Sulfur-Cycling in Methane-Rich Ecosystems: Uncovering Microbial Processes and Novel Niches

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ABSTRACT

Microbial sulfur cycling communities were investigated in two methane-rich ecosystems, terrestrial mud volcanoes (TMVs) and marine methane seeps, in order to investigate niches and processes that would likely be central to the functioning of these crucial ecosystems. Terrestrial mud volcanoes represent geochemically diverse habitats with varying sulfur sources and yet sulfur-cycling in these environments remains largely unexplored. Here we characterized the sulfur-metabolizing microorganisms and activity in 4 TMVs in Azerbaijan, supporting the presence of active sulfur-oxidizing and sulfate-reducing guilds in all 4 TMVs across a range of physiochemical conditions, with diversity of these guilds being unique to each TMV. We also found evidence for the anaerobic oxidation of methane coupled to sulfate reduction, a process which we explored further in the more tractable marine methane seeps. Diverse associations between methanotrophic archaea (ANME) and sulfate-reducing bacterial groups (SRB) often co-occur in marine methane seeps, however the ecophysiology of these different symbiotic associations has not been examined. Using a combination of molecular, geochemical and fluorescence *in situ* hybridization coupled to nano-scale secondary ion mass spectrometry (FISH-NanoSIMS) analyses of *in situ* seep sediments and methane-amended sediment incubations from diverse locations, we show that the unexplained diversity in SRB associated with ANME cells can be at least partially explained by preferential nitrate utilization by one particular partner, the seepDBB. This discovery reveals that nitrate is likely an important factor in community structuring and diversity in marine methane seep ecosystems. The thesis concludes with a study of the dynamics between ANME and their associated SRB partners. We inhibited sulfate reduction and followed the metabolic processes of the community as well as the effect of

ANME/SRB aggregate composition and growth on a cellular level by tracking ^{15}N substrate incorporation into biomass using FISH-NanoSIMS. We revealed that while sulfate-reducing bacteria gradually disappeared over time in incubations with an SRB inhibitor, the ANME archaea persisted in the form of ANME-only aggregates, which are capable of little to no growth when sulfate reduction is inhibited. These data suggest ANME are not able to synthesize new proteins when sulfate reduction is inhibited.

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Introduction

"If I could do it all over again, and relive my vision in the twenty-first century, I would be a microbial ecologist."

– E. O. Wilson

Introduction to Microbial Ecology

I would first like to familiarize you with the branch of environmental microbiology that encompasses my thesis, microbial ecology. Microbial ecology is the study of how microorganisms interact with their environment. Together along with the other residents of the environment, these components form an ecosystem. An ecosystem might be something as small as a termite gut or as large as a mud volcano, and it can be naturally occurring or something created/altered by humans like a sewage treatment plant or a polluted lake. Understanding the dynamics that exist between microorganisms and their environments can allow us to understand important factors affecting the ecosystem as a whole, such as why methane does not reach the atmosphere when large natural stores are slowly leaking upwards through the ocean floor. The study of microbial ecology can also uncover critical environmental factors that contribute to a microorganism's growth and proliferation. A good example of this is the understanding that microorganisms involved in bioremediation (the use of microorganisms to break down pollutants) often need a nitrogen source to effectively degrade oil spills (Röling et al., 2002, and references therein).

Microorganisms can be thought of as "ecosystem engineers" because of the central role they can play in an ecosystem. In fact, when you zoom out and view our planet as one giant ecosystem and see that microorganisms were responsible for the early oxygenation of earth (Kopp et al., 2005), you can appreciate just how powerful are these tiny members. In addition to oxygen, microorganisms are involved in the biogeochemical cycling of many chemical elements/molecules. Another example of a prominent biogeochemical cycle that is often the focus of microbial ecologists is the sulfur cycle, which contains components responsible for acid rain. Biogeochemical cycles often interact and the sulfur and carbon

cycles are prime among them – sulfate reduction, a biotic component of sulfur cycling is responsible for the conversion of up to 50% of organic matter back into CO_2 in anoxic marine sediments (Jörgensen, 1982; Canfield et al., 1993).

Overcoming Major Hurdles in Microbial Ecology

Because microbial ecology is primarily concerned with microorganisms in their native environment, it often involves working with microorganisms that are not in pure culture. "The great plate count anomaly" is a common phrase (coined by Staley and Konopka, 1985) used in microbial ecology that refers to the difference between the number of colony-forming cells versus those visible by microscopy from the same environmental sample. Often this difference is several orders of magnitude with 1% of visible cells producing colonies (Staley and Konopka, 1985; Connon and Giovannoni 2002), a testament to how many microbes are recalcitrant to culturing by common methods. Recent high-throughput cultivation techniques allow us to culture new species (Leadbetter, 2003, and references therein), however, owing to factors such as extreme environments and common symbiotic associations between different species, it is often the case that a microbial ecologist has to investigate a species or consortia without the convenience of having them in pure culture.

Molecular phylogenetic surveys are a pioneering technique in microbial ecology (Pace 1997; Woese and Fox 1977) that allow for the culture-independent characterization of microbial diversity in a sample. Briefly, this technique relies on the 16S ribosomal RNA gene, which is conserved in all bacteria and archaea, yet undergoes enough variation to allow for the detection of differences at the species level. By examining the diversity of

16S rRNA genes in an environmental sample, one can begin to understand the overall microbial diversity in an environment without needing to culture its members. A similar approach involves looking at the diversity of genes involved in a specific metabolic pathway, for example, sulfate reduction, in order to get an overview of the diversity of organisms potentially carrying out that metabolic pathway in a particular environment (Meyer and Kuever, 2007). These genes may not have the same phylogenetic resolution as 16S rRNA gene studies, but they allow one to focus on the diversity of a particular microbial guild, or functional group, in an environment.

Microbial ecologists can thus combine molecular phylogenetic surveys with a detailed study of the native environment of the microorganisms in order to guide investigations of microbial function and processes in that environment. Comparing multiple environments and their microbial communities reveals what environmental factors affect the microbial community composition and function and vice versa. Terrestrial mud volcanoes, which will be discussed in further detail below, provide an excellent opportunity for this type of comparative analysis as they represent discrete but similar environments, like mini-ecosystems, that can be compared and contrasted in order to gain an understanding of the dynamics between microbial communities and their environments. Surveying the variations in geochemistry in different mud volcanoes, reveals parameters that warrants further investigation. For example, variations in sulfate concentrations between mud volcanoes could prompt measurements of the diversity of microorganisms involved in sulfur cycling. Trends observed at this level of diversity could further lead to measurements of sulfate reduction rates from bulk samples, together creating an informative view of sulfur cycling in mud volcanoes.

The study of microbial processes, such as sulfate reduction, leads us to another major hurdle in microbial ecology: the coupling of function with phylogenetic identity. In the previous example, phylogenetic diversity of sulfur-cycling microorganisms as well as sulfate reduction rates were investigated. However, in that example there was no way of knowing which sulfate-reducing bacteria identified in the gene survey were responsible for the bulk sulfate reduction rate measurements. It could be the case that all of the bacteria from the gene survey were active, but more likely it would be the case that different ones were active or dominant under different conditions. Further, as previously described many functional gene studies do not provide phylogenetic resolution at the species or even genus level, so how do we begin to discern precisely who is doing what in an environmental sample?

There are several means of isolating individual cells from environmental samples in order to query their DNA for both 16S rRNA gene identity as well as functional genes (or even sequence the entire genome). One method developed by Ottesen and colleagues (2006), uses microfluidic PCR reactions in which individual cells are first isolated into discrete micro chambers. A dual PCR reaction is then performed in which both 16S rRNA and functional genes can be amplified and later sequenced, thus telling us the phylogenetic identity and metabolic capabilities of a single cell. Another method, developed by Pernthaler and colleagues (2008) begins with a specific fluorescence *in situ* hybridization (FISH) reaction followed by the attachment of microscopic magnetic beads to the targeted cells via antibodies, which bind the FISH fluorophores. A strong magnet can then be applied to the sample and all but the target cells washed away. Once an enrichment of target cells is thus attained, 16S rRNA and functional gene PCR amplification can be

performed on the target cells.

Stable isotope probing studies can be used to determine who is doing what in a sample. Environmental samples are incubated with a substrate of interest that has been labeled with a heavy isotope, which will be incorporated into the biomass of cells consuming the substrate. Incubations of environmental samples in small volumes (< 1 l) are often referred to as microcosms, and are often used in microbial ecology to measure microbial processes or the effects of various amendments or physiochemical perturbations on the microbial community. Once a microcosm has been incubated with a labeled substrate, for example, ^{15}N-ammonium or ^{13}C-carbon dioxide, there are two basic means for identifying which microorganisms consumed this substrate. In the first method, stable isotope probing (SIP; Radajewski et al., 2000), DNA or RNA is extracted from the microcosm and separated by density such that the DNA/RNA containing the heavier isotopes can be isolated. 16S rRNA surveys of this heavier DNA/RNA fraction can then be carried out to determine the phylogenetic identity of the organisms that consumed the labeled substrate. The second basic method involves first identifying the cells using a 16S rRNA FISH probe and then measuring the isotopes of the targeted cells to determine if the heavier isotopes have been incorporated from the labeled substrate. This method is particularly powerful as it allows microbial ecologists to couple function with identity in an environmental sample while still observing cell-cell interactions, such as symbioses, which will be explored further in the following section.

Microbial Ecology in Action: Thesis Overview

Microorganisms are a driving force behind many ecosystems that are relevant to

humans, from forests that provide oxygen, to the oceans, which absorb CO_2. Some ecosystems are particularly critical to humans simply because their balance maintains our status quo. Polar ice caps are such examples as they maintain the sea level. Though climate change has brought many such ecosystems to our attention, there are many whose balance we still take for granted. I would like to turn your attention to a very potent green house gas, methane, which is a central part of several naturally occurring ecosystems. This exists in large stores in the Earth's surface and escapes through various outlets on land and in the ocean. There are two prominent methane sources – mud volcanoes and marine methane seeps – that I focus on in my thesis. Mud volcanoes are a major source of methane flux to the atmosphere (6-9 Tg/year; Etiope and Milikov, 2004), and while the anaerobic oxidation of methane (AOM) is responsible for recycling up to 80% of the oceanic methane production (Reeburgh, 2007), an estimated 11-18 Tg are released annually (Bange et al., 1994). In order to better understand these crucial ecosystems, we set out to understand how their geochemistry interplays with their anaerobic inhabitants. Anaerobes specialize in breathing molecules other than oxygen, such as nitrate, manganese, iron and sulfate. We first chose to study anaerobic microorganisms in terrestrial mud volcanoes because they represent convenient and relatively discrete, self-contained environments whose geochemistry and microbial diversity and function could be compared to key factors in the functioning of these ecosystems.

Chapter 1 of my thesis focuses on terrestrial mud volcanoes. In October of 2008 we visited four terrestrial mud volcanoes in Azerbaijan, a region of very densely populated TMVs (Figure 1). This was a multidisciplinary group in which Hans-Martin Schultz, Jens Kallmeyer and Akper Feyzullayev measured geochemistry; Jens Kallmeyer, Patrick Sauer,

Casey Hubert and Martin Krueger measured bulk microbial processes; Martin Krueger conducted 16S rRNA qPCR analyses and I conducted 16S rRNA and functional gene analyses as well as fluorescence *in situ* hybridization. Among the many geochemical factors measured, sulfate was variable and in some habitats more than sufficient to potentially support active sulfate reducing bacteria (SRB). SRB are a ubiquitous class of microorganisms, which make a functional guild defined by the ability to respire sulfate (ie, to use sulfate as a terminal electron acceptor). This is an extremely environmentally relevant guild of microorganisms due to their role in bioremediation and oil field souring, both afforded by this guilds' ability to degrade a vast array of organic compounds. We measured active sulfate reduction whose magnitude clearly reflected the ambient sulfate concentrations. The surface connection to the deep biosphere and underlying hot fluids were an additional motivation for this study, and a surprising find was also the discovery of active thermophilic sulfate reduction, which we measured in several of the TMVs.

Measuring SR tells us what an environment's inhabitants are capable of doing, but it does not tell us about the diversity of species responsible for this process. We therefore surveyed the phylogenetic identity of genes specific to sulfur cycling in each TMV. What we found was that genes from organisms capable of sulfate reduction dominated the gene libraries from environments containing measureable levels of sulfate, whereas those with little or no sulfate were dominated by genes from organisms capable of sulfur oxidation. Among the sulfate-reducing bacterial genes we also found several interesting results. We

Figure 1. Sample collection from mud volcanoes in Azerbaijan, 2008.

were able to confirm the presence of thermophilic SRB and we found evidence for niche differentiation among two major types of SRB: those capable of degrading organic compounds completely to CO_2 and those which can only incompletely degrade carbon substrates, releasing acetate as their byproduct.

Although the nature of the samples made microscopy work difficult we were also able to identify some of the organisms detected by our gene targeted analyses, in association with anaerobic methane-oxidizing archaea (ANME). This example of a now famous symbiosis is highly suggestive of sulfate reduction coupled to methane oxidation, which we did indeed observe evidence for in subsequent microcosm activity experiments.

This symbiosis has been observed previously in terrestrial and marine mud volcanoes where the dominant form present appears to be a combination of ANME associated with SRB related to incomplete carbon-oxidizing SRB. We also observed examples of ANME with different families of bacteria and wanted to explore this phenomenon further.

In order to do this I would like to take you now to the bottom of the ocean (Figure 2) where vast stores of methane naturally exist as methane hydrates along continental margins, which slowly disassociate and seep into the overlying sediment. These are the sites from which the anaerobic oxidation of methane (AOM) was first characterized and indeed the sample type, much less viscous and often oil free sediment greatly facilitates their investigation via microscopy. This affords us a number of tools that get at one of the more crucial questions in microbial ecology, "Who is doing what?" You will notice in the prior investigation we were able to measure the activity of SRB and look at the diversity of SRB present via their genes. However with these tools it is not possible to say which SRB were responsible for which portions of the sulfate-reduction rates (SRR) we observed. The next two chapters of my thesis use a technique known as FISH-NanoSIMS (nanoscale secondary ion mass spectrometry), which allows us to measure labeled isotopes (from cells incubated with isotopically enriched substrates) inside individual cells previously identified via FISH (Orphan et al., 2001; Dekas and Orphan, 2011). Along with molecular and geochemical methods, we used FISH-NanoSIMS to explore the ANME/SRB symbiosis in marine methane seeps.

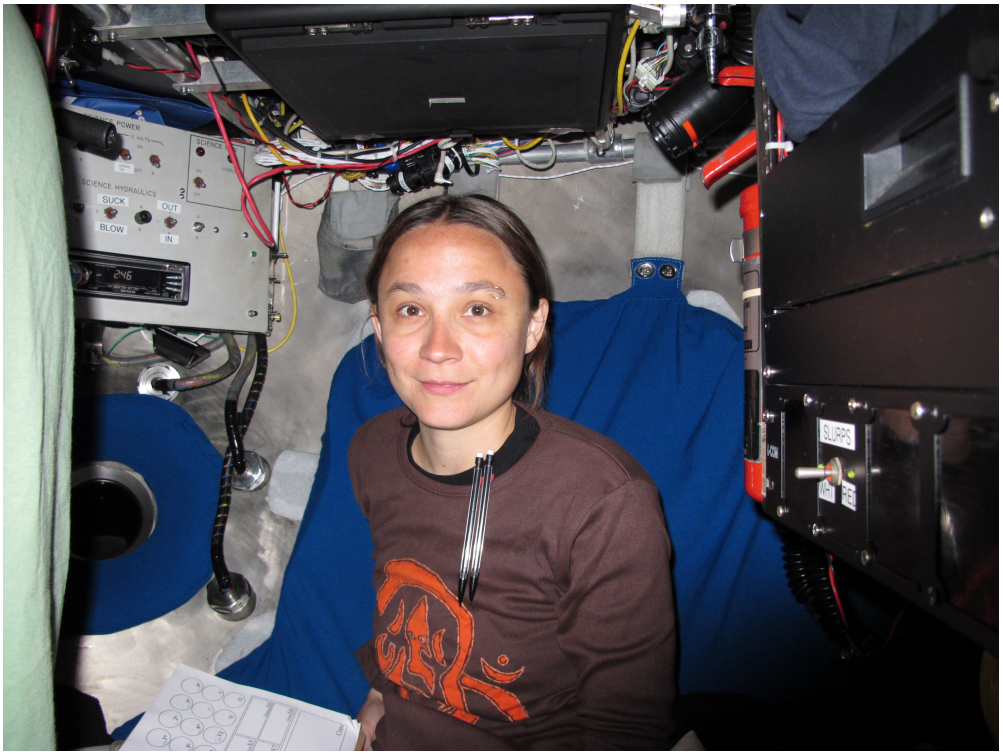

Figure 2. Sample collection from ocean floor; inside ROV Alvin, 2010.

The anaerobic oxidation of methane (AOM) is responsible for recycling up to 80% of the oceanic methane production (Reeburgh, 2007). This crucial biogeochemical process serves as a major sink for methane, a greenhouse gas with heat trapping capabilities up to 20 times stronger than CO_2 (Schiermeier, 2006). Syntrophic aggregates of ANME and SRB appear to carry out the anaerobic oxidation of methane (Orphan et al., 2001). In the following putative pathway, sulfate serves as the electron acceptor for methane (Boetius et al., 2000; Iverson and Jorgensen, 1985): $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$. However, to date, neither ANME nor SRB involved in this reaction have been grown in pure culture, and thus the pathway for AOM, including the method for electron transfer, remains unclear (Knittel and Boetius, 2009). Indeed, Milucka and colleagues (2012) recently proposed a

new pathway in which ANME-2 is capable of both the anaerobic oxidation of methane and reduction of sulfate to disulfide (or other S0 compounds), which is then scavenged by the SRB and disproportionated to sulfide and sulfate.

First we wanted to address the importance of sulfate reduction to the ANME/SRB consortia. As sulfate-reducing bacteria were initially implicated as an agent responsible for AOM (Reeburgh, 1976), multiple studies have used sulfate reduction inhibitors such as molybdate or tungstate to validate and study dynamics of AOM (Alperin and Reeburgh, 1985; Hansen et al 1998; Iversen et al., 1987; Nauhaus et al., 2005; Orcutt et al., 2008). Due to the difficulties of getting the ANME/SRB consortia into pure culture, prior sulfate-reduction inhibition studies are based on bulk geochemical measurements in which they inhibit sulfate reduction in an entire community and measure the AOM activity rate of that community (Alperin and Reeburgh, 1985; Hansen et al. 1998; Iversen et al., 1987; Nauhaus et al., 2005; Orcutt et al., 2008). Cell specific tracking of the effects of sulfate-reducing inhibitors on ANME/SRB aggregate abundance and composition can be accomplished via FISH, which can then be coupled to NanoSIMS in order to measure growth in consortia incubated with ^{15}N-labeled ammonium (Orphan et al., 2009). FISH-nanoSIMS allows the coupling of function with identity through the measurement of ^{15}N incorporation in individual cells or aggregates whose phylogenetic identity is determined via FISH. For chapter 3 of my thesis, in order to study dynamics of AOM, we inhibited sulfate reduction and followed the metabolic processes of the microcosm community as well as the effect of aggregate composition and growth on a cellular level. We found that while bacterial cells appear to decay, ANME cells persist in the form of ANME-only aggregates, which were found to exhibit little to no growth (as measured with FISH-NanoSIMS) when sulfate

reduction is inhibited. Our data suggest the growth and metabolism of ANME is tightly linked to the bacterial partner.

Once we confirmed the importance of sulfate reduction to the growth of ANME/SRB consortia in these marine methane seeps, we began to focus (Chapter 2 of my thesis) on another critical question related to this system: why do ANME associate with multiple families of SRB in one habitat? A previous study in our lab showed that the ANME in these habitats can associate with either Desulfobacteraceae (DSS) or Desulfobulbaceae (DBB) (Pernthaler et al., 2008). Gause, one of the founders of early ecology put forth the notion that ecosystems can be defined by their niches, or habitats they provide for their inhabitants (Gause, 1934). This is true for microbial ecology as well and in fact SRB are known to divide a habitat into microniches allowing their co-existence (Dar et al., 2007). Thus the apparent functional redundancy suggested by ANME coupling with multiple SRB families in the same habitat could potentially be explained by the existence of separate niches within which each of these variants of the symbiosis thrive.

The more abundant aggregate form observed was ANME/DSS, so we decided to follow this up first by seeing how ubiquitous was the ANME/DBB symbiosis. If you look closely, it is everywhere. But importantly, it is more abundant in the shallowest parts of the examined habitats, which is not where we typically expect to see a lot of ANME/SRB thriving. Various nutrients and energy sources may be more available in these shallow horizons thus we decided to measure nitrogen species in these habitats in subsequent research cruises. In several instances we saw that nitrate peaks in the shallow depth horizons below microbial mats. Upon investigation this is where the ANME/DBB also peaked and there appeared an appaprent correlation between nitrate and relative

ANME/DBB numbers.

Incubations from methane seeps were amended with ^{15}N-nitrate and followed over time. Nitrate-amended incubations showed a higher relative number of ANME/DBB at later time point than no-nitrate incubations. In order to determine if these ANME/DBB were utilizing nitrate we used FISH-NanoSIMS to compare nitrate incorporation from ANME/DBB and ANME/DSS aggregates. We found that the ANME/DBB aggregates did indeed incorporate more nitrate than their ANME/DSS counterparts. Further, this was not true for labeled ammonium incubations, suggesting that these two types of aggregates grow at similar rates but ANME/DBB simply prefers nitrate more than do ANME/DSS aggregates. These findings are interesting as they suggest that ANME/SRB aggregate types may coexist via dividing their environment based on nitrate, with ANME/DBB using nitrate either as nutrient or energy source.

In sum these data uncover novel aspects of the sulfur-cycling microbial communities in two crucial ecosystems rich in natural methane stores. As discussed further in the conclusions section, this contribution allows for a more complete understanding of not only *in situ* communities and processes but also novel factors that may be central to the ecosystem and yet were heretofore unknown.

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