane-rich and methane-poor sediments of the Beaufort Sea, Alaska. The Beaufort Sea system was sampled as part of a multi-disciplinary expedition ("Methane in the Arctic Shelf" or MIDAS) in September 2009. Microbial communities were examined by quantitative PCR analyses of 16S rRNA genes and key methane degradation genes (*pmoA* and *mcrA* involved in aerobic and anaerobic methane degradation, respectively), tag pyrosequencing of 16S rRNA genes to determine the taxonomic make up of microbes in these sediments, and sequencing of all microbial genes ("metagenomes"). The taxonomic and functional make-up of the microbial communities varied with methane concentrations, with some data suggesting higher abundances of potential methane-oxidizing archaea in methane-rich sediments. Sequence analysis of PCR amplicons revealed that most of the *mcrA* genes were from the ANME-2 group of methane oxidizers. According to metagenomic data, genes involved in methane degradation and other degradation pathways changed with sediment depth along with sulfate and methane concentrations. Most importantly, sulfate reduction genes decreased with depth while the anaerobic methane degradation gene (*mcrA*) increased along with methane concentrations. The number of potential methane degradation genes (*mcrA*) was low and inconsistent with other data indicating the large impact of methane on these sediments. The data can be reconciled if a small number of potential methane-oxidizing archaea mediates a large flux of carbon in these sediments. Our study is the first to report metagenomic data from sediments dominated by ANME-2 archaea and is one of the few to examine the entire microbial assemblage potentially involved in anaerobic methane oxidation.

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Executive summary

Methane hydrates have attracted much interest because of their potential as commercially-viable energy deposits and as a substantial potential contributor to climate change. The methane hydrates in the Arctic are especially important to examine for both reasons. The net flux of methane from methane hydrates and other sources to the atmosphere depends on methane degradation as well as methane production and release from geological sources. The goal of this project was to examine microbes and organic carbon degradation in methane-rich and methane-poor sediments of the Beaufort Sea, Alaska. These sediments and the water column were sampled as part of a multi-disciplinary cruise ("Methane in the Arctic Shelf" or MIDAS) in September 2009. Little is known about the main biotic process degrading methane in these sediments: anaerobic oxidation of methane. This process is thought to be mediated by archaea in close association with sulfate-reducing bacteria.

Microbial communities were examined by quantitative PCR analyses of 16S rRNA genes and key methane degradation genes (*pmoA* and *mcrA* involved in aerobic and anaerobic methane degradation, respectively), tag pyrosequencing of 16S rRNA genes to determine the taxonomic make up of microbes in these sediments, and sequencing of all genes ("metagenomes") of all microbes. The metagenomic data were used to examine potential methane-degrading organisms and other microbes (the "anaerobic food chain") involved in the anaerobic degradation of the entire organic carbon pool.

The taxonomic and functional make-up of microbial communities varied with methane concentrations in these cores. Bacteria made up 77% to 97% of all microbes, while archaea were 0.3-22%, depending on the method. The metagenomic approach suggested higher abundance of archaea in the methane-rich layers than in the methane-poor layers (8-9% vs. 3.3%), but this was not seen with two other approaches. Although methane-rich and methane-poor sediments had similar abundances of bacteria, archaea, and eukaryotes, they differed when examined at a taxonomic level closer to species, i.e. when the 16S rRNA genes were grouped at the $\geq$97% similarity level. A cluster analysis indicated that the low methane communities were quite different from the communities in the high methane sediments. When normalized for total sequences, we found over 5-fold more Methanosarcinales (the archaeal class containing the known anaerobic methane oxidizers) in the methane-rich sediments than in the methane-poor sediments (32.3 ± 4.9 vs. $6.0 \pm 2.3 \times 10^{-4}$ Methanosarcinales sequences per total metagenome sequences), although there was no obvious trend with depth. In contrast, sulfate reducers decreased with depth, probably due to a decrease in labile organic material down the sediment core. In support of that hypothesis, the relative abundance of Deltaproteobacteria (the bacterial class containing many sulfate reducers) was positively correlated with percent organic carbon in these sediments ($r=0.57$, $n=37$, $p<0.001$). Sequence analysis of PCR amplicons revealed that most of the *mcrA* genes were from the ANME-2 group of methane oxidizers.

Methane degradation and other degradation pathways were explored in further detail using metagenomic data from five depths in a methane-rich core. These genes changed along the depth profile as suggested by the taxonomic and biogeochemical data. Two important parts of the anaerobic food chain, however, did not vary significantly with depth. Genes for fermentation and acetogenesis were equally abundant through the sediment core profile. As expected from the other data, sulfate reduction genes decreased with depth while the anaerobic methane degradation gene (*mcrA*) increased along with methane concentrations.

Some of these genes were as abundant as expected while the relative abundance of others was surprising. The total number of fermentation genes was about equal to the single copy gene frequency, indicating the high abundance of fermenting bacteria in these sediments and the importance of this pathway in the anaerobic degradation of organic material. The number of sulfate reduction genes (roughly 10%) was lower than expected from the tag pyrosequence data (about 20% which is the percentage of Deltaproteobacteria in the total community). The number of potential methane degradation genes (*mcrA*) was about that expected from other data. Both metagenomic and QPCR analyses indicated that the abundance of *mcrA* and archaeal 16S rRNA genes were roughly equal, suggesting that a large fraction of, if not all, archaea in these sediments were involved in either methane degradation or methanogenesis.

The abundance of potential methane-oxidizing microbes in these sediments seems to be inconsistent, however, with other data indicating the large impact of methane on these sediments. The data can be reconciled if a small number of potential methane-oxidizing archaea mediates a large flux of carbon in these sediments. The transfer of methane carbon to other organic carbon pools would be facilitated if carbon respired as carbon dioxide was recaptured by anaerobic carbon dioxide fixation. In support of that hypothesis, we found that genes for anaerobic carbon dioxide fixation occur throughout the sediment profile.

Our study is the first to report metagenomic data from sediments dominated by ANME-2 archaea and is one of the few to examine the entire microbial assemblage potentially involved in anaerobic methane oxidation. The results from this study have implications for understanding rates and controls of methane oxidation in methane-rich environments.

Introduction

Methane hydrates have attracted much interest because of their potential as commercially-viable energy deposits (Boswell 2007; Boswell 2009) and as a substantial potential contributor to climate change (Archer 2007). The methane hydrates in the Arctic are especially important to examine for both reasons. Because of perennially cold temperatures, methane hydrates are found at shallower depths in the Arctic than in lower latitude systems such as the Gulf of Mexico, making them conceivably easier to extract but also potentially more susceptible to climate change. Even a small increase in the average temperature of the Arctic could substantially increase methane fluxes to the atmosphere (Archer 2007).

The net flux of methane from methane hydrates and other sources to the atmosphere depends on methane degradation as well as methane production and release from geological sources. Predictive models need a better understanding of methane degradation because environmental factors and climate change will differentially affect degradation processes, biological methane production and abiotic release from methane hydrates and geological sources. It is known that methane degradation is carried out by a limited number of microbes (Valentine 2011) which are either dependent on oxygen (aerobic methanotrophs) or not (anaerobic methanotrophs). While aerobic methanotrophy has been examined for years (Hanson and Hanson 1996), relatively little is known about anaerobic oxidation of methane, the process likely to be most important in coastal sediments and many soils rich in methane but poor in oxygen. An understanding of these microbes and how they degrade methane is crucial for improving predictive models of methane fluxes to the atmosphere.

Anaerobic methane degradation is carried out by a syntrophic relationship between archaea and sulfate-reducing bacteria, according to the following equation:

$$CH_4 + SO_4^{2-} \rightarrow HS^- + HCO_3^- + 2H_2O \qquad (1).$$

The archaea are thought to be responsible for methane degradation via reverse methanogenesis with a key enzyme being methyl coenzyme M reductase, which is often traced by following a gene for a highly conserved subunit (*mcrA*). This reaction is thermodynamically more favorable when the end products of reverse methanogenesis, hydrogen gas (H_2) and acetate, are drawn down to low levels by the sulfate-reducer bacterial partner. The anaerobic methane degraders belong to three clades: ANME-1, ANME-2, and ANME-3. ANME-1 is distantly related to organisms in the orders Methanosarcinales and Methanomicrobiales while ANME-2 and ANME-3 are related to Methanosarcinales (Knittel and Boetius 2009). Although defined as methane oxidizers, it is unclear if ANME archaea are also capable of methanogenesis (Lloyd et al. 2011; Thauer 2011). One study, however, found methanogenesis to be only 10% of methane oxidation in anoxic sediments (Orcutt et al. 2008). These organisms and many others are part of a complex group of organisms, the “anaerobic food chain”, which are responsible for degrading organic material in the absence of oxygen. While the individual steps in the anaerobic food chain are known, few studies have explored relationships among them in a single environment.

The general goal of this NETL project was to gain more insights into methane degradation through analysis of samples taken from methane-rich sediments off the coast of Alaska in the Beaufort Sea (Fig. 1). The samples were collected as part of a multi-disciplinary cruise ("Methane in the Arctic Shelf" or MIDAS) in September 2009, led by Rick Coffin at the Naval Research Lab (NRL). Although the proposal submitted to NETL mentioned that we would measure methane degradation, we learned that Professor Dr. Tina Treude (IFM-GEOMAR, Kiel, Germany) planned to carry out the same measurements while also examining sulfate reduction. Consequently, we decided to focus on the other objectives of the project: to use molecular methods or "cultivation-independent" approaches in order to gain insights into methanotrophic microbes in these sediments. This project was helped immensely by our successful proposal, submitted in April 2010 to the DOE Community Sequencing Program (CSP), to obtain support for tag and metagenomic sequencing of Beaufort Sea sediments. We received the last of the sequence data in June 2011.

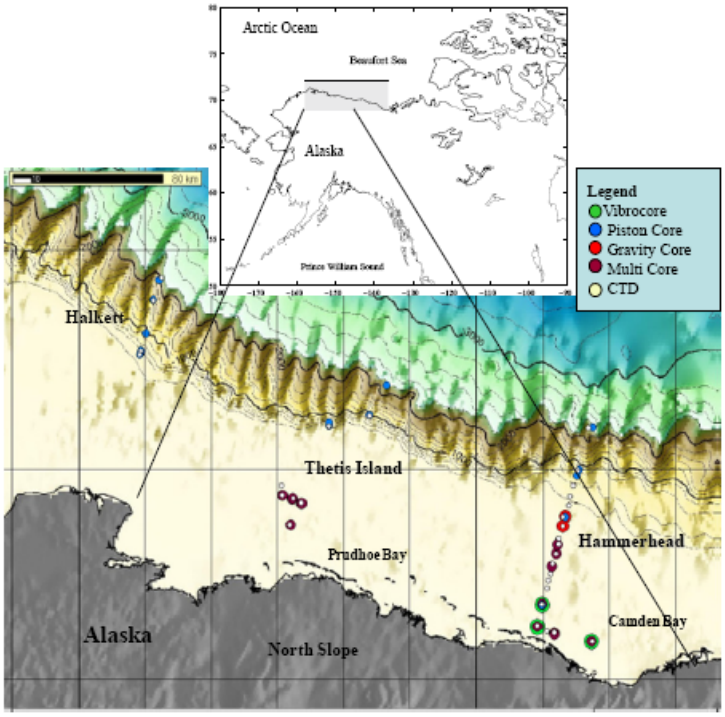

Figure 1. Sampling locations for the MITAS expedition in the Beaufort Sea. Taken from Coffin et al. (2011).

In addition Coffin and his colleagues at NRL, this project involved two investigators at the University of Delaware. Matt Cottrell participated on the MIDAS expedition and was heavily involved in the laboratory work (done with a technician, Liying Yu) and in the analyses of the sequence data discussed below. Tom Hanson joined this effort to analyze the metagenomic sequence data as part of the CSP effort.

Methods

Sample collection The 12 day expedition was on the USCGC Polar Sea in the Alaskan North Slope coastal waters during September 2009. We focused on sampling sediments collected by piston coring, vibracoring, and multi-core, with some samples taken from the water column. The locations for sediment coring were chosen based on historic and ship-board high-resolution seismic profiling to detect pockets of gas from dissociating hydrates. About 15 cores were taken in four regions: Hammerhead offshore, Hammerhead nearshore, Thetis Island, and Halkett. Once brought onboard, the cores were subsampled in collaboration with MIDAS colleagues.

DNA extraction DNA was extracted from sediments using a FastPrep-24 homogenizer with the FastDNA Spin Kit for Soil (MP Biomedicals) following the protocol supplied by the manufacturer. A 200 µL aliquot of sediment was mixed with the phosphate and MT buffers provided with the kit, and the sample was homogenized for 40 sec at a power setting of 6 to lyse the cells. The homogenate was then centrifuged at 14,000 x g for 10 min to pellet debris and the supernatant was transferred to another microcentrifuge tube. The protein precipitation solution included in the kit was added and the sample was then centrifuged at 14,000 x g for 5 min. The supernatant was then transferred to a 15 mL polypropylene centrifuge tube, combined with 1 mL of the binding matrix provided in the kit and mixed gently for 2 min. After the matrix was allowed to settle for 3 min a 500 µL aliquot of the supernatant was discarded. The binding matrix was then resuspended and transferred to the spin filter device provided in the kit. The sample was centrifuged at 14,000 x g for 1 min and the filtrate was discarded. The filter was rinsed with SEWS-M buffer, centrifuged at 14, 000 x g for 1 min and then briefly air-dried for 5 min. Finally, the DNA was eluted from the spin filter with deionized water.

DNA for pyrosequencing was prepared by further purifying the extract using agarose gel electrophoresis. The extract was electrophoresed on a 1% agarose gel and the DNA was purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen) following the protocol provided by the manufacturer.

DNA for metagenomic sequencing was prepared by pooling 20 - 40 extracts generated using the MP Biomedicals kit and further purified using cesium chloride (CsCl) gradient centrifugation. Six g of solid CsCl, the DNA extract and 5 mL of Tris-EDTA buffer (pH 8) were combined in a 15 mL centrifuge tube and then mixed until the CsCl dissolved. SYBR Safe stain (0.6 uL, Invitrogen) was added to the sample and then transferred to a polyallomer centrifuge tube (Seton Scientific) and centrifuged for 16 h at 25°C in a TV867 rotor (Sorval). The sample was viewed using blue light (Dark Reader, Clare Chemical Research) and the DNA band was removed by puncturing the centrifuge tube wall with a hypodermic needle. The sample was then split into three 0.3 mL aliquots. Deionized water (0.6 mL), 1 µL of a glycogen solution (New England BioLabs), 90 µL of sodium acetate (5 M) and 0.9 mL of isopropanol were then added to each aliquot. The sample was mixed and then centrifuged at 14,000 rpm for 1 h. The supernatant was poured off, the sample was rinsed with 0.5 mL of ethanol, and the sample was centrifuged again for 5 min. The final supernatant was poured off and the sample was air-dried. The DNA was then resuspended in 50 µL of deionized water.

Pyrosequencing analyses Sequences of 16S rRNA genes were amplified using primers 926F and 1392R (Table 1). Twenty-microliter PCR reactions were performed in duplicate and pooled to minimize PCR bias using 0.4 µl Advantage GC 2 Polymerase Mix (Advantage-2 GC PCR Kit, Clonetech), 4 µl 5X GC PCR buffer, 2 µl 5M GC Melt Solution , 0.4 µl 10mM dNTP mix (MBI Fermentas), 1.0 µl of each 25 nM primer, and 10 ng sample DNA. The thermal cycler protocol was 95°C for 3 min, 25 cycles of 95°C for 30 s, 50°C for 45 s, and 68°C for 90 s, and a final 10-min extension at 68°C. PCR Amplicons were purified using SPRI Beads and quantified using a Qubit flurometer (Invitrogen). Samples were diluted to10 ng/µl and mixed in equal concentrations. Emulsion PCR and sequencing of the PCR amplicons were performed following the Roche 454 GS FLX Titanium technology manufacturer's instructions

Table 1. Primers for PCR analyses and sequencing used by this project.

Purpose	Gene Target	Name	Reference
Tag pyrosequencing of all microbes	16S rRNA	926F 1392R	
Enumerate all bacteria by QPCR	16S rRNA	BACT 1369F PROK 1541R	(Suzuki et al. 2001)
Enumerate all archaea by QPCR	16S rRNA	ARCH915F PROK 1059R	(Suzuki et al. 2001)
Aerobic methane degradation	pmoA	A189f A682r	(Losekann et al. 2007)
Anaerobic methane degradation	mcrA	ME1 fwd ME3rev	(Losekann et al. 2007)

Quantification of 16S rRNA and mcrA gene abundances The abundance of 16S rRNA, *mcrA*, and *pmoA*, genes in Beaufort Sea sediments was determined by real time quantitative PCR (QPCR) using the primers given in Table 1. Standard curves were generated using plasmid clones. Plasmid DNA was linearized with PstI restriction enzyme and quantified using the picogreen assay (Invitrogen).

Quantitative PCR was performed in triplicate using 1 µl of environmental sample DNA or plasmid DNA solution and the SpeedSTAR HS DNA Polymerase (Takara Bio) with 0.1X SYBR Green I (Invitrogen) in a total reaction volume of 12.5 µl. Thermal

cycling and quantification of bacterial 16S rRNA genes and *mcrA* genes was performed using a Rotor Gene 6000 (Corbett Life Sciences) instrument programmed with the following conditions for bacterial 16S rRNA genes: 95°C for 10 min; followed by 30–40 cycles of denaturation at 95°C for 15 s, primer annealing at the primer-specific annealing temperature for 30 s, and polymerase extension at 72°C for 30 s. The conditions for *mcrA* gene amplification were: 95°C for 10 min; followed by 35-40 cycles of denaturation at 95°C for 15 s, primer annealing at the primer-specific annealing temperature for 30 s, and polymerase extension at 72°C for 30 s. Quantification of archaeal 16S rRNA genes was performed using a model 7500 Real Time PCR System (Applied Biosystems) instrument programmed with the following conditions: 95°C for 10 min; followed by 30–40 cycles of denaturation at 95°C for 15 s, primer annealing at the primer-specific annealing temperature for 45 s, and polymerase extension at 72°C for 45 s. Final primer concentrations were 0.2 mM. Amplification efficiencies ranged from 82% - 86%. Amplification specificity was evaluated by the presence of a single peak in post-amplification dissociation curves.

The specificity of PCR reactions for *pmoA* and *mcrA* genes was also explored by cloning and sequencing (by the Sanger method) 48 and 95 amplicons from each reaction, respectively. Samples for this test were taken from 150 and 240 centimeters below the seafloor (cmbsf). Only six of the sequences from the *pmoA* PCR were similar to known *pmoA* genes according to BLAST analyses and were not analyzed further. Similar analyses of the *mcrA* amplicons indicated that 87 sequences were similar to known *mcrA* genes. Of these, 81 sequences could be conceptually translated to proteins for further analyses (see below).

Metagenomic sequencing The metagenome sequencing was done on 454-FLX with Titanium reagents according to standard manufacturer's protocols. The libraries were made with the 454-Rapid protocol.

Data analysis Pyrosequencing tags were analyzed using the software tool Pyrotagger (http://pyrotagger.jgi-psf.org) using a 200 bp sequence length threshold as described previously (Engelbrektson et al. 2010). The number of sequences was normalized by random resampling 100 times using the sample function in R (http://www.r-project.org/) to get 1256 sequences per sample Three samples with <1256 sequences were removed. Percentages were arcsin transformed before statistical analyses.

Metagenomic analyses were primarily done in MG-RAST (http://metagenomics.anl.gov/) or by local BLAST analyses. The following single copy genes were used for determining if the metagenomes were biased: CTP synthase [EC:6.3.4.2], DNA-directed RNA polymerase subunit beta [EC:2.7.7.6], isoleucyl-tRNA synthetase [EC:6.1.1.5], large subunit ribosomal protein L2, leucyl-tRNA synthetase [EC:6.1.1.4], recombination protein RecA. For exploring the relative number of fermenting and sulfate-reducing bacteria, we used the frequency of genes for lactate dehydrogenase [EC:1.1.1.27] and adenylylsulfate reductase, subunit A [EC:1.8.99.2],

respectively. Acetogenic bacteria were assessed by the gene for carbon monoxide dehydrogenase / acetyl-CoA synthase subunit beta [EC:1.2.7.4 1.2.99.2 2.3.1.169].

The overall statistics for the metagenomic sequencing effort are given in Table 2.

Phylogenetic analysis of *mcrA* amplicons Nucleotide sequences were aligned according to the amino acid alignment generated by the transAlign.pl script (Bininda-Emonds 2005). CLUSTALW was used to align the conceptual translations and the codons were then aligned to the corresponding amino acids, which avoids introducing any frame shifts into the alignment. Reference *mcrA* genes included sequences reported by (Hallam et al. 2003), (Jiang et al. 2011) and sequences identified by BLAST analysis of the amplicons using the GenBank nr database (March 2012). The *mrtA* gene of *Methanothermobacter marburgensis* was used as the out group. The tree was produced by the RAxML program using the GTRGAMMA model and 20 maximum likelihood trees (Stamatakis 2006). Confidence values for branch nodes were estimated from 100 bootstrap replicates.

Table 2. Summary of metagenomic sequencing of a methane-rich core (PC12). "PostQC" refers to statistics after quality control. Depth is given as centimeters below the seafloor (cmbsf).

	Depth (cmbsf)					
	20	90	170	260	485	Total
Upload size (bp)	5.7E+08	6.1E+08	1.0E+09	3.8E+08	4.5E+08	3.0E+09
Upload Sequences	1.6E+06	1.8E+06	2.6E+06	1.5E+06	1.5E+06	9.1E+06
PostQC Size (bp)	4.1E+08	4.2E+08	5.0E+08	2.7E+08	4.4E+08	2.0E+09
PostQC: Sequences	1.1E+06	1.2E+06	1.2E+06	1.0E+06	1.4E+06	6.0E+06
Predicted Proteins	1.1E+06	1.2E+06	1.2E+06	9.6E+05	1.4E+06	5.8E+06
Predicted rRNA genes	1.3E+05	1.5E+05	1.7E+05	1.7E+05	2.3E+05	8.4E+05

Results and Discussion

The goal of this project was to examine microbes and carbon degradation processes in methane-rich and methane-poor sediments of the Beaufort Sea, Alaska. About 15 cores were taken in this region, each sampled roughly every 10 cm through the length of the core, in parallel with several biogeochemical analyses. Although many cores were taken, we focused our analyses on one core with low methane concentrations (PC10) and another (PC12) with high concentrations.

As typical for these sediments, sulfate concentrations decreased with depth in each core, especially in the methane-rich core (Fig. 2). In that core, sulfate concentrations became unmeasurable by about 150 cmbsf. Methane was below detection limits throughout core PC10 and in the upper 150 cm of core PC12. In PC12, however, methane concentrations increased greatly to nearly 15 mM in sediments deeper than about 150 cmbsf. The methane data, along with preliminary molecular analyses (see below), led us to focus on PC10 and PC12 for detailed analyses.

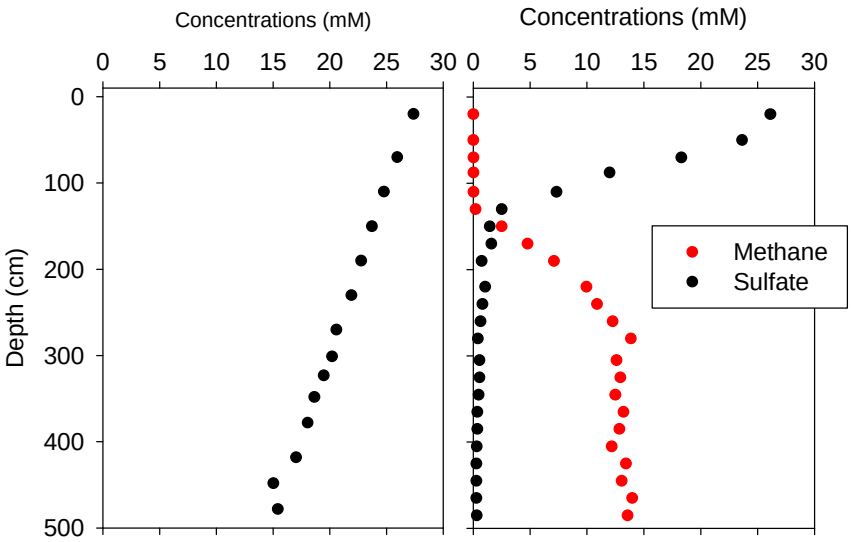

Figure 2. Concentrations of sulfate and methane in PC10 (left) with no significant methane and in PC12 (right) with high methane concentrations in the bottom half of the core. Data from R. Coffin (Coffin et al. 2011).

The microbial communities in these two cores were quantitatively dissected at various levels of resolution, ranging from the abundance of the major domains of life (archaea, bacteria and eukaryotes) to the genes involved in methane cycling.

Community structure in low and high methane sediments The taxonomic makeup (community structure) of microbes in the low methane (PC10) and high methane (PC12) cores was determined by tag pyrosequencing of the 16S rRNA gene and by quantitative PCR analyses. Forty samples analyzed from PC10 and 12 resulting in a total of 212,224 pyrosequences. We will first focus on PC12 because metagenomic data are also available for this core.

Starting with the highest phylogenetic level, all of the data sets indicated the expected dominance of bacteria in these cores (Table 3). Bacteria made up 77% to

97% of all microbes, depending on the method. The QPCR approach indicated that archaea made up slightly over 20% of all microbes, but this estimate is much higher than seen by the other approaches. The lowest estimate of archaeal abundance came from the tag pyrosequencing approach, which may reflect biases in the PCR primers. The metagenomic approach (all genes) suggested higher abundance of archaea in the methane-rich layers than in the methane-poor layer of PC12 (8-9% vs. 3.3% at 20 cm), but this was not seen with the two other approaches.

Table 3. Taxonomic composition of a methane-rich core (PC12) as determined by three independent analyses. The data are mean and standard deviation (SD) for all depths. For the metagenomic data, composition was deduced from all genes and from only the 16S rRNA genes. The abundance of eukaryotes was not determined (ND) by QPCR.

	Metagenomic				Tag data		QPCR	
	All genes	SD	16S rRNA	SD	16S rRNA	SD	16S rRNA	SD
Bacteria	77.2	4.3	79	7.0	97.0	3.2	78.4	10.7
Archaea	5.3	1.9	5	1.7	0.30	0.18	21.6	10.7
Eukaryota	4.4	3.0	11	4.8	2.7	3.1	ND	ND
Other	13.1	0.7	6	1.2	0	0	ND	ND

Although the cores had similar levels of the three kingdoms of life (bacteria, archaea, and eukaryotes), they differed when examined at a taxonomic level closer to species, i.e. when the 16S rRNA genes were grouped at the $\geq$97% similarity level. A cluster analysis indicated that the low methane core communities were quite different from the communities in the high methane core sediments (Fig. 3). Communities at all depths in the low-methane core differed from those in the high-methane core. The communities in the top layers of PC12 which did not have high methane concentrations were somewhat different from those deeper in the sediment core with high methane concentrations. Community structure was significantly correlated with both methane ($r=0.23$, $p<0.001$) and sulfate concentrations ($r=0.19$, $p<0.003$), according to partial Mantel tests.

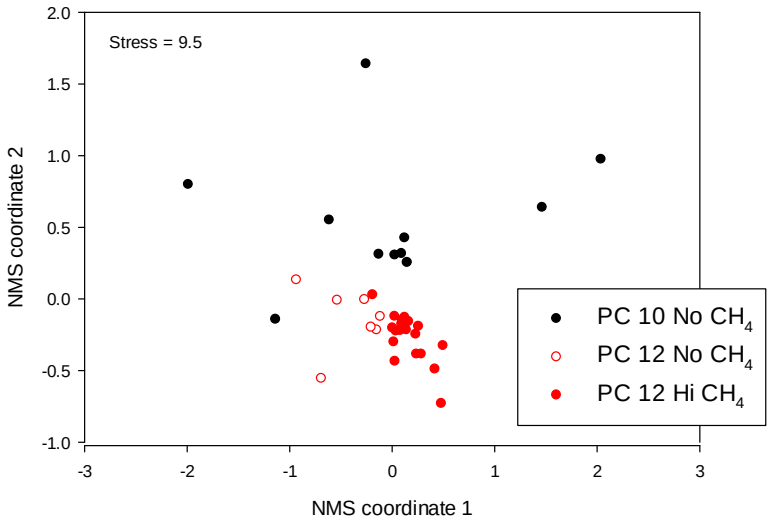

Figure 3 Nonmetric multi-dimensional scaling analysis of bacterial community structure (defined by tag sequence data) depending methane concentrations.

We next examined the relative abundance of potential partners involved in methane degradation. Tag pyrosequencing indicated that total archaea were more abundant in the methane-rich core than the methane-poor core (0.41% vs. 0.14% of the total community), but this difference was not seen in the QPCR data (data not shown). We then counted the number of archaea in the Methanosarcinales, the archaeal order with the known anaerobic methane degraders, in the tag pyrosequence data. When normalized for total sequences, Methanosarcinales sequences were encountered over 5-fold more frequently in the methane-rich sediments than in the methane-poor sediments (32.3 $\pm$ 4.9 vs. 6.0 $\pm$ 2.3 X 10^{-4} Methanosarcinales sequences per total metagenome sequences), although there was no obvious trend with depth, even in the methane-rich core (Fig. 4).

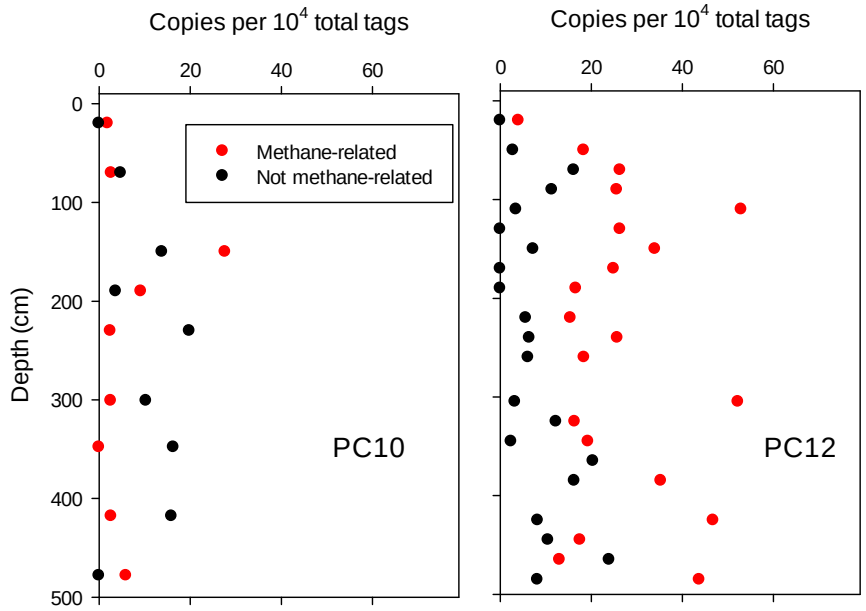

Figure 4. The relative number of archaea in the Methanosarcinales (red circles) and other archaea (black) in the methane-poor core (left) and methane-rich core (right), according to tag sequence data.

However, the other potential partner in methane-degradation, the sulfate-reducers, did change with depth, although not in relationship to methane concentration. Bacteria in the Deltraproteobacteria, which includes many known sulfate-reducers, were abundant in the upper part of both sediments cores, making up 20-35% of the total community according to the tag pyrosequence data. These percentages then decreased with depth within the core to as low as 5% (Fig. 5). Perhaps some of the decrease seen in PC12 can be explained by the corresponding decrease in sulfate concentrations (Fig. 1), but these potential sulfate reducers also decreased substantially with depth in PC10 where sulfate concentrations varied only slightly. Rather than sulfate, we suspect the decrease in potential sulfate-reducing bacteria is due to a decrease in labile organic material down the sediment core. In support of that hypothesis, the relative abundance of Deltaproteobacteria was positively correlated with percent organic carbon in these sediments ($r=0.57$, $n=37$, $p<0.001$).

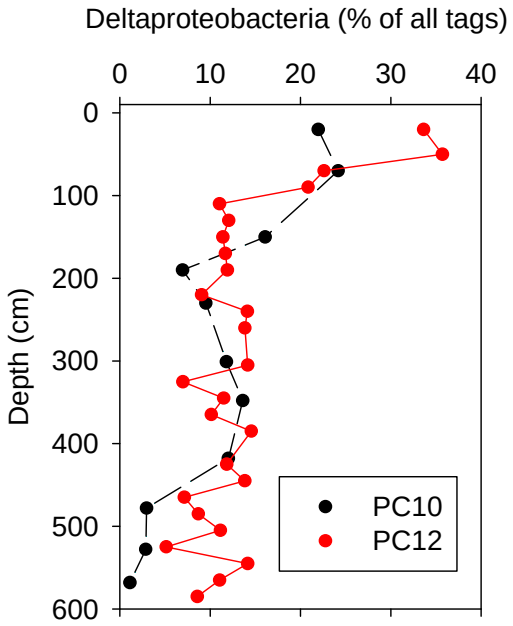

Figure 5. Relative abundance of potential sulfate-reducing bacteria (Deltaproteobacteria) in the methane-poor core (PC10) and the methane-rich core (PC12), according to 16S rRNA tag sequencing.

Phylogenetic analysis of *mcrA* gene sequences The *mcrA* gene encodes a protein found in archaea that carry out either methanogenesis or anaerobic methane oxidation or potentially both. However, these two types of *mcrA* are readily distinguished by comparing new sequences to sequences from known methanogenic and methane-oxidizing archaea. This type of phylogenetic analysis indicated that nearly all of the *mcrA* sequences from these sediments were most similar to *mcrA* genes from ANME-2 while only three sequences (out of 81) were similar to ANME-1 sequences (Fig. 6). Only two sequences (79-150cm and 86-150cm) were most similar to genes from known

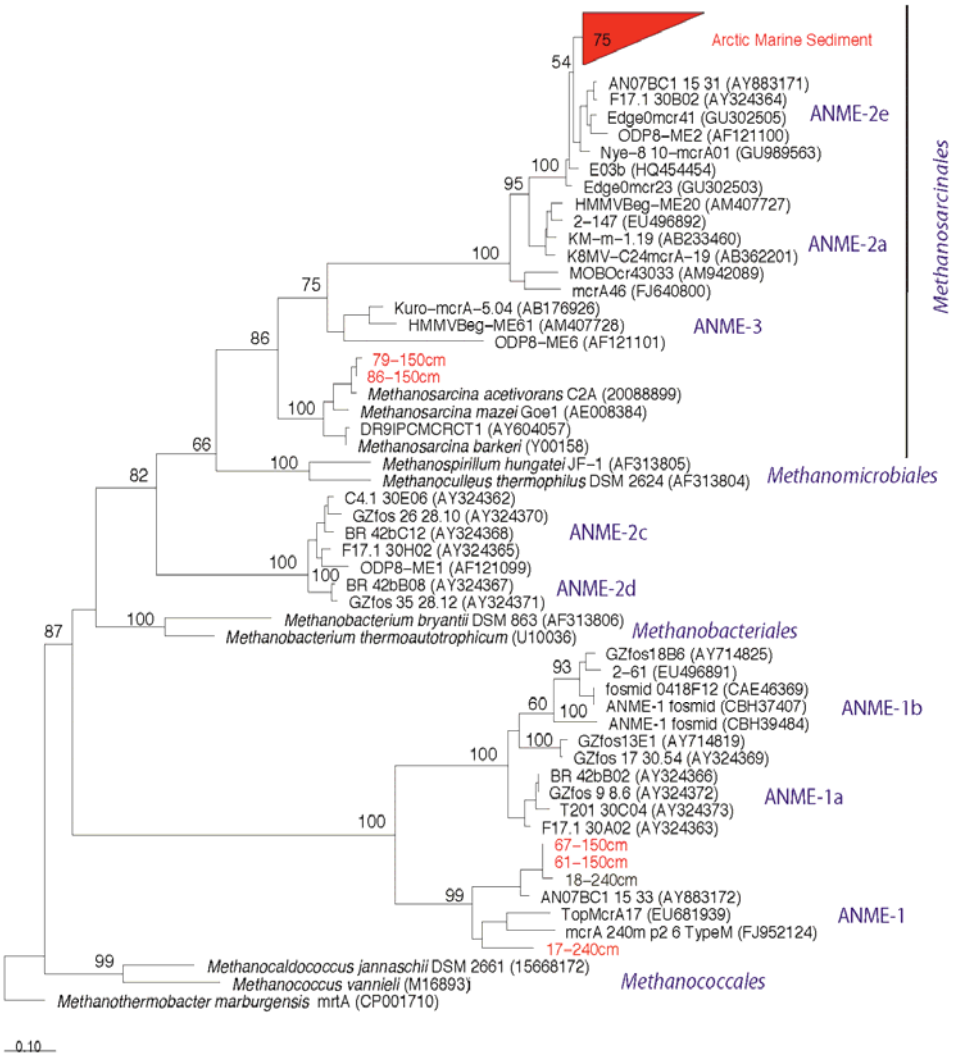

Figure 6 Phylogenetic analysis of a key gene (*mcrA*) in anaerobic methane oxidation (ANME). Sequences from this study are indicated by red labels. The red wedge represents 75 sequences of which 67 are unique. Values at the nodes are the number of times the node occurred in 100 bootstrap replicate trees. Only values greater than 50 are shown. GenBank accessions are in parentheses. The scale bar indicates a sequence divergence equal to one base change per 100 nucleotides.

methanogenic archaea not in one of the ANME groups. These data suggest that most of the archaea with *mcrA* genes were involved in anaerobic methane degradation rather than methanogenesis, although it is possible that ANME archaea also produce methane (Lloyd et al. 2011; Thauer 2011).

Methane-related and other genes in Beaufort Sea sediments We next examined the potential for methane degradation and other microbial processes as revealed by metagenomic data. We were limited to five samples for this analysis. To choose these samples, we used data on methane concentrations and other geochemical properties and results from qualitative screening for methane-related genes. The screening was based on a PCR assay for the presence or absence of genes involved in aerobic (*pmoA*) and anaerobic methane degradation (*mcrA*). Although this assay indicated the presence of *pmoA* in many samples, which would be difficult to explain, subsequent sequence analysis indicated that these were false-positives; *pmoA* was not present in these cores. In contrast, sequence analysis indicated that *mcrA* was present in anoxic layers of sediment cores with high methane concentrations (see above). Based on these results, we chose five samples in the high methane core (PC12) for metagenomic sequencing.

The 16S rRNA genes in the metagenomic data indicated these communities differed depending on methane concentrations and undoubtedly other biogeochemical properties as well (data not shown), similar to what was seen in the pyrosequence data. Of more interest, the communities also could be distinguished based on the abundance of functional genes, those coding for enzymes and other proteins (Fig. 7). The two communities in high methane sediments (260 and 485 cm) were most closely associated whereas the other communities were distinct.

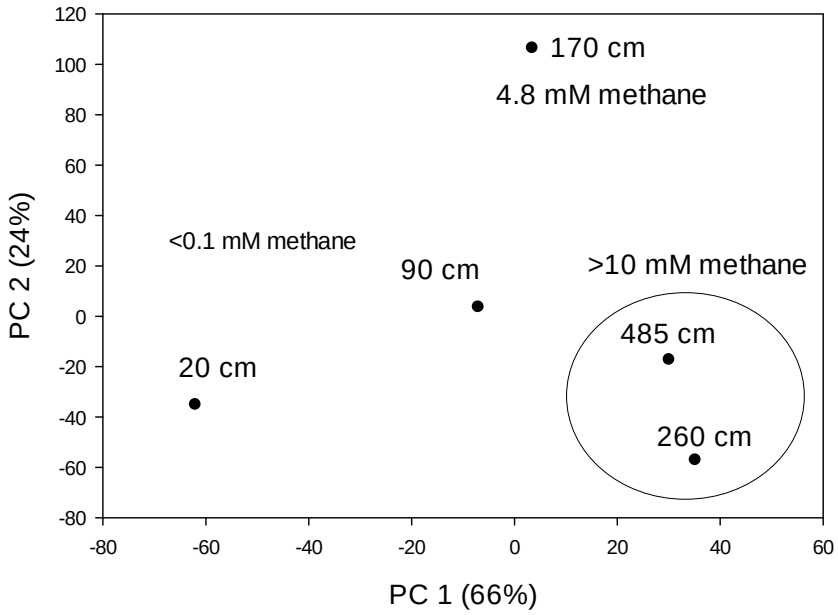

Figure 7 Principle component analysis of microbial communities from various depths in the methane-rich core (PC12). Communities were defined by all genes in their meta-genomes. Two components explained 90% of the variation in the data.

We next analyzed genes connected to methane degradation and, more generally, the oxidation of organic material in sediments. Before doing so, we examined the number of single copy genes (six in total) to determine whether the metagenomes were biased along the depth profile. We found that the single copy gene abundance did

not vary significantly with depth (overall average of 189 ± 24 copies per Gbp), indicating that the metagenomic samples are not biased with regard to depth.

Genes related to methane degradation and other degradation pathways changed along the depth profile as suggested by the taxonomic and biogeochemical data. Two important parts of the anaerobic food chain, however, did not vary significantly with depth. Fermentation genes appeared to be equally abundant through the sediment core profile (Fig. 8) as were those for acetogenesis (72 ± 12 copies per Gbp, data not shown). As expected from the other data, sulfate reduction genes decreased with depth while the anaerobic methane degradation gene (*mcrA*) increased.

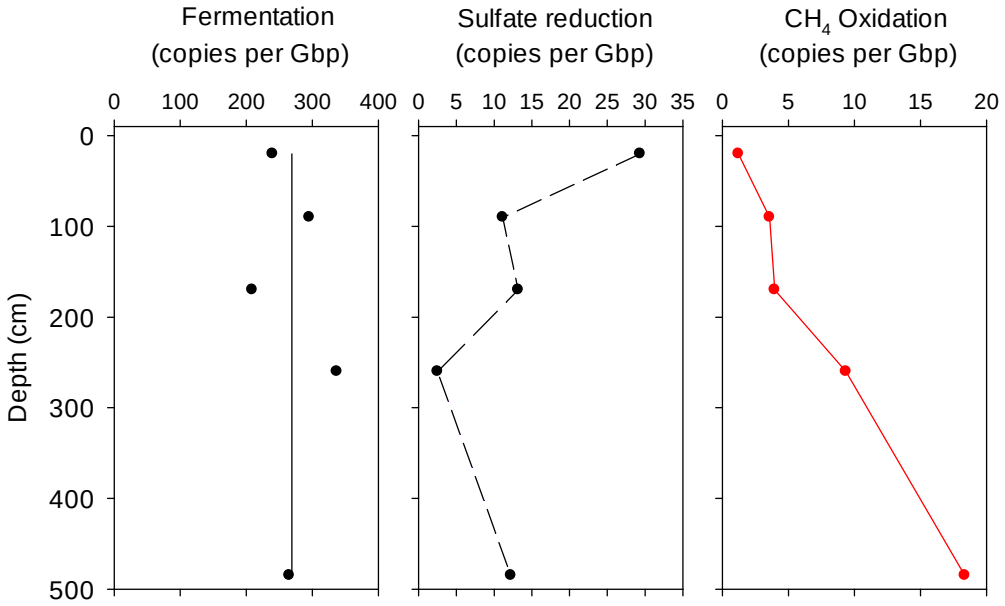

Figure 8 Relative number of genes involved in fermentation, sulfate reduction and potential anaerobic methane degradation (*mcrA*).

Some of these genes were as abundant as expected while the relative abundance of other genes was surprising (Fig. 9). The total number of fermentation genes was about equal to the number of single copy genes, indicating the high abundance of fermenting bacteria in these sediments, consistent with the known importance of this pathway in the anaerobic degradation of organic material. The number of sulfate reduction genes (roughly 10%) was lower than expected from the tag pyrosequence data (about 20% which is the fraction of Deltaproteobacteria making up the total community). The number of potential methane degradation genes (*mcrA*) was about that expected from other data. Both metagenomic (see above) and QPCR analyses (data not shown) indicated that the abundance of *mcrA* and archaeal 16S

rRNA genes were roughly equal, suggesting that a large fraction of, if not all, archaea in these sediments were involved in either methane degradation or methanogenesis.

The abundance of potential methane-oxidizing microbes seems to be inconsistent, however, with other data indicating the impact of methane on these sediments. The number of potential methane-oxidizing archaea was rather small even in methane-rich sediments, accounting for about 7% to 20% of total microbial abundance (Figure 9; Table 3), depending on depth and the detection method. In contrast, fluxes of methane are similar to those for sulfate in these sediments (Coffin et al., in prep). These data imply that methane carbon dominates carbon fluxes in this environment, if sulfate is the main terminal electron acceptor, as is usually the case in

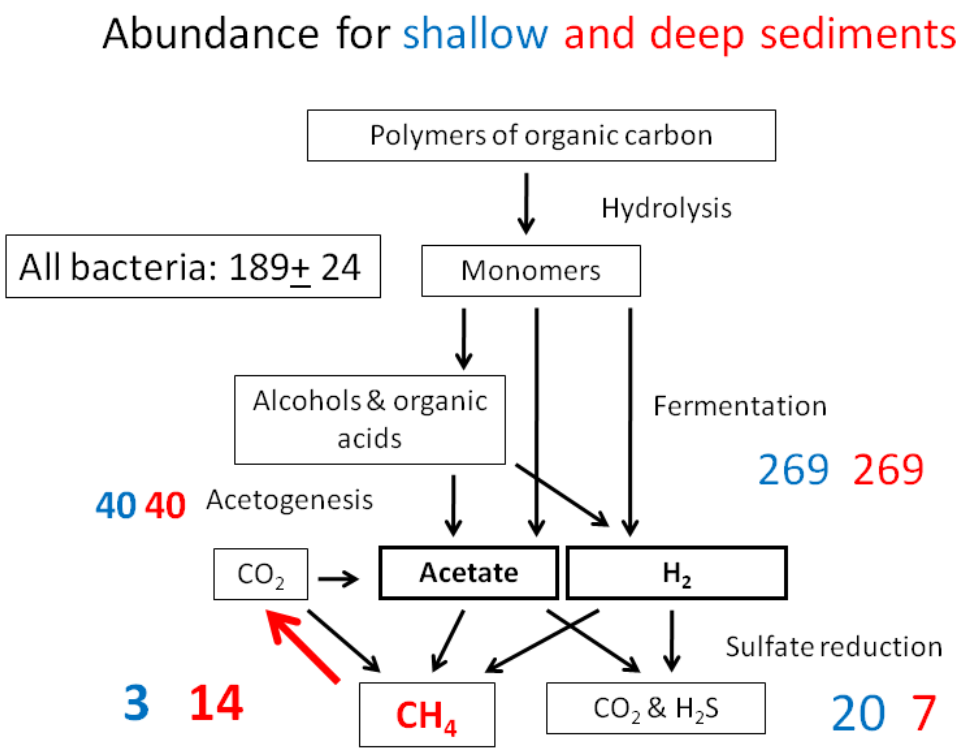

Figure 9. Relative abundance of key genes in the anaerobic food chain. Methane concentrations were low in shallow sediments (20 and 90 cmbsf) but high in the deep sediments of PC12 (260 and 485 cmbsf). Numbers are sequences per Gbp.

marine environments (reviewed by (Canfield et al. 2005) and (Kirchman 2012)). In support of the importance of methane, we observed a significant effect of methane concentrations on bacterial and archaeal community structure (see above). What is harder to reconcile is the 14C data indicating that methane input into organic carbon is

high in these sediments (Coffin et al., in prep). The only known mechanism to explain the 14C data is incorporation of methane carbon into microbial biomass and then subsequent transfer of that microbial carbon into other organisms and the detrital organic carbon pool.

The data can be reconciled if a small number of potential methane-oxidizing archaea mediates a large flux of carbon in these sediments. The transfer of methane carbon to other organic carbon pools would be facilitated if carbon respired as carbon dioxide was recaptured by anaerobic carbon dioxide fixation. In support of that hypothesis, we found that genes for anaerobic carbon dioxide fixation occur throughout the sediment profile.

Conclusion

Our study is the first to report metagenomic data from sediments dominated by ANME-2 organisms and is one of the few to examine the entire microbial assemblage potentially involved in anaerobic methane oxidation. Other studies have reported genomic data from anaerobic methane oxidizing archaea in the ANME-1 group (Hallam et al. 2004; Meyerdierks et al. 2010; Meyerdierks et al. 2005), and a recent metagenomic survey similar to ours also found that most ANME sequences were related to those from ANME-1 (Havelsrud et al. 2011). Using a PCR-based approach another study also found mostly ANME-1 (Lloyd et al. 2011). In contrast, the archaea in the Arctic sediments we examined appeared to be mainly from ANME-2, similar to what was found in a cold seep off the Oregon coast (Hydrate Ridge) and in some microbial mats in the Black Sea (Knittel et al. 2005; Treude et al. 2007). Further analyses are needed to explore possible differences in genomes of ANME-1 (Hallam et al. 2004; Meyerdierks et al. 2010; Meyerdierks et al. 2005) and ANME-2 (this study).

Further work is necessary to determine how methane oxidation by these two ANME groups differs. Previous studies suggest that ANME-2 forms tight physical consortia with sulfate-reducing bacteria whereas ANME-1 methane oxidizers occur as single cells (Knittel and Boetius 2009), suggesting that the relationship between methane oxidation and sulfate reduction would differ depending on whether ANME-1 or ANME-2 is most abundant. The two groups appear also to differ in 13C fractionation, which would affect interpretation of stable isotope data (Treude et al. 2007). All of these observations suggest that the regulation and probably rates of anaerobic methane oxidation vary with the relative abundance and activity of ANME-1 versus ANME-2.

An advantage of the metagenomic approach used here is that it provides insights into the entire community of bacteria and archaea required to completely oxidize methane and organic carbon in anoxic environments. Our strategy in analyzing these data is similar to that used in a recent study (Lauro et al. 2011), although that study did not focus on the anaerobic food chain. Our analyses confirm the importance of fermentation in providing substrates used by sulfate-reducing bacteria, acetogenic bacteria, and ultimately methanogens and anaerobic methane degraders. Potential

sulfate-reducing bacteria also appear to be abundant although their abundance decreased with depth.

In contrast to the high numbers of fermentation genes and potential sulfate reducing bacteria, numbers of potential methane-oxidizing archaea and a key gene (*mcrA*) are low even in methane-rich sediments. This low number of microbes apparently mediates a large flux of carbon, seemingly sufficient to support sulfate reduction, which is usually the main pathway for organic carbon mineralization in marine anaerobic sediments. This study highlights the need to examine the entire microbial community in order to understand methane degradation and thus net fluxes of methane to the atmosphere.

References

Archer, D. 2007. Methane hydrate stability and anthropogenic climate change. Biogeosciences 4**:** 521-544.

Bininda-Emonds, O. 2005. transAlign: using amino acids to facilitate the multiple alignment of protein-coding DNA sequences. Bmc Bioinformatics 6**:** 156.

Boswell, R. 2007. Resource potential of methane hydrate coming into focus. Journal of Petroleum Science and Engineering 56**:** 9-13.

Boswell, R. 2009. Is Gas Hydrate Energy Within Reach? Science 325**:** 957-958.

Canfield, D. E., B. Thamdrup, and E. Kristensen. 2005. Aquatic Geomicrobiology Elsevier Academic Press. 640 pp.

Coffin, R. B., L. J. Hamdan, J. P. Smith, R. Plummer, L. Millholland, R. Larson, and W. Wood. 2011. Beaufort Sea Methane Hydrate Exploration: Energy and Climate Change. NRL/MR/6110--11-9330.

Engelbrektson, A., V. Kunin, K. C. Wrighton, N. Zvenigorodsky, F. Chen, H. Ochman, and P. Hugenholtz. 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J. 4**:** 642-647.

Hallam, S. J., P. R. Girguis, C. M. Preston, P. M. Richardson, and E. F. Delong. 2003. Identification of Methyl Coenzyme M Reductase A (mcrA) Genes Associated with Methane-Oxidizing Archaea. Appl. Environ. Microbiol. 69**:** 5483-5491.

Hallam, S. J., N. Putnam, C. M. Preston, J. C. Detter, D. Rokhsar, P. M. Richardson, and E. F. Delong. 2004. Reverse methanogenesis: Testing the hypothesis with environmental genomics. Science 305**:** 1457-1462.

Hanson, R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. Microbiol. Rev. 60**:** 439-471.

Havelsrud, O. E., T. H. A. Haverkamp, T. Kristensen, K. S. Jakobsen, and A. G. Rike. 2011. A metagenomic study of methanotrophic microorganisms in Coal Oil Point seep sediments. BMC Microbiol. 11**:** 221.

Jiang, L., Y. Zheng, J. Chen, X. Xiao, and F. Wang. 2011. Stratification of Archaeal communities in shallow sediments of the Pearl River Estuary, Southern China. Antonie Van Leeuwenhoek 99**:** 739-751.

Kirchman, D. L. 2012. Processes in Microbial Ecology. Oxford University Press. 312 pp.

Knittel, K., and A. Boetius. 2009. Anaerobic oxidation of methane: Progress with an unknown process. Annu. Rev. Microbiol. 63**:** 311-334.

Knittel, K., T. Losekann, A. Boetius, R. Kort, and R. Amann. 2005. Diversity and distribution of methanotrophic archaea at cold seeps. Appl. Environ. Microbiol. 71**:** 467-479.

Lauro, F. M., M. Z. Demaere, S. Yau, M. V. Brown, C. Ng, D. Wilkins, M. J. Raftery, J. a. E. Gibson, C. Andrews-Pfannkoch et al. 2011. An integrative study of a meromictic lake ecosystem in Antarctica. ISME J. 5**:** 879-895.

Lloyd, K. G., M. J. Alperin, and A. Teske. 2011. Environmental evidence for net methane production and oxidation in putative ANaerobic MEthanotrophic (ANME) archaea. Environ. Microbiol. 13**:** 2548-2564.

Losekann, T., K. Knittel, T. Nadalig, B. Fuchs, H. Niemann, A. Boetius, and R. Amann. 2007. Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby mud volcano, Barents Sea. Appl. Environ. Microbiol. 73**:** 3348-3362.

Meyerdierks, A., M. Kube, I. Kostadinov, H. Teeling, F. O. Glöckner, R. Reinhardt, and R. Amann. 2010. Metagenome and mRNA expression analyses of anaerobic methanotrophic archaea of the ANME-1 group. Environ. Microbiol. 12**:** 422-439.

Meyerdierks, A., M. Kube, T. Lombardot, K. Knittel, M. Bauer, F. O. Glockner, R. Reinhardt, and R. Amann. 2005. Insights into the genomes of archaea mediating the anaerobic oxidation of methane. Environ. Microbiol. 7**:** 1937-1951.

Orcutt, B., V. Samarkin, A. Boetius, and S. Joye. 2008. On the relationship between methane production and oxidation by anaerobic methanotrophic communities from cold seeps of the Gulf of Mexico. Environ. Microbiol. 10**:** 1108-1117.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22**:** 2688-2690.

Suzuki, M. T., O. Beja, L. T. Taylor, and E. Delong. 2001. Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environ. Microbiol. 3**:** 323-342.

Thauer, R. K. 2011. Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO(2). Current Opinion In Microbiology 14**:** 292-299.

Treude, T., V. Orphan, K. Knittel, A. Gieseke, C. H. House, and A. Boetius. 2007. Consumption of methane and CO_2 by methanotrophic microbial mats from gas seeps of the anoxic Black Sea. Appl. Environ. Microbiol. 73**:** 2271-2283.

Valentine, D. L. 2011. Emerging Topics in Marine Methane Biogeochemistry. Annu. Rev. Mar. Sci. 3**:** 147-171.

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