AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Marine sediments contain an abundance of methane that is biologically produced and plays a significant role in the global carbon cycle. Microbes responsible for the carbon cycle in marine sediments, and the processes that they carry out, need to be characterized in order to fully understand the role of this large methane reservoir in the global carbon cycle. The objective of this research was to describe the identity, distribution, and the factors that control distributions of microbes in three biogeochemical zones that are defined by methane in marine sediments, namely: the sulfate-methane transition (SMT), the gas hydrate occurrence zone (GHOZ), and the free gas zone (FGZ). Sediments from the Cascadia margins, Indian Ocean, Andaman Sea, and Ulleung Basin were examined. Fracture-dominated SMT environments from the Pacific and Indian Oceans harbored unique macroscopic biofilms composed of ANME-1 and *Deltaproteobacteria*. These biofilms contained 1-2 orders of magnitude more cells cm^{-3} than the surrounding sediment. The Andaman Sea sediments occur in a unique forearc basin that contains biogenic methane; yet, the organic carbon content here is lower than similar environments. Sediments from the Andaman Sea contained 1-2 orders of magnitude fewer cells cm^{-3} than typical hydrate-containing sediments and members of the Firmicutes such as *Bacillus* species dominated the microbial community. Statistical

analysis of the molecular data using non-metric multidimensional scaling (NMS) and multi-response permutation procedures indicated that the GHOZ in the Andaman Sea contains a microbial community distinct from communities above and below the GHOZ. The measured abiotic variables most closely associated with the community structure were the concentration of organic carbon and variables associated with increasing depth. The Ulleung Basin sediments from above and below the SMT contained *Deltaproteobacteria* and the marine benthic group-B. NMS and cluster analysis identified two distinct microbial communities in the GHOZ of the Ulleung Basin. The microbial communities in the GHOZ that were typically closer to layers that contained higher hydrate saturation had indicator taxa related to *Vibrio*-type species. NMS ordinations also indicated that microbial communities from all three zones (SMT, GHOZ, or FGZ) were distinct from each another. Future refinements of total subsurface cellular abundance will benefit by including the cell abundance terms reported here. In addition, the biogeography of methane-containing sediments presented here will aid in understanding the carbon cycle in marine sediments by identifying environmental constraints on microbial taxa.

Geomicrobiology of Marine Sediment Containing Methane

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Brandon R. Briggs, Author

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1. INTRODUCTION

Methane

Methane is a greenhouse gas that is 33 times more potent on a volume basis than carbon dioxide, and thus, atmospheric variations in methane content can significantly influence global climate (Mitchell, 1989). Presently the total flux of methane to the atmosphere is 500 Tg yr^{-1} (Reeburgh, 2007). Anthropogenic methane emissions account for 69% of the total methane flux and are responsible for the dramatic increase in atmospheric methane concentration from pre-industrial 700 ppb to about 1700 ppb today (Schnoor, 2007). Seventy-eight percent of anthropogenic methane emissions are the result of habitat creation that is amenable to biological production of methane (Colwell & Ussler, 2010).

Biological production of methane is accomplished by microbial degradation of organic matter under anoxic conditions and is the largest natural source of methane. Environments that support natural biogenic methane production such as wetlands, tundra, and swamps emit 115 Tg yr^{-1} and are the major natural sources of methane. Marine sediments are the largest reservoir for methane yet they emit only 5 Tg yr^{-1} of methane into the atmosphere (Colwell & Ussler, 2010). This is, in part, because of both physical and biological controls on methane flux from marine sediments.

Methane hydrates and sediments rich in methane

Marine sediments contain an estimated 500 to 2500 gigatons of methane carbon (Milkov, 2004). Depending on the temperature, pressure, salinity, and methane concentrations the methane can reside as free gas, dissolved gas, or as a gas hydrate (frozen crystalline solid composed of rigid cages of water molecules enclosing the methane or other small molecular weight gasses) (Sloan, 2003). Gas hydrates have been found or inferred to be present along the continental margins of all oceans as well as in inland seas, lakes, and permafrost regions (see http://walrus.wr.usgs.gov/globalhydrate for an updated inventory) (Trehu*, et al.*, 2006).

Since the discovery of gas hydrates, several areas of research related to these geological features have been undertaken. The depletion of traditional fossil fuels has prompted research into alternative or "non-conventional" fuels. Although only a small fraction of methane trapped within gas hydrate may be accessible as a resource, it might still represent a significant contribution to our fossil energy reserves. Research has focused on possible ways to recover the methane. For example, the Mallik Project was a test well in the Mackenzie delta in far northwest Canada aimed at characterizing the hydrate and then trying to extract the methane. In a preliminary experiment, the scientists were able to capture the methane both after reducing the pressure of the well and after recirculating warm water through the well (Kerr, 2004).

Methane is also known to be a potent greenhouse gas and, accordingly, researchers are exploring the extent to which a release of methane from gas hydrates may play a role in global climate change (Dickens, 2003; Sloan, 2003). There is evidence that

in the past large amounts of methane were released into the atmosphere. Dickens asserts that during the Paleocene/Eocene thermal maximum, hydrates dissociated, causing an immense amount of carbon to be released into the ocean and atmosphere (Dickens, 2003). Benthic foraminifera $\delta^{18}O$ records appear to demonstrate that a sudden increase of seafloor temperatures occurred just prior to a large ^{12}C input into the oceans (Dickens, 2003). A modern example of gas hydrate dissociating due to temperature fluctuations has been documented along the West Spitsbergen continental margin. The West Spitsbergen current has warmed by $1^{o}C$ over the past 30 years and recently 250 plumes of methane bubbles have been discovered emanating from the seabed along the landward limit of the hydrate stability (Westbrook*, et al.*, 2009). A large-scale simulation of this area suggests that the methane plumes are dissociating from hydrate deposits (Reagan & Moridis, 2009). This research strongly suggests that there is potential for substantial methane hydrate dissociation in the future, if bottom water temperature was to rise.

Researchers are also looking at hydrate-saturated sediments and their role in seafloor stability. Gas hydrates are present in large masses on continental slopes. If a large amount of hydrate dissociated releasing the trapped gas, then the sediment could collapse causing an underwater slide or slump (Paull*, et al.*, 2002). Among other threats, communication cables can be severed by such underwater slides. There is evidence of very large slides in locations rich in gas hydrates; however, it is uncertain if the gas hydrate destabilization actually triggered the slide (Kvenvolden, 1999).

Microbes involved in the subseafloor carbon cycle

Stable isotope analysis has shown that methane hydrate systems are often composed of biologically produced methane (Kvenvolden, 1995). Microorganisms that can decompose organic matter to produce methane in a process called methanogenesis have been found in gas hydrate bearing sediment (Wellsbury*, et al.*, 1997; Reed*, et al.*, 2002; Mikucki*, et al.*, 2003; Newberry*, et al.*, 2004; Inagaki*, et al.*, 2006). The biological production of methane has been attributed to a group within the Archaeal Domain called methanogens. Example phyla of methanogens are *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales,* and *Methanopyrales* (Madigan*, et al.*, 2000). Methanogens are strict anaerobes that commonly occupy anoxic, sulfate-deficient environments such as swamps, tundra, hydrothermal vents, landfill sites, sewage, and marine sediments. The environments that they occupy exhibit large temperature ranges from as high as 122^{o}C (*Methanogenium kandleri* strain 116) (Takai*, et al.*, 2008) to below 0^{o}C (*Methanogenium frigidum*) (Madigan*, et al.*, 2000).

Methanogens mediate methane production by either reduction of C1 compounds or disproportionation reactions of three classes of substrates: CO_2 –type substrates, methyl substrates, and acetate. CO_2 –type substrates include CO_2 (reaction 1), CO, and formate (Madigan*, et al.*, 2000). The reduction of these C1 compounds depends on hydrogen to provide electrons for CO_2 reduction. The second class of methanogenic substrates, methyl substrates, includes methanol, methylamine, methylmercaptan, and dimethylsulfide. These substrates can be reduced to methane using hydrogen as an electron donor (reaction 2). In the absence of hydrogen, some methanol can be oxidized

to CO_2 to generate electrons needed to reduce additional methanol molecules (reaction 3). The most common methanogenic disproportionation reaction uses acetate (reaction 4) and most methane in nature is produced from acetoclastic methanogenesis (Ferry, 1999). This process is through a modified reverse acetyl-CoA pathway that produces electrons from the oxidation of the carbonyl-group to CO_2 (Ferry, 1999).

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ $\Delta G^{0'} = -131$ kJ/ mol CH_4 (reaction 1)

$CH_3OH + H_2 \rightarrow CH_4 + H_2O$ $\Delta G^{0'} = -112$ kJ/ mol CH_4 (reaction 2)

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ $\Delta G^{0'} = -319$ kJ/ mol CH_4 (reaction 3)

$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ $\Delta G^{0'} = -36$ kJ/ mol CH_4 (reaction 4)

Despite the large production of methane in marine sediments very little methane is released into the water column and even less to the atmosphere. This is, in part, because of a consortium of sulfate reducing bacteria (SRB) and archaeal anaerobic methanotrophs (ANME) that work symbiotically to remove methane from sediments and anoxic water column reservoirs (Hinrichs & Boetius, 2002; Valentine, 2002; Alperin & Hoehler, 2009). Globally, anaerobic oxidation of methane (AOM) (reaction 5) is estimated to oxidize 304 Tg of methane per year (Hinrichs & Boetius, 2002), a quantity estimated to be 90% of methane that is produced in marine sediments (Reeburgh, 2007). However, the efficiency of this biofilter is dependent on the flux of methane. In diffusively controlled marine sediments the AOM consortia oxidize 100% of the methane to carbon dioxide (Niewohner*, et al.*, 1998). However, seeps are dominated by fluid advection and AOM is unable to oxidize all of the methane because the methane seepage is faster than the AOM metabolic processes. For example, anaerobic processes consume

only 37% of methane from the Haakon-Mosby mud volcano (Niemann*, et al.*, 2006) leaving a large proportion of the methane to escape into the overlying water.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \Delta G^{0'} = -16 \text{ kJ/ mol } CH_4 \quad \text{(reaction 5)}$$

Evidence of AOM came from geochemical analysis such as methane profiles (Martens & Berner, 1977), radiotracer experiments (Iversen & Jorgensen, 1985), and stable carbon isotope data (Alperin*, et al.*, 1988) (reviewed in (Reeburgh, 2007)). In 2000, the microorganisms responsible for AOM were observed using fluorescence in-situ hybridization (FISH) (Boetius*, et al.*, 2000). Phylogenetic analysis of the AOM consortia has shown that there are three groups of methane-oxidizing archaea (ANME-1, 2, and 3) (Orphan*, et al.*, 2002; Knittel*, et al.*, 2005; Losekann*, et al.*, 2007). These organisms have ^{13}C-depleted lipids, which indicate that the ^{13}C-depleted methane is a carbon source for the AOM consortia (Orphan*, et al.*, 2001; House*, et al.*, 2009). In addition, metagenomic and protein studies indicate that these organisms are likely involved in AOM (Kruger*, et al.*, 2003; Hallam*, et al.*, 2004). These reports document that microbes are present in the deep biosphere, and are responsible for the production and consumption of methane. Yet, we are still trying to understand fundamental aspects about the microbial processes that cycle carbon in gas hydrate-rich sediments. Knowledge of the microbial distributions in methane containing marine environments will help to elucidate the biological processes that mediate methane presence and their role in the global carbon cycle.

General subseafloor microbial cellular abundance

In general, most microbial biomass can be found near the seafloor where organic carbon is more readily available; biomass then decreases with increased depth below the seafloor (Parkes*, et al.*, 1994; Parkes*, et al.*, 2005). In 1994, Parkes *et al*. obtained cell counts from five sites in the Pacific Ocean which revealed a logarithmic decrease of cell numbers with depth. Subsequently, based on the Parkes *et al.* (1994) data, Whitman *et al*. (1998) calculated the total abundance of oceanic subsurface cells to be 3.5×10^{30}, which represents 303 Pg of carbon (Whitman*, et al.*, 1998).

While the Whitman *et al.* (1998) paper has been widely cited, revisions of these numbers are needed because they were based on a limited number of subseafloor samples. The cell count data that was reported by Parkes *et al*. (1994) was obtained from only five sites along continental margins in the Pacific Ocean where the total organic carbon can range from 1-11% TOC (Meister*, et al.*, 2005). It has been proposed that the logarithmic decrease of cells with depth is likely due to decreasing organic carbon quality and availability (Parkes*, et al.*, 2000). The organic content of sediments along margins is significantly higher than the abyssal plains (0.01-0.5%) (D'Hondt*, et al.*, 2009). Recent data from the South Pacific Gyre sediments indicate that cell counts are 3-4 orders of magnitude lower than on typical continental margins (D'Hondt*, et al.*, 2009). Addition of these data prompts a reconsideration of the total subsurface cellular abundance calculated by Whitman *et al*. (1998). In addition, further refinements of cell abundance in the subsurface will have to include the oceanic crust, which may harbor significant cell

abundance and diversity (Cowen*, et al.*, 2003; Santelli*, et al.*, 2008; Orcutt*, et al.*, 2011; Smith*, et al.*, 2011).

Furthermore, the typical relationship of decreasing cell numbers with increasing depth can depend on seafloor geology. Rebata-Landa and Santamarina (2006) define regions in subsurface strata that constrain microbial survival and activity based on factors such as sediment grain size and pore-throat size (Rebata-Landa & Santamarina, 2006). They propose three regions of microbial activity ('active and motile', 'trapped inside pores', and 'dead') based on pore space and mechanical interactions. In this model, active cells exist in sediments with large pore-throats and dead cells occur when the compressed sediment punctures the cell membrane. Field observations of microbes in the subsurface support this model (Parkes*, et al.*, 2005). Inagaki *et al*. (2003) observed that ash layers from the Sea of Okhotsk contained significantly higher biomass concentrations than intervening clay layers (Inagaki*, et al.*, 2003). The ash layers have larger grain sizes than the clay layers thereby increasing the habitable pore space. Likewise, in the terrestrial subsurface Phelps *et al.* (1994) observed lower cell density and activity in clay-rich sediment than sandy sediment with more habitable space (Phelps*, et al.*, 1994). In addition, the increased space allows for greater fluid flow and delivery of electron acceptors and donors. The introduction of oxidants to the subseafloor coincides with increased biomass (D'Hondt*, et al.*, 2004).

Microbial distributions in geochemical zones defined by methane

In shallow marine sediments the distribution of microbial taxa is dependent on the presence of more electrochemically positive electron acceptors. The remineralization of organic material proceeds in a sequence of redox reactions that cascade from high to low free energy yield until the electron acceptor source is depleted (Kappler*, et al.*, 2005). This sequence of redox reactions manifest as zoned sediment that reflects the dominant microbial community growing at a particular depth. While the geochemical zones of electron acceptors defines the microbial distributions in shallow sediments, in deep marine sediments geochemical zones defined by methane are the dominant features that may control deep microbial distributions.

Methane in marine sediment often produces three distinct zones based on the presence of sulfate, gas hydrate, or free methane gas. The first geochemical zone is defined as a chemical boundary where sulfate ion concentrations are depleted and methane concentration begins to increase; this boundary is known as the sulfate methane transition (SMT) (Figure 1-1A) (Borowski*, et al.*, 1999). The location of the SMT can occur at the sediment water interface, or as deep as several meters, depending on the upward flux of methane (Borowski*, et al.*, 1996). This important geochemical zone is the site where the consortia of SRB and ANME symbiotically carry out AOM.

The AOM consortium is restricted to the SMT because of the dependence on sulfate and methane; however, field and bioreactor studies identify general environmental trends that control the distribution and quantities of different ANME groups. ANME-1 dominate sediments overlying a methane-rich brine pool in the Gulf of Mexico (Lloyd*, et*

al., 2006), and microbial mats from the Black Sea (Knittel*, et al.*, 2005). Most cold seep studies indicate the dominance of ANME-2 or ANME-3 in the top 10 cm of the sediment (Knittel & Boetius, 2009). These studies indicate that ANME-1 are typically found in areas that have more methane and less sulfate, while ANME-2 are found in areas with more sulfate and less methane (Girguis*, et al.*, 2005). In addition, these studies have highlighted environmental conditions that appear to control the cell concentrations where AOM occurs. In advective systems such as Hydrate Ridge and the Black Sea, ample methane supply allows for greater biomass ($>10^{10}$ cells cm^{-3}) at or above the sediment water interface (Knittel*, et al.*, 2005), despite the energetic limitations of AOM (Dale*, et al.*, 2008). However, when the AOM consortia occur meters below the seafloor, biomass is less than or equal to 10^6 cells cm^{-3} (Niemann*, et al.*, 2005). Only recently has our team reported an exception to this biomass restriction in the subsurface (see Chapter 2) (Briggs*, et al.*, 2011).

Below the SMT, if the temperature, pressure, and methane concentration are favorable gas hydrate will form. The second geochemical zone of interest is the gas hydrate occurrence zone (GHOZ), which is defined by the presence of gas hydrate (Figure 1-1B). Microbiological investigations of the GHOZ have taken place at Blake Ridge (Wellsbury*, et al.*, 2000), Nankai Trough (Reed*, et al.*, 2002), Cascadia Margin (Bidle*, et al.*, 1999; Marchesi*, et al.*, 2001; Inagaki*, et al.*, 2006; Colwell*, et al.*, 2008; Nunoura*, et al.*, 2008), and the Peru Margin (Fry*, et al.*, 2006; Inagaki*, et al.*, 2006; Sorensen & Teske, 2006; Biddle*, et al.*, 2008). These studies indicate that the microbes in sediments that contain hydrates appear to be distinct from those in non-hydrate sediments

obtained along the Pacific Ocean margins (Inagaki*, et al.*, 2006). Using clone libraries to study community diversity Inagaki *et al*. (2006) found that gas hydrate saturated sediment is typically dominated by the bacterial candidate division JS1 and the Deep-Sea Archaeal Group (Inagaki*, et al.*, 2006). Non-gas hydrate areas are dominated by the bacterial phylum Chloroflexi and the Marine Crenarchaeotic Group I (Inagaki*, et al.*, 2006). Furthermore, these studies verified the presence of methanogens (Mikucki*, et al.*, 2003; Kendall*, et al.*, 2006; Colwell*, et al.*, 2008).

Underneath the GHOZ methane occurs as free gas. The lower boundary of gas hydrate stability is commonly imaged as a bottom-simulating reflector (BSR) in seismic data (Figure 1-1C). The BSR is characterized as a change in reflectance of a seismic section indicating the boundary where hydrate is no longer stable and methane exists as a free gas (Trehu*, et al.*, 2006). The term free gas zone (FGZ) is used elsewhere in this thesis to describe this location. Early expeditions did not drill through the BSR because of safety concerns about overpressure. Therefore, few studies have described the microbiology at the BSR.

One of the first microbiological studies on sediments in the BSR was by Reed *et al.* (2002) from the Nankai Trough (Reed*, et al.*, 2002). Reed *et al.* found 10^4 to 10^5 cells g^{-1} of sediment and a diverse assemblage of Archaeal and Bacterial 16S rRNA gene sequences belonging to *Euryarchaeota, Crenarchaeota, Planctomycetes, Firmicutes, Beta* and *Deltaproteobacteria*. A study from the Cascadia margin that focused on the functional gene *mcrA* (key functional gene for methanogenesis) found an increase of methanogens at the BSR (Yoshioka*, et al.*, 2010). Likewise, Wellsbury *et al*. (1997)

suggested an increase in methanogenesis at the BSR based on increased acetate concentrations and acetoclastic methanogenesis enrichments (Wellsbury*, et al.*, 1997).

The above studies provide some generalizations about the microbiology in gas hydrate-containing sediment. Most studies have found a diverse assemblage of both Archaea and Bacteria including methanogens, but in low abundance (Newberry*, et al.*, 2004; Biddle*, et al.*, 2008; Colwell*, et al.*, 2008). Inagaki *et al*. compared the microbial communities in hydrate and non-hydrate cores along the Pacific Ocean Margins and found that distinctive microbial communities occur in sediments that contain methane hydrates (Inagaki*, et al.*, 2006).

The geochemical zones defined by methane (SMT, GHOZ, FGZ) can be tested to see if they contain distinct microbial communities. Large-scale community diversity assays that have been reported did not consider the microbial diversity in discrete sediment layers where gas hydrates form (Marchesi*, et al.*, 2001; Newberry*, et al.*, 2004; Inagaki*, et al.*, 2006; Hamdan*, et al.*, 2008; Nunoura*, et al.*, 2008; Yoshioka*, et al.*, 2010). Studies that have considered the effect of gas hydrate on the microbial distributions within a single site have been limited in scope. Reed *et al.* (2002) considered the microbial diversity above, within and below the GHOZ but only had one sample from each layer (Reed*, et al.*, 2002). None of the above studies have considered the microbial communities in the context of their environmental conditions. Higher-resolution studies that consider the microbial communities in the geochemical zones defined by methane and associated abiotic characteristics of the sediment are needed to elucidate the biogeographic patterns.

Molecular techniques to study subsurface communities

Traditionally, microbial studies used pure culture models. With respect to the specific microbes responsible for producing and consuming methane, current culture-based methods are of limited value for detecting cells, because most environmental bacteria will not grow using current culturing techniques (Staley & Konopka, 1985; Cragg*, et al.*, 1990). In order to fully understand microbial populations, molecular ecology techniques are required for the analysis of microbial signatures in environmental settings.

The most commonly used microbial signature is DNA, and techniques have been developed to obtain the DNA by lysing the microbes in the sediment, followed by subsequent chemical purification. Once the DNA is obtained a researcher can gain information to help identify an organism and to infer its metabolic capabilities, based on previous reports of similar organisms. Although there are several limitations to using molecular techniques for characterizing environmental microbes (e.g., low biomass, inefficient lysing of cells, DNA degradation, poor primer efficiency, and co-extraction of PCR inhibitors), it is still possible to detect DNA in such complex samples with the right tools and procedures (Inagaki*, et al.*, 2004).

No one DNA extraction technique has been shown to be the best for all environments. Inefficiency in DNA extractions is often linked to either incomplete lysing of microbial cells or the loss of DNA during purification steps. Lipp *et al.* (2008) determined lysing efficiency of different DNA extraction procedures by visually

searching the sediment for intact cells before and after lysing (Lipp*, et al.*, 2008). Increasing the lysing efficiency led to the development of a DNA extraction technique that obtained more Archaeal DNA as shown by comparing molecular and lipid data.

Techniques have been developed that analyze DNA after it has been obtained. One technique that is commonly used is terminal restriction fragment length polymorphism (T-RFLP) (Liu*, et al.*, 1997; Sakano*, et al.*, 2002; Reardon*, et al.*, 2004; Denaro*, et al.*, 2005). This technique amplifies 16S rDNA with fluorescently labeled primers. The amplicon is then digested with a restriction enzyme, and size fractionated on a polyacrylamide gel. The output data appear as a series of peaks on an electropherogram, with each peak representing a different phylotype. T-RFLP is valuable in determining the number and relative abundance of phylotypes, allowing for large data sets that compare biodiversity of differing habitats and environments. However, more than one species can be present for each phylotype (Dunbar*, et al.*, 2001). Specific species that can significantly alter the environment may not be identified, and species richness can be underestimated (Bent*, et al.*, 2007).

DNA sequencing provides more detailed information on the organisms than T-RFLP and this information can be used to identify functional capabilities and phylogenetic relationships. Traditional Sanger sequencing is very time consuming and costly because it relies on clone library construction to isolate individual DNA amplicons. Recently, a push for faster and cheaper sequencing technologies has produced the Illumina and Roche 454 platforms. These platforms rely on an emulsion PCR, which isolates individual DNA amplicons inside oil droplets. In addition, they are

faster and produce many more reads than Sanger sequencing. However, Sanger sequencing can produce about 800 bp reads, an advantage over the Illumina and 454 platforms that only produce about 100 bp and 400 bp, respectively. This shorter length may not allow for distinguishing among closely related taxa.

Microarray technologies have been developed that can quickly identify microbial taxa present in a sample. The PhyloChip version G3 is a microarray technology that has probes for 59,000 microbial taxa (Hazen*, et al.*, 2010). For each taxon there are additional probes that have mismatches for a total of 1,100,000 probes on this array. Thus, known taxa and closely related taxa can be detected. The PhyloChip works by hybridizing amplified environmental DNA to the probes on the chip. This parallel hybridization approach reduces sampling bias and the impact of dominant organisms in detecting less abundant organisms; therefore, it is more sensitive to rare microbes than typical sequencing approaches (DeSantis*, et al.*, 2007). However, as the PhyloChip relies on detecting sequences related to those in existing databases, it is limited in detecting highly novel microorganisms. Approaches that use both PhyloChip analysis and DNA sequencing to analyze samples can produce data sets that cover both novel and rare microbes.

These methods of microbial community characterization can be applied to methane hydrate systems. While each technique has its disadvantages, used in conjunction with each other, the weakness can be overcome. T-RFLP is relatively inexpensive and rapid compared to other techniques for diversity measurements and can be used to narrow the number of important samples for further analysis. PhyloChip and

DNA sequencing can then be used for more detailed investigations. By combining these techniques with geochemical data the relationships that microbes have to environmental factors in methane-rich sediments can be determined.

Statistical analysis of microbial communities

Statistical analysis of microbial communities is becoming more common with the advent of large molecular datasets. These datasets are often complex, making it difficult to identify trends in microbial distributions. Ordination is a statistical tool used to simplify complex datasets by offering a graphical representation of the microbial taxa similarities among multiple samples.

A common ordination technique is non-metric multidimensional scaling (NMS). This procedure calculates a dissimilarity matrix, which is a pair-wise comparison based on a distance measure (Sorenson, etc.). Samples are randomly placed in 1-3 dimensions and the Euclidean distance is calculated between samples. The elements in the dissimilarity matrix (Sorenson distance) are ranked in ascending order and plotted against the ordination distance (Euclidean distance). The stress value is a measure of the departure from monotonicity in the Sorenson versus Euclidean distance plot. A value of 0 indicates perfect monotonicity and that the NMS plot perfectly depicts the true trends. However, a stress value of 0 is often difficult to obtain; therefore, the NMS plot is then rearranged and again the monotonicity is re-checked. After multiple iterations the lowest stress value can be attainted and samples containing common microbes will cluster

together in NMS space (Rees*, et al.*, 2004; Osborne*, et al.*, 2006). Ordination of the environmental data obtained on the same samples can be overlaid on the ordination of taxa to determine which, if any, of the environmental parameters are related to the observed statistical groupings of samples. This approach is valuable for exploratory evaluations of microbial community data from environmental settings (Ramette, 2007).

Objectives

This dissertation expands on current knowledge of microbial biogeography in sediments that contain methane. Specifically, the objective of this research was to describe the cell abundance, biogeographic patterns, and the factors that control distributions of microbes in the biogeochemical zones that are defined by methane in marine sediments: SMT, GHOZ, and FGZ.

To investigate the microbial biogeography in subseafloor geochemical zones samples were obtained from the northeastern Pacific, the Andaman Sea, and the Ulleung Basin (Figure 1.2). Each of these sites contains gas hydrate-saturated sediment and geochemical measurements have been made on corresponding samples, allowing for the microbiology to be placed in the larger environmental context.

Northern Cascadia margin (NCM) is in the Pacific Ocean off the coast of Vancouver Island (Figure 1.2A). The area of this investigation is on the accretionary prism of the Cascadia subduction zone and was extensively studied during two drilling campaigns: Ocean Drilling Program (ODP) Leg 146 in 1992 and Integrated Ocean

Drilling Program (IODP) Expedition 311 in 2005. In addition, Pacific Geosciences Center Cruise, PGC0807, sampled the active cold methane seep Bullseye Vent in 2008. PGC0807 collected sediment samples from six sites using a piston core. At four sites subseafloor biofilm samples were retrieved. These biofilm samples are described in Chapter 2.

In the Indian Ocean, biofilm samples were collected from cores obtained from the Krishna-Godavari (KG) Basin (Site 20A) and the Mahanadi Basin (Site 18A) during the National Gas Hydrate Program Expedition 01 (NGHP01) (Figure 1.2B). These sites are located on a passive margin and the biofilms examined are described in Chapter 2. Site 17A in the Andaman Sea is located in a back-arc basin and is unique in that the geothermal gradient is low allowing gas hydrate to exist down to 600 meters below seafloor. The microbial diversity of 43 sediment samples that transition through the GHOZ were characterized in Site 17A and presented in Chapter 3.

The Ulleung Basin is a back-arc basin located in the East Sea between Japan and South Korea (Figure 1.2C). The Ulleung Basin Gas Hydrate Expedition 02 (UBGH02) was a deep drilling effort focused on characterizing gas hydrate deposits. Sites 1-1 and 10 were chosen for microbiological analysis because samples were obtained from all three geochemical zones (SMT, GHOZ, FGZ) at both sites. The results from the Ulleung Basin research are described in Chapter 4.

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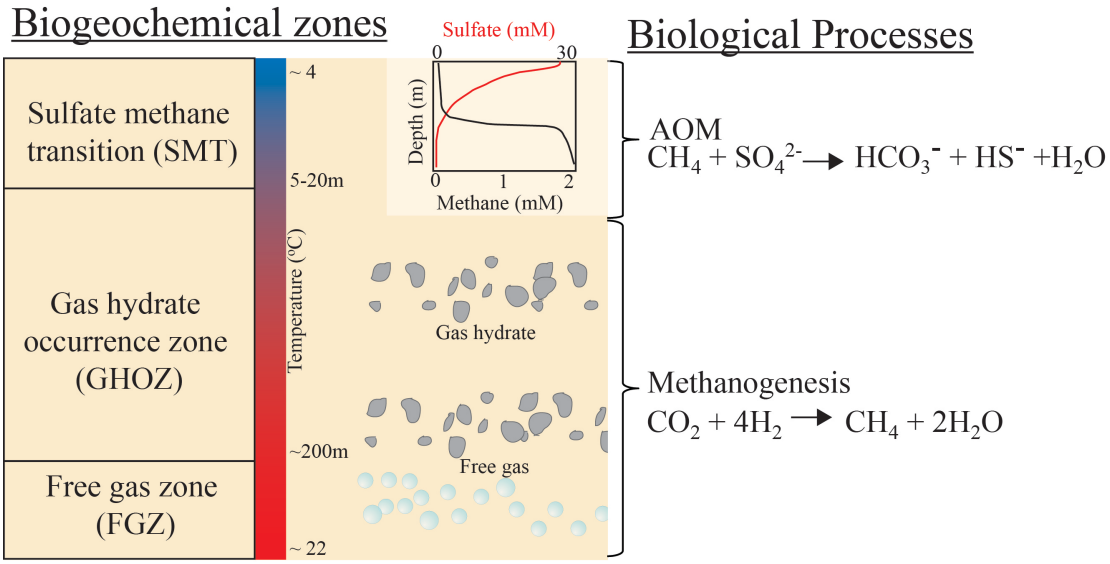

Figure 1.1. Biogeochemical zones defined by methane found in subseafloor environments and the biological processes that can occur in each zone (A), the sulfate methane transition is the depth where sulfate and methane converge (B), the gas hydrate occurrence zone defines the presence of gas hydrate (C), the free gas zone occurs when hydrate is no longer stable because of rising temperature.

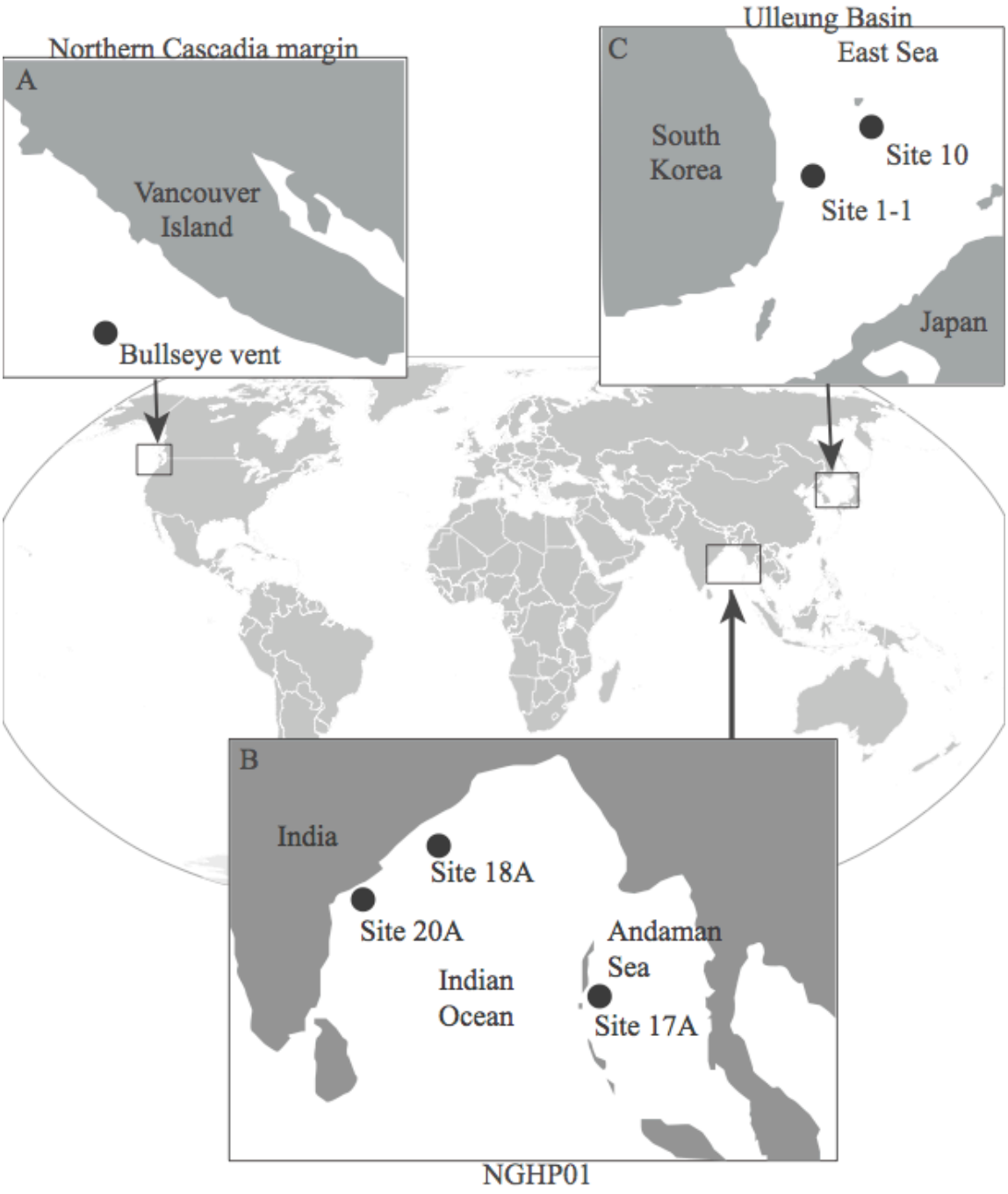
Northern Cascadia margin
A
Vancouver Island
Bullseye vent
Ulleung Basin
East Sea
C
South Korea
Site 10
Site 1-1
Japan
B
India
Site 18A
Site 20A
Indian Ocean
Andaman Sea
Site 17A
NGHP01

Figure 1.2. Locations of microbial biogeography studies presented in this dissertation (A), northern Cascadia margin (B), the Indian Ocean and Andaman Sea (C), and the Ulleung Basin in the East Sea.

2. MACROSCOPIC BIOFILMS IN FRACTURE-DOMINATED SEDIMENT THAT ANAEROBICALLY OXIDIZE METHANE

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Abstract

Methane release from seafloor sediments is moderated, in part, by the anaerobic oxidation of methane (AOM) performed by consortia of archaea and bacteria. These consortia occur as isolated cells and aggregates within the sulfate methane transition (SMT) of diffusion and seep-dominant environments. Here we report on a new SMT setting, where the AOM consortium occurs as macroscopic pink to orange biofilms within subseafloor fractures. Biofilm samples recovered from the Indian and northeast Pacific Oceans had a cellular abundance of 10^7 to 10^8 cells cm^{-3}. This cell density is 2-3 orders of magnitude greater than that in the surrounding sediments. Sequencing of bacterial 16S rRNA genes indicated that the bacterial component is dominated by *Deltaproteobacteria*, candidate division WS3, and *Chloroflexi*, representing 46%, 15% and 10% of clones, respectively. In addition, major archaeal taxa found in the biofilm were related to ANME-1, *Thermoplasmatales*, and *Desulfurococcales*, representing 73%, 11%, and 10% of archaeal clones, respectively. All major taxa were similar to sequences previously reported from cold seep environments. PhyloChip microarray analysis detected all bacterial phyla identified by the clone library plus an additional 44 phyla. However, sequencing detected more archaea than the PhyloChip within the phyla of *Methanosarcinales* and *Desulfurococcales*. The stable carbon isotope composition of the biofilm from the SMT (-35 to -43‰) suggests the production of the biofilm is associated with AOM. These biofilms are a novel, but apparently widespread, aggregation of cells represented by the ANME-1 clade that occur in methane-rich marine sediments.

Introduction

Marine sediments contain an estimated 500-10,000 Gt (1 Gt = 10^{15} g) of methane carbon, primarily in the form of gas hydrate (Milkov, 2004). In the past, this reservoir may have been a significant greenhouse gas source to the atmosphere (Dickens, 2003). Presently, however, oceanic methane contributions (including that from marine gas hydrate) accounts for <2% of the global atmospheric methane flux (Reeburgh, 2007), with most sedimentary methane being microbially oxidized to carbon dioxide in anoxic sediments by the anaerobic oxidation of methane (AOM). Globally, AOM consumes ~90% of methane produced in marine sediments (Reeburgh, 2007).

Environmental genomic and stable isotope studies have linked methane-oxidizing archaea (ANME-1, 2, and 3) to AOM (Boetius*, et al.*, 2000; Orphan*, et al.*, 2002). These anaerobic methanotrophs often form aggregates with sulfate-reducing *Deltaproteobacteria* where they are believed to symbiotically reduce sulfate and oxidize methane by the net reaction:

$$SO_4^{-2} + CH_4 \rightarrow HS^- + HCO_3^- + H_2O.$$

AOM consortia depend on sulfate and methane and thus typically occur at the sulfate methane transition (SMT), a biogeochemical horizon where seawater sulfate and methane from the underlying anoxic sediments converge. SMT environments are classified as diffusion or seep-dominant environments (Alperin & Hoehler, 2009). Diffusion-dominant SMTs are well-defined, occur as deep as several meters below the surface, have moderate methane concentrations (~1 mM), and reduced sulfate concentrations (Alperin & Hoehler, 2010). In these environments, AOM aggregates consist of fewer than 10^6

cells cm^{-3} and consume methane at a rate of 0.001 to 1 nmol cm^{-3} day^{-1} (Niemann*, et al.*, 2005; Sivan*, et al.*, 2007). In contrast to diffusion-dominant environments advective seep-dominant environments, such as Hydrate Ridge offshore of Oregon (USA) and the Black Sea, have near-surface SMTs. The advective flow of methane in these environments often overwhelms the AOM biofilter. For example, anaerobic processes consume only 37% of methane from the Haakon-Mosby mud volcano (Niemann*, et al.*, 2006) leaving a large proportion of the methane to escape into the overlying water. Seep-dominant environments contain ample sulfate and methane that support biofilms or mats with cell densities of the AOM aggregates that exceed 10^{10} cells cm^{-3} (Knittel*, et al.*, 2005). AOM occurs in both diffusion and seep-dominant SMT environments; however, the microbial mechanisms and processes may be different for each environment (Alperin & Hoehler, 2009).

Within each SMT environment, gradients of sulfate and methane are the controlling factors for the types of anaerobic methanotrophs present. For example, ANME-1 are typically found in areas with high methane and limited sulfate concentrations (Girguis*, et al.*, 2005). Examples include a methane-rich brine pool in the Gulf of Mexico (Lloyd*, et al.*, 2006) and microbial mats in the Black Sea (Knittel*, et al.*, 2005; Reitner*, et al.*, 2005). In contrast, ANME-2 and ANME-3 are found in areas with more sulfate and lower methane concentrations such as in the top 10 cm of sediments at cold seeps (Knittel & Boetius, 2009).

We have found macroscopic biofilms from subseafloor fractures intersecting SMTs between depths of 0.5 to 19 meters below seafloor (mbsf) in gas hydrate-bearing

continental margins of the northeastern Pacific Ocean and the Indian Ocean. The objective of this study was to determine the cell concentrations, phylogenetic relationships, and the isotopic composition of the microbes from this novel fracture-dominant SMT environment. These objectives were achieved by sampling visible biofilm from fractures in split sediment cores for molecular analysis, stable isotope analysis, or epifluorescent microscopy.

Materials and Methods

Site descriptions. Macroscopic pink to orange subseafloor biofilms (typically 0.5 cm^3 per occurrence) were collected from sediment fractures that were within or near the SMT in three marine methane-bearing settings: Hydrate Ridge (offshore of Oregon, USA), northern Cascadia Margin (offshore of Vancouver Island, Canada) and the Indian Ocean (offshore of India) (Figure 2.1) (Table 2.1).

Samples from northern Cascadia Margin (NCM) were collected during the Integrated Ocean Drilling Program (IODP) Expedition 311 (EXP 311) and the Pacific Geosciences Center (PGC) Cruise PGC2008007 (referred to herein as PGC0807). During PGC0807, the active cold seep Bullseye Vent was sampled. Bullseye Vent is part of a larger seep field covering an area of about 2 by 4 km on the mid slope of the NCM. Four large seeps varying between 10 to several 100 m in diameter have been identified within this seep field (Riedel*, et al.*, 2002).

Hydrate Ridge is a 25 by 15 km ridge in the Cascadia accretionary complex. Drilling in this area has verified the presence of methane hydrate (Trehu*, et al.*, 2004) and active vents that expel methane into the overlying water column creating bubble plumes hundreds of meters high (Kulm*, et al.*, 1986; Suess*, et al.*, 1999). A biofilm was collected from the Hydrate Ridge area during the coring of the Ocean Drilling Program (ODP) Leg 204 Site 1251A (Trehu*, et al.*, 2003). Site 1251A is characterized by a shallow (0.5 mbsf) SMT (Trehu*, et al.*, 2003).

In the Indian Ocean, biofilm samples were collected from cores obtained from the Krishna-Godavari (KG) Basin (Site 20A) and the Mahanadi Basin (Site 18A) during the National Gas Hydrate Program Expedition 01 (NGHP01). The KG Basin is in a passive margin setting that shows a thick alluvium sediment accumulation deposited since the Miocene in a growth fault environment (Gupta, 2006). Sediment input in this region has been dominated by the Krishna and Godavari river systems producing sedimentation rates ~20-25 cm ka^{-1} (Rao*, et al.*, 1994). The high sedimentation rate and rich source of organic carbon (1.5–2.0 wt% TOC) make the site a good candidate for methane generation in shallow subsurface sediments (Kundu*, et al.*, 2008).

*Sample collection***.** Biofilm and proximal sediments (~10 g of sediment adjacent to, but lacking visible biofilm and hereafter referred to as "reference" samples) were collected during NGHP01 and PGC0807 and preserved for molecular analysis (Table 2.1). Samples were retrieved using an Advanced Piston Core during the NGHP01 or by piston core during the PGC0807 expedition. The IODP guidelines for obtaining high quality microbiology cores were adhered to on both expeditions. The cores were split onboard

and if a biofilm was identified in the split core a sterile spatula was used to transfer the biofilm into a sterile 2 ml microcentrifuge tube. During NGHP01, the tubes were immediately frozen at -80º C. During the PGC0807 expedition, 1 ml of RNA Later® was added to the microcentrifuge tubes, which were kept at 4º C for 24 h and stored at -80º C thereafter. Biofilm samples for stable carbon isotope analysis collected during EXP 311 were preserved at -20º C. Biofilms obtained for epifluorescent microscopy were collected during ODP Leg 204 site 1251A. Cells were counted from a photomicrograph of the stained biofilm taken aboard the *JOIDES Resolution* immediately after the biofilm was collected. The direct count procedure is detailed in the Initial Reports of the ODP Leg 204 (Trehu*, et al.*, 2003) but briefly, cells were suspended in a solution containing 0.01% acridine orange and 0.25% formaldehyde, stained for 5 min, and then filtered through a 0.2 μm pore size filter. The area of the image (1.2×10^{-2} mm^2) was calculated based on the scale bar shown in the photomicrograph (Figure F23 in reference (Trehu*, et al.*, 2003)). These counts estimate the cellular abundance in the biofilm assuming 10 mg of the biofilm cells were evenly distributed on the filter (M. Delwiche, personal communication).

Geochemistry. Pore water analysis was performed on whole-round core samples collected during EXP 311 (NCM) and NGHP01 (Indian Ocean). The surface of each whole-round core sample was carefully scraped with a clean spatula to remove potential contamination from seawater and sediment smearing in the borehole. The cleaned sediment was placed into a titanium squeezer, modified after the stainless-steel squeezer of Manheim *et al.* (Manheim & Sayles, 1974). Gauge pressures up to 15 MPa were

applied using a laboratory hydraulic press to extract pore water. Interstitial water was passed through a prewashed Whatman No.1 filter fitted above a titanium screen, filtered through a 0.2 μm Gelman polysulfone disposable filter, and subsequently extruded into a precleaned (10% HCl) 50 mL plastic syringe attached to the bottom of the squeezer assembly (Riedel*, et al.*, 2006). During PGC0807, pore water for sulfate analysis was extracted by pressure-squeezing (~3 bar) and filter-sterilized with 0.2 μm acrodisc polyethersulfone (PES) syringe filters (Pall Corporation) (Pohlman*, et al.*, 2008).

Sulfate (SO_4^{2-}) concentration was measured with a Dionex DX-120 (EXP 311) or a Metrohm 861 (NGHP01 and PGC0807) advanced ion chromatograph (Riedel*, et al.*, 2006; Pohlman*, et al.*, 2008). Removal of H_2S was done by nitrogen bubbling of the sample (EXP 311) or by adding $Cd(NO_3)_2$ aliquots (NGHP01 and PGC0807) immediately after interstitial water collection.

Samples for pore water methane concentration were collected as 3 cm^3 sediment plugs from split cores and stored at -20º C in 20 ml serum vials sealed with 1 cm thick butyl rubber septa. Methane concentrations were determined by the headspace equilibration technique and gas chromatography-flame ionization detection using a Hewlett Packard 6890 Plus (GC3) during EXP 311, and NGHP01, and a Shimadzu GC-14A during PGC0807.

Samples for stable carbon isotope analysis of the biofilm were placed in silver cups, acidified with 10% HCl to dissolve all carbonaceous material, dried at 50º C for 24 hrs and then combusted with a Fisons EA-1100 Elemental Analyzer (EA) interfaced to a

Thermo Finnigan Delta Plus IRMS. The carbon isotope ratios are reported in the standard δ-notation relative to the Vienna PeeDee Belemnite (V-PDB) standard.

Nucleic acid extraction and purification. Total DNA was extracted from reference samples (10 g) using a PowerMax soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA). The Mo Bio protocol was modified initially by suspending the sediment in artificial seawater (6 g $MgSO_4$, 30 g NaCl, 2 g KCl L^{-1}) with occasional shaking at room temperature for 1 h prior to using the PowerMax Soil kit. The sediment/seawater mixture was centrifuged at 8000 x g for 10 min and then the sediment was transferred into the bead beating tubes of the PowerMax Soil kit along with two 0.25" steel balls (MP Biomedicals, Solon, OH) and subsequently the extraction procedure followed PowerMax Soil kit instructions. DNA was extracted from biofilm samples (~1 g) using a PowerSoil DNA extraction kit (Mo Bio Laboratories) according to the manufacturer's recommendations. The eluted DNA from all Mo Bio kits was concentrated to 50 μl using a Montage PCR spin column (Millipore, Billerica, MA). The amount of DNA in each sample was measured using a Qubit (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's recommendations.

Terminal restriction fragment length polymorphism analysis. To determine microbial diversity between samples, terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial and archaeal rRNA genes was performed on samples from both India and NCM. Each DNA suspension was amplified using the general bacterial primers 27F-FAM and 926R (Edlund*, et al.*, 2008) or general archaeal primers 21F-FAM and 958R (DeLong, 1992) in a MasterCycler thermocycler (Eppendorf, Westbury, NY). Each 20 μl

PCR mixture contained 1.25 units of AmpliTaq Gold LD (ABI, Foster City, CA), 1x PCR buffer, 4 mM $MgCl_2$, 800 μM of each dNTP, 0.5 μM of each primer, and 8 μg of BSA. PCR conditions consisted of an initial denaturation step of 5 min at 95º C followed by 35 cycles of 40 s at 95º C, 40 s at 50º C, 60 s at 72º C, and a final elongation step of 5 min at 72º C (Roussel*, et al.*, 2009). Thirty-five cycles were required to amplify the low levels of DNA in the reference samples. This is consistent with other studies having low biomass (Roussel*, et al.*, 2009). Products were combined from three PCR runs per DNA sample and purified with Montage PCR spin columns (Millipore, Billerica, MA).

One hundred ng of amplified and combined DNA products were digested using the restriction enzyme *HaeIII* (Newberry*, et al.*, 2004). Digestions were run according to the manufacturer's specifications (Fermentas, Glen Burnie, MD) by incubating the restriction digest for 3 hrs at 37º C followed by heat inactivation at 80º C for 20 min. The size of the restricted samples was determined by capillary gel electrophoresis using an ABI Prism 3100 Genetic Analyzer at the Oregon State University Center for Genome Research and Biotechnology (CGRB).

Clone library construction and phylogenetic analysis. Clones were created from both biofilm samples from India (site 18A and 20A) and the biofilm sample from PGC0807-C19. Bacterial clone libraries were constructed using non-labeled bacterial specific forward 27F and universal reverse 1492R primers that target 16S rRNA gene (Harrison*, et al.*, 2009). The archaeal 16S rRNA gene was amplified using archaeon-specific forward 21F and universal reverse 1492R primers (Takai*, et al.*, 2001). PCR amplification of the 16S rRNA gene used the same protocol as for T-RFLP analysis.

PCR products of the correct size were cloned using pCR® 2.1 TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones from each library were sequenced at the CGRB or at the Genome Center at Washington University (St. Louis, USA).

Sequence analysis was performed using Geneious (Drummond*, et al.*, 2009) and Mothur freeware (Schloss*, et al.*, 2009). Sequences with >95% similarity were assigned to the same phylotype (Roussel*, et al.*, 2009). Representative phylotypes were then sequenced in the reverse direction. The sequences were aligned in Mothur then imported into FastTree to create a phylogenetic tree (Price*, et al.*, 2009). The tree was visualized on the Interactive Tree of Life (ITOL) website (http://itol.embl.de/). In addition, phylotypes were digested in silico with *HaeIII* and the end fragment sizes were compared to terminal restriction fragments (TRFs) obtained from T-RFLP analysis.

PhyloChip. PhyloChip version G3 sample preparation and data analysis were performed on biofilm and reference samples from Site 18A (India) and PGC0807-C19 (NCM) as previously described (Hazen*, et al.*, 2010), except that 100 ng of internal spike DNA, 200 ng of bacterial 16S rRNA gene amplicons, and 50 ng of archaeal 16S rRNA gene amplicons were hybridized to the array.

Nucleotide sequence accession numbers. The nucleotide sequences of the rRNA gene clones were deposited in the GenBank database under the following accession numbers: HQ700668-HQ700686, HQ711382-HQ711400.

Results

Geochemistry. Sulfate and methane profiles for each of the study sites are illustrated in Figure 2.2. The SMT depth is defined as the depth where sulfate and methane concentrations converge at minimum values. The depth of the SMT in the Indian Ocean sediments was 18 to 20 mbsf, and at the NCM it ranged from 1.4 to 5 mbsf. The depth of the biofilm occurrence typically corresponded to the SMT depth (Figure 2.2). However, PGC0807-C6 also had three biofilm occurrences above the SMT. The $\delta^{13}C$ of the biofilm samples ranged from -35 to -43‰.

Nucleic acid extractions and cell counts. DNA was extracted from the biofilm and reference samples collected from the NCM and offshore India. Concentrations of DNA extracted from the reference samples ranged between 0.2 to 2.5 ng g^{-1} of sediment, whereas total DNA extracted from the biofilms ranged between 27 and 119 ng g^{-1} (wet weight). Based on the DNA yield from the biofilm and reference samples, and assuming an average DNA content of 2 fg $cell^{-1}$ (Bakken & Olsen, 1989; Takai*, et al.*, 2001), between 1.3 to 5.9 x10^7 cells g^{-1} and 1 to 1.3 x10^5 cells g^{-1} were present in biofilm and reference samples, respectively. The cellular abundance counted from the epifluorescent image of a biofilm taken from the Hydrate Ridge sample was 2.9 x 10^8 cell g^{-1}.

Molecular phylogenetic analysis. Microbial community fingerprints recovered from the DNA assemblages of the biofilm and reference samples were examined by PCR-mediated T-RFLP analysis. Identification of taxa is more difficult with T-RFLP than with other methods; however, a benefit of this approach is that it allows comparison of the

prominent DNA sequences obtained from all of the samples. A total of 133 archaeal terminal restriction fragments (TRFs) and 148 bacterial TRFs were detected. Fifty-two archaeal and 56 bacterial TRFs were shared between the biofilm and reference samples. 16S rRNA gene sequences obtained from clone libraries were digested in silico and then compared to TRFs. This enabled the identification of peaks in the T-RFLP electropherogram that corresponded to ANME-1, *Thermoplasmatales*, and *Crenarchaeales* TRFs (Figure A.1). We were unable to identify any of the other peaks in the electropherograms using this method and must rely on the clone library and PhyloChip results for additional data.

A total of 228 bacterial clones and 161 archaeal clones were sequenced from the biofilm samples. A similarity analysis with 95% similarity cutoff indicated 33 distinctive bacterial phylotypes and 19 distinctive archaeal phylotypes. Full 16S rRNA gene sequences were obtained for each of the representative phylotypes and used in phylogenetic analysis.

The major bacterial groups recovered from the biofilm were the *Deltaproteobacteria*, and *Chloroflexi*, representing 71% and 10% of the clones, respectively. In addition, candidate division JS1, candidate division WS3, *Gammaproteobacteria*, *Deferribacteres*, and OP11 were represented in smaller quantities (21% of the clones) (Figure A.2). The biofilms from NCM were mainly composed of *Proteobacteria*, while the biofilms from the Indian Ocean were composed of all previously mentioned phyla. Within the *Deltaproteobacteria,* sequences were related to putative sulfate reducing bacteria (SRB). The sequence "slm_bac_110" was the most

frequently obtained SRB phylotype (38% of total clones), and this sequence is closely related to the HydGH-Bac13 sequence (96.6% similarity) detected in a cold seep from Hydrate Ridge (accession number: AM229187). Sequences that clustered within the *Chloroflexi* phylum did not have a dominant phylotype with one clone detected for each phylotype, all clustering with the *Dehalococcoides* class. One phylotype ("slm_bac_40") was detected in the *Deferribacteres* phylum and had a 97.6% similarity to a phylotype that was detected in the Kazan mud volcano (Pachiadaki*, et al.*, 2010).

The archaeal diversity within the biofilm contained both *Euryarchaeota* and *Crenarchaeota* for both NCM and India. *Euryarchaeota* sequences were related to ANME-1, *Thermoplasmatales*, and *Methanosarcinales* representing 73%, 11%, and 2% of total archaeal clones, respectively. The *Crenarchaeota* sequences were related to *Desulfurococcales* and *Crenarchaeales*, representing 10% and 3% of the total archaeal clones, respectively (Figure A.3). Four phylotypes of ANME-1 were detected; however, two sequences ("slm_arc_201" and "slm_arc_400") were the most abundant making up 94% of the ANME phylotypes. The sequence "slm_arc_201" was predominantly found in the NCM (29 clones from NCM, 1 clone from India), while the sequence "slm_arc_400" was exclusively found in Indian samples (42 clones). The sequence "slm_arc_201" was closely related to the sequence Arch125 (99%) detected in Aarhus Bay (Aquilina*, et al.*, 2010). The sequence "slm_arc_400" was related to the sequence GBa1r013 (95.6%) detected in the Guaymas Basin (Teske*, et al.*, 2002). "Slm_arc_400" and "slm_arc_201" are 94% similar to each other. No other ANME group was detected.

PhyloChip. A total of 132, 491, 178, and 193 phylotypes were detected on the PhyloChip from the NCM biofilm, NCM reference, Indian Ocean biofilm, and Indian Ocean reference samples, respectively (Figure 2.3). All seven bacterial phyla that were detected in the clone library were also detected in the PhyloChip. In addition, 44 phyla were detected on the PhyloChip but not in the clone library. Of the additional 44 phyla detected, nine were unclassified phyla. Only five archaeal phyla were detected, one of which was an unclassified phylum. The PhyloChip detected *Archaeoglobi* that was not detected in the clone library. Although, the PhyloChip detected ANME-1 cells in the biofilm and reference samples from India it failed to detect *Methanosarcinales* and *Desulfurococcales*. The general observation here that the PhyloChip detected more taxa than a library composed of a few hundred clones is consistent with prior research findings where the two methods were compared in an analysis of microbial populations in urban aerosols, subsurface soil, and subsurface water samples that contained 417, 485, and 253 clones, respectively (DeSantis*, et al.*, 2007).

Discussion

Unique macroscopic subseafloor biofilms found in fractures in NCM and Indian Ocean sediments intersecting the SMT were characterized by culture-independent techniques. Most biofilms reported in our study were located at the SMT, a geochemical indicator of AOM (Reeburgh, 1976; Boetius*, et al.*, 2000). The presence of the ANME-1/SRB consortia within the biofilm and the SMT suggest that this biofilm is affiliated

with AOM. At site PGC0807-C6, biofilm was found above the SMT (Figure 2.2). However, the DNA concentration of that biofilm (0.5 ng g^{-1}) was similar to the surrounding sediment that lacked biofilm (0.2 to 2.5 ng g^{-1} of sediment). While we do not know the biological characteristics of these non-SMT biofilms, as molecular analyses were not performed, it is plausible that these are remnant biofilms from a time when the methane flux was higher and the SMT was located nearer the seafloor. This explanation is consistent with the highly dynamic nature of methane seepage at this site.

More ^{13}C-depleted values for the biofilm (-35 to -43‰) than the surrounding sediment (-25.7‰) (Kaneko*, et al.*, 2010) provides further evidence that the biofilm was synthesized within an active SMT. Assimilation of ^{13}C-depleted methane carbon or autotrophic fixation of CO_2 by the AOM consortium results in ^{13}C-depleted microbial biomass (Wegener*, et al.*, 2008). However, previous isotopic values for lipids from the AOM consortia are typically more ^{13}C-depleted (-60‰) (Alperin & Hoehler, 2009) and more consistent with the isotopic value of the carbon in the methane itself. Metabolism of the organic carbon coupled to sulfate reduction could produce the observed isotopic values (Alperin & Hoehler, 2009). It is also possible that during sampling of the biofilms significant amounts of surrounding sediment was mixed with the biofilm thereby generating a "mixed" sediment-biofilm isotopic ratio.

Three molecular techniques were used to determine the composition of the microbial communities: T-RFLP, clone libraries, and PhyloChip. Each technique depends upon the efficiency of DNA extraction from cells and then amplification of the resulting DNA. T-RFLP is a rapid and relatively inexpensive technique useful in

identifying trends of dominant taxa but is not sensitive to species level differences and multiple taxa can be represented by individual TRFs (Dunbar*, et al.*, 2001). Both clone libraries and the PhyloChip are more labor intensive but provide much higher resolution of closely related taxa. The PhyloChip version G3 is a parallel hybridization microarray technology with probes for 59,000 microbial taxa (Hazen*, et al.*, 2010). For each taxon there are additional probes that have mismatches yielding a total of 1,100,000 probes. Thus, known taxa and closely related taxa can be detected simultaneously and the array is more sensitive to rare microbes than typical sequencing approaches (DeSantis*, et al.*, 2007). Our results were consistent with this observation. As a probe-based approach the PhyloChip has limitations in detecting novel organisms. Archaeal 16S rRNA gene databases are notoriously underrepresented compared to bacterial 16S rRNA gene databases. Therefore, clone libraries can sometimes detect novel archaeal taxa that would be missed by any array technology.

T-RFLP was performed on all of the samples allowing for a direct comparison. An in silico approach of comparing TRFs to the clone library was only able to identify three of the Phyla that were detected in the clone libraries and on the PhyloChip (Figure 2.3). Takishita *et al*. (Takishita*, et al.*, 2009) used the same in silico approach and were able to identify TRFs that correspond to the ANME-2 group from a cold seep environment. Our investigation was able to detect TRFs that correspond to ANME-1 in all biofilm samples and this finding was consistent with both of the other molecular ecology methods used.

The presence of ANME-1 is significant because microbial biogeography studies have shown a variety of environmental factors that influence the distribution of the AOM consortia. From studies conducted to date, the main controlling factors appear to be the availability of methane and sulfate (Nauhaus, *et al.*, 2005). For instance, bioreactor studies have shown that ANME-1 are enriched in high methane, low sulfate environments while ANME-2 are present in high sulfate, low methane environments (Girguis, *et al.*, 2005). Field studies of sediments overlying a methane-rich brine pool in the Gulf of Mexico and microbial mats of the Black Sea offer additional evidence for the localization of ANME groups according to sulfate and methane concentrations (Lloyd, *et al.*, 2006). In the Black Sea mats the outer (lower methane) black portion is primarily composed of ANME-2, while the inner (higher methane) pink portion is composed of ANME-1 (Reitner, *et al.*, 2005). The biofilms reported herein are also pink to orange and contain ANME-1 suggesting some commonalities between the fracture-dominant environments and the conditions that foster growth of the Black Sea mats. The dominant sequences that were recovered from both NCM and Indian Ocean biofilms were similar to sequences found in advective cold seeps where there is typically ample supply of methane (Pachiadaki, *et al.*, 2010).

Our results show that 10^7 to 10^8 cells cm^{-3} can occur within these fracture-dominant environments. In diffusion-dominant environments the AOM consortia densities are typically low, with 10^6 cells cm^{-3} (Niemann, *et al.*, 2005). In contrast, seep-dominant environments such as Eel River Basin (Hinrichs, *et al.*, 1999; Orphan, *et al.*, 2001), Hydrate Ridge (Boetius, *et al.*, 2000; Knittel, *et al.*, 2005), the Black Sea

(Michaelis, *et al.*, 2002; Reitner, *et al.*, 2005), and Gulf of Mexico (Lloyd, *et al.*, 2006) contain $>10^{10}$ AOM consortia cells cm^{-3}. For example, in the Black Sea the AOM consortium produces macroscopic biomass seen as columnar structures in the methane laden anoxic bottom waters that extend as much as 4 m into the water (Michaelis, *et al.*, 2002; Knittel, *et al.*, 2005). Thus, despite energetic constraints, the AOM consortia have been shown to produce macroscopic quantities of biomass in seep-dominant environments. The question then is how can AOM produce macroscopic quantities of biofilm in fracture-dominant environments where the physical and energetic constraints are presumably more austere than in seep-dominant environments?

Field observations of microbes in the subsurface indicate that the sediment grain size can be a controlling factor for cell densities. Inagaki *et al*. (Inagaki, *et al.*, 2003) observed that ash layers from the Sea of Okhotsk contained significantly higher biomass concentrations than intervening clay layers. Likewise, Phelps *et al.* (Phelps, *et al.*, 1994) also observed lower cell density and activity in clay-rich sediment than sandy sediment. The ash and sand layers that harbor greater cell densities have larger grain sizes than the clay layers. The mechanism that allows for higher cell densities is unknown; although, Inagaki *et al*. (Inagaki, *et al.*, 2003) hypothesized that habitable space and/or fluid flow and delivery of electron acceptors and donors could explain the higher levels of cells observed in locations with higher pore space. The fractured systems that we have studied may function in a manner similar to geological media with large pore space available for microbial colonization. Still, other explanations for variable cell densities in subsurface

media include water activity or the mineralogy of the sediment (van Loosdrecht*, et al.*, 1990; Takai*, et al.*, 2001).

The AOM consortia are restricted to environments with both sulfate and methane. Seep-dominant SMT environments have higher sulfate and methane concentrations that occur coincident with higher abundance of cells involved with AOM. We have not measured methane or sulfate flux in the fractures where we collected the biofilms; however, fractures in the sediment may be preferred flow paths for fluids (Weinberger & Brown, 2006). If advective flux of methane and sulfate occurs in these environments, most would probably travel through the fractures making them the most likely location for higher biomass.

Based on the preceding discussion and observations of biofilms in fractures, we postulate that fractures are pathways for advective methane flux. Support for gas migration through the NCM fractures comes from geophysical measurements obtained during EXP 311 at Bullseye Vent, the location of some of the biofilms that we collected. Electrical resistivity tools imaged several steeply dipping fractures with elevated resistivity values caused by high gas hydrate saturation in the fractures (Riedel*, et al.*, 2006). That these fractures are preferred flow paths for fluid advection is also supported by observations of methane bubbles at the seafloor and acoustic images of bubble plumes rising from the seafloor near our NCM biofilm sampling sites (Zyla*, et al.*, 2010). The model presented by Riedel *et al*. (Riedel*, et al.*, 2006) describes Bullseye Vent as a complex subsurface network of fractures partially filled with gas hydrate and feeding methane upwards towards the seafloor. Such fractures may increase habitable space that

is otherwise lacking in sediments and provide a supply of methane that creates a favorable condition for higher biomass accumulation. The biofilms from the Indian Ocean were also collected from what appeared to be fractures; however, there is no geophysical evidence for fracturing.

AOM consortia occur in environmental settings that range from diffusion to seep-dominant SMTs (Alperin & Hoehler, 2009). Fracture-dominant SMT settings contain macroscopic biofilms comprised of ANME-1 (Figure 2.4A). The distribution of ANME groups is, in part, controlled by methane and sulfate concentrations (Girguis*, et al.*, 2005). In the Black Sea mats, the outer (lower methane) is primarily composed of ANME-2, while the inner (higher methane) is composed of ANME-1 (Reitner*, et al.*, 2005) (Figure 2.4B). The Black Sea is a seep-dominant SMT and biomass can exceed 10^{10} cells cm^{-3}. In contrast, diffusion-dominant SMTs typically contain microcolonies of ANME-2 and their sulfate reducing symbiont (Figure 2.4C).

In summary, it appears that fracture-dominant SMT constitutes a previously unreported setting where the AOM consortia resides and produces macroscopic biofilms. Their presence may be associated with characteristics typical of fractures in the seafloor and as such may represent an alternative condition where AOM may occur (i.e., not diffusive or seep-dominant). If indeed these fractures are conduits for methane flow we would also expect to see higher AOM rates in a subsurface fractured SMT compared to a subsurface diffusive-dominant SMT. Additional studies will be needed to verify this hypothesis.

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Table 2.1. Summary of biofilms examined in this study including the analyses that were performed on each sample. Isotopic: Isotopic composition of carbon; PC: PhyloChip; CL: clone library; tR: terminal restriction fragment length polymorphism (T-RFLP); Microscopic: epifluorescent image obtained during Ocean Drilling Program Expedition Leg 201 by Mark Delwiche and used for cell counts.

Location	Site	Analysis	Latitude	Longitude	Biofilm Depth (mbsf)[a]	SMT (mbsf)[a]
northern Cascadia Margin	1325B	Isotopic	48°39.150'N	126°59.650'W	1.35	1.4
	1327D	Isotopic	48°42.484'N	126°51.367'W	7.2	7.6
	Stn06	CL, tR	48°40.194'N	126°50.945'W	3.84	5.02
	Stn18	tR	48°39.690'N	126°55.244'W	2.87	2.85
	Stn19	PC, CL, tR	48°39.640'N	126°55.105'W	2.15	2.29
Hydrate Ridge	1251A	Microscopic	44°34.219'N	125°4.452'W	0.47	0.5
Indian Ocean	20A	CL, tR	15°48.567'N	81°50.576'E	18	16.5
	18A	PC, CL, tR	19°09.145'N	85°46.375'E	19.8	19.8

[a]meters below seafloor

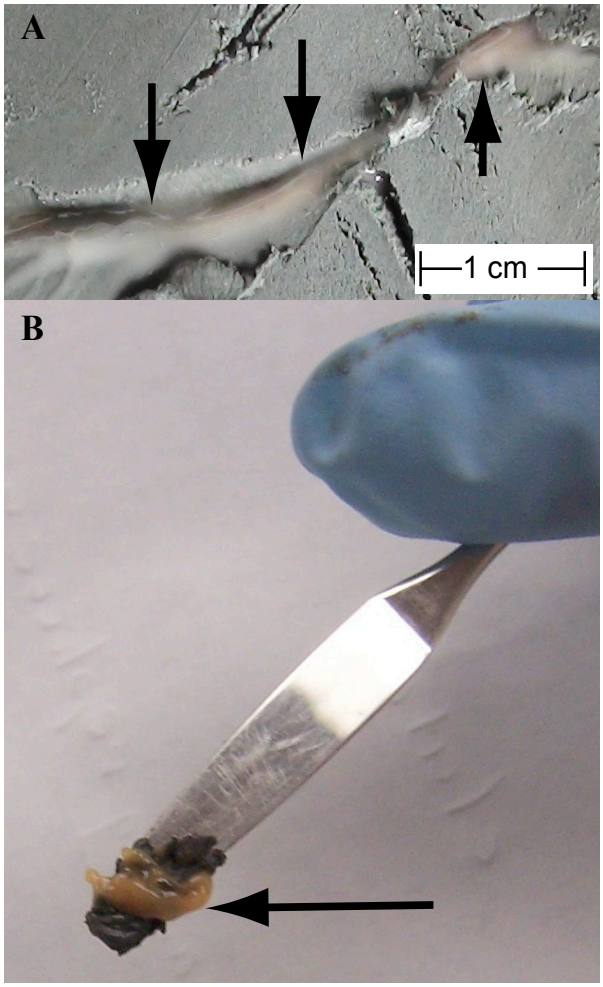

Figure 2.1. Photograph of a biofilm collected from a fracture in the Indian Ocean at 19 meters below seafloor (see table 2.1). Arrows point to the orange biomass. A) Biofilm within the fracture before sampling. B) Biofilm that has been scraped out of the fracture using a spatula. The spatula blade is approximately 2 cm long.

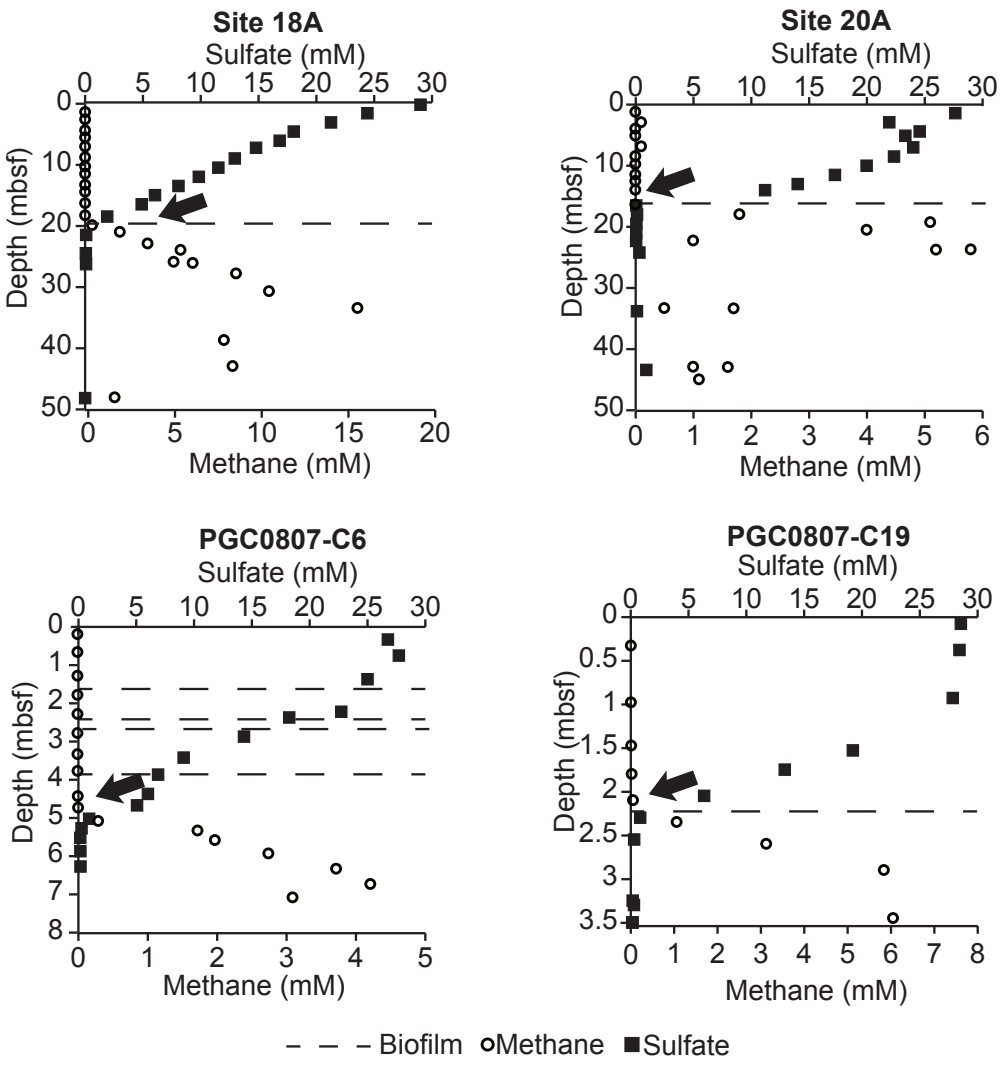

Figure 2.2. Sulfate and methane profiles depicting the SMT depth (noted by arrows). Dashed lines show the depths where biofilm was recovered from fractures in the respective cores. The depth is measured in meters below seafloor (mbsf) and the scale varies for the different sites shown.

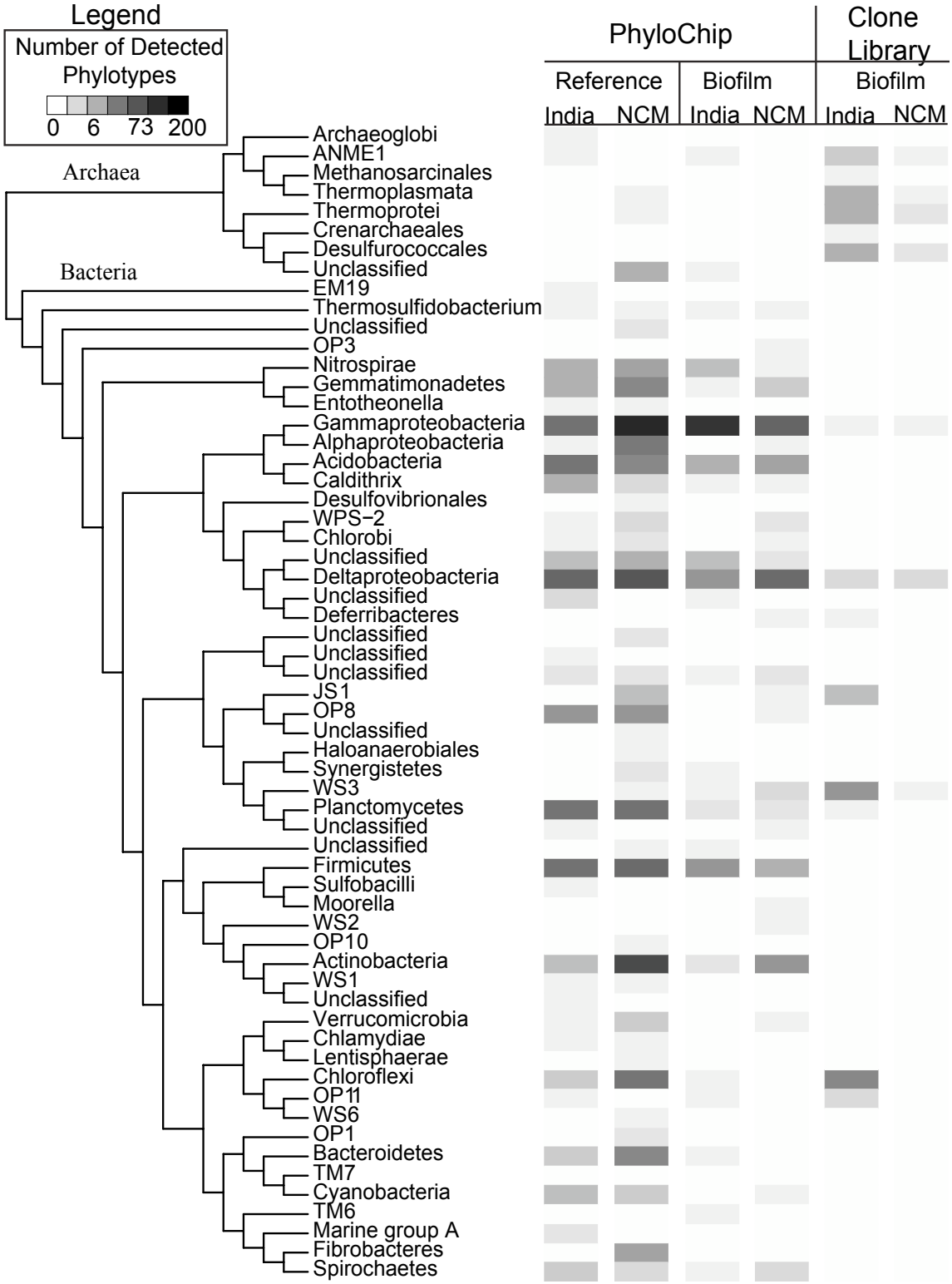
Legend
Number of Detected Phylotypes
0 6 73 200
Archaea
Bacteria
PhyloChip
Clone Library
Reference
Biofilm
Biofilm
India NCM India NCM India NCM
Archaeoglobi
ANME1
Methanosarcinales
Thermoplasmata
Thermoprotei
Crenarchaeales
Desulfurococcales
Unclassified
EM19
Thermosulfidobacterium
Unclassified
OP3
Nitrospirae
Gemmatimonadetes
Entotheonella
Gammaproteobacteria
Alphaproteobacteria
Acidobacteria
Caldithrix
Desulfovibrionales
WPS−2
Chlorobi
Unclassified
Deltaproteobacteria
Unclassified
Deferribacteres
Unclassified
Unclassified
Unclassified
JS1
OP8
Unclassified
Haloanaerobiales
Synergistetes
WS3
Planctomycetes
Unclassified
Unclassified
Firmicutes
Sulfobacilli
Moorella
WS2
OP10
Actinobacteria
WS1
Unclassified
Verrucomicrobia
Chlamydiae
Lentisphaerae
Chloroflexi
OP11
WS6
OP1
Bacteroidetes
TM7
Cyanobacteria
TM6
Marine group A
Fibrobacteres
Spirochaetes

Figure 2.3. Phylogenetic tree of Archaeal and Bacterial phyla detected using PhyloChip and clone library analyses of reference and biofilm samples acquired from different locations examined in this study. The number of phylotypes that were detected in each classification using the respective analytical method are shown using a heat map.

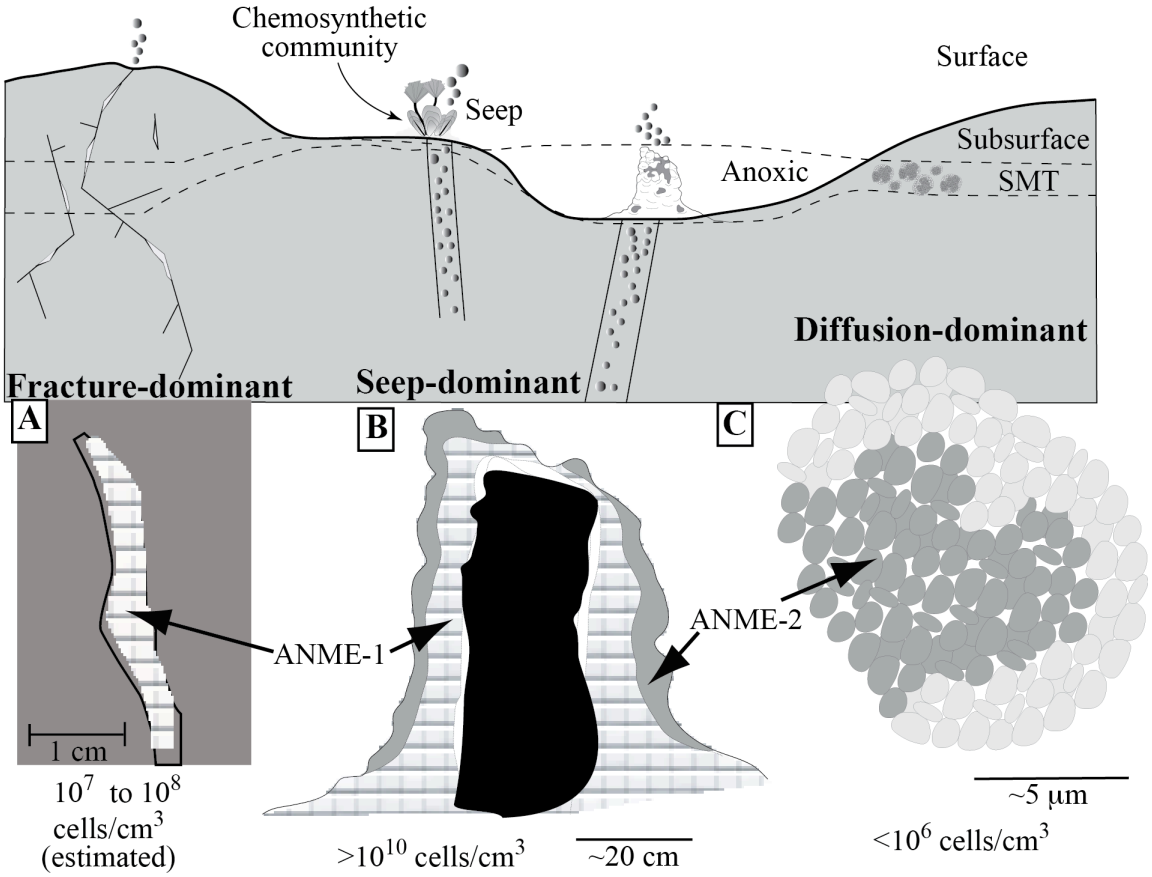

Figure 2.4. Conceptual model of biomass and ANME groups found in fracture, seep, and diffusion-dominant sulfate methane environments. A) Representation of a biofilm in a fracture. B) Representation of the Black Sea microbial mats. D) Representation of the microcolonies found in diffusion-dominant environments.

3. BACTERIAL DOMINANCE IN SUBSEAFLOOR SEDIMENTS CHARACTERIZED BY METHANE HYDRATES

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In Review

Abstract

The degradation of organic carbon in subseafloor sediments on continental margins contributes to the largest reservoir of methane. Sediments in the Andaman Sea are composed of ~1% marine-derived organic carbon and biogenic methane is present as the hydrate form. Our objective was to determine the microbial abundance and diversity in sediments that transition the gas hydrate occurrence zone (GHOZ) in the Andaman Sea. Microscopic cell enumeration revealed that most sediment layers harbored relatively low microbial abundance (10^3 to 10^5 cells cm^{-3}). Archaeal genes were never detected despite the use of multiple molecular methods. This is consistent with the lack of methanogenic archaeal biomarker lipids and the water column origin of detected archaeal lipids. Statistical analysis of terminal restriction fragment length polymorphisms found that the microbial communities from above, within, and below the GHOZ were distinct from each other and that GHOZ samples were correlated to an increase in inorganic carbon. Primer-tagged pyrosequences of bacterial 16S rRNA genes showed that members of *Firmicutes* are predominant in all zones. Compared to other seafloor settings that contain biogenic methane, this deep subseafloor habitat has a unique microbial community and the low cell abundance detected can help refine global subseafloor microbial biomass.

Introduction

Methane hydrate, a crystalline mineral composed of methane surrounded by water, is a defining geologic feature in subsurface sediments along many continental margins. Hydrates are estimated to hold a total of 500-2,500 gigatons of methane carbon (Milkov, 2004) and it is important to consider this methane in computational models of the global carbon cycle and in estimates of accessible energy resources.

Since the late 1980s, we have known that gas hydrate systems often harbor biologically produced methane (Kvenvolden, 1995; Whiticar, 1999), yet we are still trying to understand fundamental aspects about the microbial processes that cycle the carbon in gas hydrate-rich sediments. Microbes responsible for the generation of the methane that exists in gas hydrates need to be characterized in order to fully understand the ecological and biogeochemical role of the largest methane reservoir on the planet (c.f. Reeburgh, 2007). Furthermore, a more complete accounting of the types of microbes present and the diversity of Bacteria and Archaea that occur in and near sediments where gas hydrates exist is needed to develop conceptual models of the biological representatives of these globally significant hydrocarbon reservoirs.

In 2006, the National Gas Hydrates Program (NGHP) Expedition 01 drilled a 700 meter below seafloor (mbsf) hole in the Andaman Sea (Collett*, et al.*, 2008). The low geothermal gradient (21.0º C/ km) at this site allows methane hydrate to exist deep into the sediment column, making the methane hydrate deposits found at this location among the deepest discovered so far. In addition, the Andaman Sea sediments are unique because the total organic carbon (TOC) is less than 1% by weight (Johnson*, et al.*, 2009),

while other hydrate-containing sediments along continental margins contain 1-11% TOC (Meister*, et al.*, 2005). The combination of a thick gas hydrate zone extending deep into the sediments and the low to intermediate organic carbon concentrations in the Andaman Sea sediments offered the chance to examine the microbiology in a hydrate-bearing system considerably different than most that have already been explored through previous scientific ocean drilling.

Past research has considered the presence of microorganisms in gas hydrate-bearing sediments contributing to an understanding of the importance of microbes in these systems. Reed *et al.* (2002) reported the microbial diversity above, within and below the gas hydrate occurrence zone (GHOZ), but they only created a single clone library from a single sample from each layer (Reed*, et al.*, 2002). Inagaki *et al*. used statistical analysis to compare the microbial communities in areas characterized by the presence or absence of gas hydrate along the Pacific Ocean Margins and found that distinctive microbial communities may occur in sediments that contain methane hydrates as opposed to those that lack hydrates (Inagaki*, et al.*, 2006). Nunoura *et al*. used clone libraries to describe the microbial communities at several sites along the gas hydrate-bearing Cascadia margin and found a dominance of the bacterial candidate division JS1 and the Deep Sea Archaeal Group (DSAG) (Nunoura*, et al.*, 2008). In addition, methanogens have been detected by both culture-dependent and culture-independent methods in methane hydrate-containing sediment (Marchesi*, et al.*, 2001; Mikucki*, et al.*, 2003; Newberry*, et al.*, 2004; Colwell*, et al.*, 2008; Yoshioka*, et al.*, 2010). These studies indicate that a diverse assemblage of bacteria and archaea are present in methane

hydrate-containing areas. However, higher-resolution studies on the effect of the GHOZ on microbial distributions remain elusive. This information is needed to understand the biogeography of hydrate-containing sediments and identify microbes that are potentially involved with carbon cycling in the deep subseafloor biosphere.

Our objective was to compare the abundance and diversity of microbial communities from above, within, and below sediment that contained methane hydrate in the Andaman Sea, a setting made unique by the notable depth of gas hydrates and also by the limited quantities of organic carbon buried in the sediments. To accomplish the scientific goal, we enumerated SYBR Green I stained cells and used several molecular ecological techniques along with statistical community analyses of microbial distribution and diversity.

Materials and Methods

Site description and sampling. Sediment samples were obtained from the Andaman Sea at Site 17A (10º 45.1912' N, 93º 6.7365' E) during the NGHP Expedition 01. This site is located in a forearc basin, and contains mainly marine deposited nanofossil ooze that accumulated at a rate of 5.6 cm kyr^{-1} (Raju, 2005; Collett*, et al.*, 2008). Geologic horizons demarcate the GHOZ as evidenced by increasing porosity from 50% to 63% at 250 mbsf and a change in lithology from calcareous to siliceous ooze. The depth of the seismically observed bottom-simulating reflector (BSR), below which methane is believed to exist as a free gas, is estimated at approximately 600 mbsf.

Forty-three core samples were collected from various depths that span 20-700 mbsf, with the deepest sample corresponding to an age of 12.35 Ma (Collett*, et al.*, 2008). These samples were collected as whole round cores and stored onboard at -80º C, before being shipped frozen overnight in dry shippers to Oregon State University for molecular microbiological analyses. In the laboratory, the frozen cores were sub-sampled by paring the outer layer of sediment core with an alcohol and flame-sterilized chisel. The outer layer of the core was discarded and the inner core (subcore) was used for the subsequent experiments such as DNA and lipid extractions. Fluorescent micro-sphere beads were used during drilling to identify possible contamination of the cored sediment samples, and these were not detected in the subcore material, thus ensuring minimal contamination.

Geochemistry. Stable carbon isotope ratio determinations of C_1, C_2, and CO_2 were made on a continuous flow–isotope ratio mass spectrometer (Finnigan MAT 252, GC-CF-IRMS) at the School of Earth and Ocean Sciences (SOES), University of Victoria, Canada. Samples were introduced by syringe into a SRI gas chromatograph (GC) via a gas sample valve (loop volumes: 10, 100 or 200 μL). Analytes are separated at 40°C on a 30 m GS-Q column (0.32 mm ID) with a carrier gas flow of 1.8 mL/min ultra-high purity helium. After gas partitioning on the GC, the gas then passed through a CuO/Pt microcombustion oven at 850^{o}C to convert the hydrocarbon gases to carbon dioxide and water. The combusted sample products were passed through a Nafion™ tube to remove water from the combustion and any in the carrier gas. The purified CO_2/He pulse was scaled by an open-split interface, and then transferred into the GC-CF-IRMS. Isotope

ratios were referenced to the conventional PeeDee Belemnite (PDB) standard through a known CO_2 isotope standard that was added at the open split to the sample runs several times during the analysis. For stable carbon isotope ratio measurements on the sample CO_2, the gas was partitioned on the GC as above. The microcombustion oven was bypassed for the CO_2 measurements, but the gas stream was dried, split and measured by CF-IRMS in a manner similar to the light hydrocarbons.

Cell counts. To determine the abundance of cells with intact DNA in the sediment, 1 g of frozen subcore samples were put into 9 ml of 4% paraformaldehyde/ phosphate buffered saline (PBS) solution (10% w/v) and fixed overnight at 4°C. Subsequently, the subcore and solution slurry was washed twice with PBS and finally suspended in PBS/EtOH (50:50) solution. Fixed microbial cells in the sediment slurry was filtered using a 0.22 μm-pore black polycarbonate membrane (Millipore Billerica, MA), stained with SYBR Green I, and enumerated with an automatic epifluorescent microscope and image analysis system as previously described (Morono*, et al.*, 2009; Morono & Inagaki, 2010).

Nucleic acid extraction. Total DNA was extracted from 10 g of sediment using a PowerMax Soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) as described previously (Lipp*, et al.*, 2008; Briggs*, et al.*, 2011).

In addition, to verify our extraction method, two additional extractions were performed on 10 g of subcored material from 13 samples within the GHOZ as described previously (Lipp*, et al.*, 2008). The additional extractions included a freezer mill homogenization for 6 cycles at 2 min of homogenization and 1 min of rest prior to bead beating with the Mo Bio PowerMax Soil kit, referred to hereafter as MoBio FM DNA

extraction. The second extraction included the freezer mill homogenization before bead beating and a proteinase K digestion (10 mg for 2 hr at 50°C with gentle rocking) after bead beating, referred to hereafter as MoBio FM PK DNA extraction.

The derived DNA from all extractions was concentrated to 50 μl using a Montage PCR spin column (Millipore, Billerica, MA). The amount of DNA in each sample was measured using a Qubit fluorometer (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's recommendations.

Nucleic acid amplification. To estimate copy numbers of archaeal and bacterial 16S rRNA genes, quantitative PCR (QPCR) was conducted with Power SYBR green PCR Master Mix on an Applied Biosystems 7300 real-time PCR system according to the manufacturer's instructions. The 16S rRNA gene primers used were 27F and 338R for bacterial or 806F and 958R for archaeal as reported elsewhere (Lipp*, et al.*, 2008).

Archaeal 16S rRNA genes were below QPCR detection limits; therefore, PCR was performed with the additional primers 21F (DeLong, 1992) and 1492R (Hazen*, et al.*, 2010) in attempt to detect archaeal 16S rRNA genes. All amplifications were performed in a GeneAmp(R) PCR System 9700 (Applied Biosystems, Foster City, CA). Each 20 μl PCR mixture contained 1.25 units of ExTaq (Takara, Otsu, Japan), 1× PCR buffer, 800 μM of each dNTP, and 0.5 μM of each primer. PCR conditions consisted of an initial denaturation step of 5 min at 95° C followed by 30 cycles of 40 s at 95° C, 40 s at 50°C, 60 s at 72° C, and a final elongation step of 5 min at 72° C. Products were combined from three PCR runs per DNA sample and purified with Montage PCR spin columns (Millipore, Billerica, MA).

Archaeal 16S rRNA genes were still not detected; therefore, additional steps were taken to assess the presence of these genes. A nested PCR was attempted, where the first PCR consisted of the primers 21F and 1492R with the same conditions as above. The PCR product was diluted 1:10 and a subsequent PCR reaction was performed with the primers 21F and 958R (Roussel*, et al.*, 2009). In addition, purified DNA and negative controls were amplified by multiple displacement amplification (MDA) using phi29 polymerase of the Illustra Genomiphi Phi V2 Kit (GE Healthcare Bioscience, Tokyo, Japan), with the addition of SYBR Green I to monitor amplification by real-time PCR as previously described (Lipp*, et al.*, 2008). All MDA-amplifications from negative controls were negative. The above PCR and nested PCR for archaeal 16S rRNA genes was then performed again using the MDA product.

Terminal restriction fragment length polymorphism analysis. To determine microbial diversity between samples, terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA genes was performed on 43 core samples as previously described (Briggs*, et al.*, 2011), with the exception that the restriction enzyme *Msp*I was used to digest amplified DNA products (Opatkiewicz*, et al.*, 2009). The size of the restricted samples was determined by capillary gel electrophoresis using an ABI Prism 3100 Genetic Analyzer at the Oregon State University Center for Genome Research and Biotechnology (CGRB). Analysis of the terminal restriction fragments (TRFs) was performed as previously described (Colwell*, et al.*, 2011).

Statistical analysis. Multivariate statistical analysis was performed using PC-ORD version 5.0 (mJm Software) on T-RFLP data to determine whether measured

environmental parameters affected the microbial distributions as previously reported elsewhere (McCune & Mefford, 2006; Colwell*, et al.*, 2011). Briefly, nonmetric multidimensional scaling (NMS) ordination techniques (Kruskal, 1964) with Sørensen distance measures (Bray & Curtis, 1957) were used to identify differences in community composition for each sample and multi-response permutation procedures (MRPP) (Mielke & Berry, 2001) using the Sørensen distance were used to test the strength and statistical significance of group membership between above, within, and below the GHOZ. Environmental parameters were overlaid on this ordination as a bi-plot. The environmental parameters used were carbon content (total carbon, organic carbon, inorganic carbon, and carbon to nitrogen ratio) (Johnson*, et al.*, 2009), chloride concentration, grain density, porosity, and resistivity (Collett*, et al.*, 2008).

Pyrosequencing. Primer-tagged pyrosequences of bacterial 16S rRNA genes were created at the Marine Biological Laboratory (Woods Hole, MA) using the Roche Titanium platform as previously described (German*, et al.*, 2010). Sequences were passed through quality filters and assigned taxonomy using the Global Alignment for Sequence Taxonomy (GAST) process (Huse*, et al.*, 2008), which incorporates the RDP II taxonomy as previously done (German*, et al.*, 2010). Sequences have been deposited in the VAMPS database (https://vamps.mbl.edu).

Lipid Analysis. Lipids were extracted from 2-4 g of homogenized freeze-dried sediment from 6 samples above the GHOZ, 6 samples within the GHOZ, and 3 samples below the GHOZ by microwave using a mixture of dichloromethane (DCM)/methanol (MeOH) (3/1, v/v) (Kornilova & Rosell-Mele, 2003). The total lipid extract was partitioned into

apolar (hexane/DCM (9:1; v/v)) and polar (DCM/MeOH (1:1; v/v)) fractions over activated silica. An internal standard was added to the polar fraction after extraction to quantify the glycerol dialkyl glycerol tetraethers (GDGTs) (Huguet*, et al.*, 2006; Rethore*, et al.*, 2007).

The dry polar fractions were redissolved in hexane/n-propanol (99/1, v/v) and filtered through 0.50 μm PTFE filters (Advantec). A Dionex P680 HPLC system coupled to a Thermo Finnigan TSQ Quantum Discovery Max quadrupole mass spectrometer with an atmospheric-pressure chemical ionization (APCI) interface was used to analyze the lipids (c.f Escala*, et al.*, 2007).

Additional measurements were done by gas chromatography–mass spectrometry (GC-MS) analysis to search for archaeol and hydroxyarcheaol as methanogen biomarkers (c.f. Hinrichs*, et al.*, 1999) using an Agilent 7890A chromatograph coupled to a series 5975 mass spectrometer. After injection of 1 μL of sample, in split/splitless mode, compounds were separated through an Agilent DB-5MS capillary column (30 m × 0.25 mm internal diameter × 25 μm film thickness) with a 5 m precolumn. Oven temperature was held at 80 ºC for 1 min, increased to 120 ºC at a rate of 20 ºC min^{-1}, then to 320 ºC at 6 ºC min^{-1} and held at this value for 30 min. Helium was used as carrier gas at a constant flow of 1.5 mL min^{-1}. The mass spectrometer parameters were set as follows to generate positive ion spectra: injector 310 ºC, source 250 ºC, Ms Quad 150 ºC and ionization energy 70 eV, scan range from 50-650 m/z. The glycerol compounds were derivatized online with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Results

Geochemistry. The methane carbon isotopic composition was the most ^{13}C depleted near the seafloor with a $\delta^{13}C$ value of -80 ‰. The value gradually became more ^{13}C enriched with depth until about 300 mbsf after which the $\delta^{13}C$ value remained about -67 ‰. Propane, considered to be of thermogenic origin, occurs below 550 mbsf (Collett*, et al.*, 2008). These values indicate the methane is from a biogenic source, while below 550 mbsf the presence of propane indicates some input from a thermogenic source (Whiticar, 1999).

Cell counts. Counts of Sybr Green I stained cells revealed that most sediment layers harbored relatively low cell concentrations (10^3 cells cm^{-3} to 10^5 cells cm^{-3}), as compared to those previously reported from the organic-rich subseafloor sediments (Figure 3.1). Seven samples contained cell abundance below the detection limit of 10^2 cells cm^{-3}.

Nucleic acid extractions and amplification. The MoBio DNA extraction produced the highest quantity of DNA as measured on the Qubit (0.5-6.8 ng g^{-1} of sediment). DNA yields for both the MoBio FM and MoBio FM PK DNA extractions were consistently lower at 0.3-0.9 ng $gram^{-1}$ of sediment (Figure B.1). Furthermore, both bacterial and archaeal 16S rRNA genes were not detected in the MoBio FM and MoBio FM PK DNA extractions. Bacterial 16S rRNA genes were detected from the MoBio DNA extraction and DNA from this extraction was used for all future analyses.

Bacterial 16S rRNA genes were detected in all samples. In marked contrast, archaeal 16S rRNA genes were never detected despite the use of multiple primers (Table

B.1), QPCR, MDA, and nested PCR all of which were used in an effort to detect these microbes.

Diversity of subseafloor microbial communities. The amplification of DNA from 31 samples produced enough DNA (100 ng) for T-RFLP analysis. A total of 111 TRFs were identified in the 31 samples with 58 TRFs above the GHOZ, 62 within the GHOZ, and 57 below the GHOZ. Only 17 TRFs were shared between all three zones. Twenty-eight, 27, and 28 phylotypes were shared above and within, within and below, and above and below the GHOZ, respectively. The in-silico digestion of cloned sequences compared to TRFs identified the peak at 154 bp as most likely being related to the genus *Bacillus*. In all samples within the GHOZ, the TRF at 154 bp was the most prominent peak. This TRF was present, although peak height was much smaller, in samples above the GHOZ. It was not detected below the GHOZ (Figure B.2).

A total of 72,523 primer-tagged pyrosequencing reads were classified using GAST after the poor quality sequences were discarded. Firmicutes were the dominant phylum with 52%, 53%, and 74% of reads from above, within, and below the GHOZ (Table 3.1). Other significant phyla detected were *Actinobacteria, Bacteroidetes*, *Proteobacteria*, and other bacterial phyla that could not be assigned to a phylum. The *Gamma-* and *Beta-proteobacteria* seemed predominant in the GHOZ consisting of ~21% and ~17% of the reads, respectively (Table 3.1). While, *Bacteroidetes* decreased in frequency in the GHOZ to ~2%. *Actinobacteria* and *Alpha-proteobacteria* had the highest frequency above the GHOZ (Table 3.1).

Analyses were conducted for both GDGTs and glycerol dialkyl nonitol tetraethers

(GDNTs, archaeol and hydroxyarchaeol); however, only GDGTs were detected. Isoprenoid GDGTs of archaeal origin ranged between 0.36 and 6.7 mg g^{-1} of sediment being highest at 20.5 and 626.2 mbsf and lowest at 684 mbsf. The caldarchaeol to crenarchaeol (cal/cren) ratio was always below 0.7 and displayed only small changes, suggesting little contribution from methanogenic archaea (Figure 3.2). Moreover, TEX_{86} values calculated from those extracted archaeal lipid compositions, which is a biomarker proxy for the paleo-temperature in the water column (e.g., Schouten*, et al.*, 2002; Wuchter*, et al.*, 2004), indicate the temperature range of the surface water (24-30ºC) (Figure 3.2). Given the absence of detectable archaeal DNA in the sediment examined, both DNA- and lipid-based analyses consistently suggest that most GDGTs were relict biomarkers derived from the past water column activity and that little or no GDGT production took place in the sediment.

Statistical analysis of microbial community structure. NMS ordination and MRPP ($A=0.08$, $p<0.05$) of T-RFLP profiles indicated three distinct communities based on whether microbes were present above, within, or below the GHOZ (Figure 3.3). The samples from within and below the GHOZ separated along axis 1 on the NMS plot. A bi-plot overlaid on the ordination identified environmental parameters that correlate to the community structure. The environmental parameters that had an $r^2 > 0.1$ to axis 1 were organic carbon ($r^2 = 0.243$), inorganic carbon ($r^2 = 0.225$), and total carbon ($r^2 = 0.122$). Samples aligned with the vectors typically have higher values for that particular environmental parameter. There were no environmental variables measured that had a higher correlation than 0.1 to axis 2 (axis 2 is not shown in Figure 3.3). Samples from

above and within the GHOZ were distinguished by axis 3 and the measured environmental parameters related to axis 3 were grain density (r^2=0.108), resistivity (r^2=0.158) and depth (r^2=0.362). Taken together, the three axes explained 81.5% of the variance. This statistical analysis indicated that all three geochemical zones contained distinct microbial communities and that organic carbon and unmeasured variables associated with depth had the highest correlation to the axes in the NMS plot.

Discussion

Biogenic methane is a significant fraction of the methane that occurs in marine sediments where gas hydrates are present. The relationships between microbial distributions, cellular quantities, and the pore water geochemistry and physical properties in gas hydrate environments are becoming more apparent. This study in the Andaman Sea, where methane hydrates extend deep into the sediments and where the total organic carbon concentrations are low to intermediate, complements existing studies in gas hydrate-containing marine sediments by using geochemical, lipid, and molecular techniques to describe the microbial biogeography in relation to the environment.

Cell counts. Site 17A is located in a forearc basin that contains biogenic methane in the gas hydrate form. Thus, the expected cell abundance should reflect other hydrate-containing margins. The typical cell abundance in methane hydrate-containing margins follows the general trend described by Parkes *et al.*, with the highest biomass (10^9 cells cm^{-3}) near the surface and a logarithmic decrease with increasing depth (Parkes*, et al.*, 1994; Inagaki*, et al.*, 2006; Morono*, et al.*, 2009). The sediment layers at Site 17A are

unique for a gas-hydrate containing location in that they harbor 1-2 orders of magnitude fewer cells and there is no discernable decrease with depth. The reason for this trend (relatively high cell numbers at depth) is enigmatic; however, studies that have described discrepancies from the Parkes *et al.* (1994) data have proposed carbon availability and/or lithology (i.e., resistivity, grain size, pore-connectivity) to be key drivers in cell densities (Coolen*, et al.*, 2002; Inagaki*, et al.*, 2003; D'Hondt*, et al.*, 2004; Parkes*, et al.*, 2005).

Continental margins and abyssal plains are opposite end- members on a continuum of buried carbon concentrations and sediment grain sizes. Continental margins typically contain 1-11% organic carbon (Meister*, et al.*, 2005), sandy sized sediment, and a depth integrated average of 10^7 cells cm^{-3} (Parkes*, et al.*, 1994). On the other hand, abyssal plains contain less than 0.25% organic carbon, clay sized sediment, and a depth integrated average of 10^3 cells cm^{-3} (D'Hondt*, et al.*, 2009). Site 17A is intermediate on these scales with 1% organic carbon, silt-sized nanofossil ooze, and an average 10^4 cells cm^{-3}. Another intermediate site on these scales, SPG12, was located on the edge of the South Pacific Gyre (SPG) and contains sediment that is dominated by nanofossil ooze with an intermediate organic (0.5-1%) carbon content (D'Hondt*, et al.*, 2009). The cell density for site SPG12 has only been reported for the top 10 m; however, if we assume a continuous logarithmic decrease with depth as is the case for many marine subsurface sites the trend bisects the cell counts from Site 17A (Figure 3.1). This relationship of cell density based on carbon content and grain size has considerable importance on the estimation of global prokaryotic microbial abundance (Whitman*, et al.*, 1998; Fry*, et al.*, 2009).

Statistical analysis of T-RFLP data. Statistical analysis based on molecular data aids in the interpretation of microbial community trends and factors that control those trends. NMS is a data visualization tool that can depict relatedness of microbial communities (Culman*, et al.*, 2008). Samples that contain similar microbes (yielding similar TRFs) will cluster together in the NMS ordination (Rees*, et al.*, 2004; Osborne*, et al.*, 2006; Colwell*, et al.*, 2011). Based on T-RFLP data at Site 17A, a distinct microbial community existed within the GHOZ relative to microbes obtained from sediment layers above and below this zone. However, it is difficult to determine if the presence of gas hydrate significantly affects the community or if one or more other variables affect the community.

One statistical method that can be used to identify environmental trends related to community structure is a bi-plot overlaid on an NMS plot. A bi-plot contains vectors that represent each measured variable. The length and angle of a vector indicates the strength and direction of the relationship with respect to the primary axes in the NMS plot. The T-RFLP patterns for samples obtained from the GHOZ at Site 17A were correlated with an increase in inorganic carbon and a decrease in organic carbon (Figure 3.3). Bacterial community structure from sediment in the North Sea was reported to also correlate to organic carbon concentration (Sapp*, et al.*, 2010). In addition, the highest correlated variable to axis three was depth (r^2=0.362). In marine subsurface sediments, many environmental conditions change as a function of depth such as, temperature, pressure, and depositional age. Data analysis using bi-plots is limited by the availability of measured variables; unmeasured variables may have higher correlations.

Microbial taxa. Our results from primer-tagged pyrosequencing indicate that Site 17A has very low diversity and is dominated by bacterial taxa related to *Firmicutes* (also supported by clone library analysis, see supplemental text). Other investigations of the microbiology in methane hydrate-containing areas have taken place at Blake Ridge (Wellsbury*, et al.*, 2000), Nankai Trough (Reed*, et al.*, 2002), Cascadia Margin (Bidle*, et al.*, 1999; Marchesi*, et al.*, 2001; Nunoura*, et al.*, 2008), and the Peru Margin (Fry*, et al.*, 2006; Sorensen & Teske, 2006; Biddle*, et al.*, 2008) and they have found a diverse assemblage of both bacteria and archaea. Along the Pacific Ocean margins, microbes in sediments that contain methane hydrates appear to be distinct from those in non-hydrate sediments obtained along the Pacific Ocean margins (Inagaki*, et al.*, 2006). Using clone libraries to study community diversity Inagaki *et al*. found that hydrate saturated sediment is typically dominated by the bacterial candidate division JS1 and the Deep-Sea Archaeal Group (Inagaki*, et al.*, 2006). The bacterial phylum *Chloroflexi* and the Marine Crenarchaeotic Group I dominate non-hydrate areas (Inagaki*, et al.*, 2006). *Firmicutes* have been found in gas hydrate environments, but typically only as a minor phylotype (Inagaki*, et al.*, 2006). *Firmicutes* related to the genus *Bacillus* have been found to be highly abundant in the hypersaline anoxic sediments of the Mediterranean Sea, possibly due to adaptive mechanisms to high salt concentrations (Sass*, et al.*, 2008). The reason for the dominance of *Firmicutes* at Site 17A is unknown; however, the ability to form spores, a trait of *Bacillus* sp., may be beneficial in these austere sediments. *Bacillus* species have been reported to survive in geologic environments for long periods of time as spores (Vreeland*, et al.*, 2000).

Abundance of bacteria vs. archaea. The sediment samples from Site 17A did not have detectable levels of archaea, as determined by DNA based techniques. The absolute quantity of archaea or bacteria in subseafloor environments has been difficult to determine. Culture independent methods such as QPCR, catalyzed reporter deposition fluorescence in-situ hybridization (CARD-FISH), intact polar lipids (IPLs), and slot-blot hybridization assays have been performed and each technique produced a different result (Schippers*, et al.*, 2005; Biddle*, et al.*, 2006; Schippers & Neretin, 2006; Lipp*, et al.*, 2008). However, unlike Site 17A, all studies performed with the Peru margin sediments were able to detect both bacteria and archaea. Similarly, other studies from hydrate bearing sediments have detected archaea with culture dependent and independent methods (Marchesi*, et al.*, 2001; Reed*, et al.*, 2002; Mikucki*, et al.*, 2003; Mauclaire*, et al.*, 2004; Kendall*, et al.*, 2006; Colwell*, et al.*, 2008). While the question of archaeal versus bacterial dominance remained to be verified with multiple methods in the future, these studies suggest that archaea are present in methane hydrate-containing environments and would be expected at Site 17A.

Possible reasons for an inability to detect archaea could be the biases created during DNA-based approaches. DNA extraction procedures can be less efficient at lysing the rigid cell wall structure of archaeal cells from sediment (Lipp*, et al.*, 2008). To minimize this bias we used a modified DNA extraction procedure for obtaining archaeal cells from deep subseafloor sediments (Lipp*, et al.*, 2008). Lipp *et al.* determined the lysing efficiency of three methods and used lipid analysis as an independent approximation of bacterial and archaeal abundance. They found that bacteria seem to

dominate surface layers; however, archaea dominate the deeper subseafloor sedimentary biosphere (Lipp*, et al.*, 2008). We used the three methods developed by Lipp *et al.* on the samples from the GHOZ at Site 17A, and found that the more vigorous cell lysing steps reduced DNA yield, whereas Lipp *et al.* (2008) detected more archaea using this method. QPCR failed to detect both archaeal and bacterial 16S rRNA genes with the more vigorous cell lysing steps suggesting that the bacterial DNA at Site 17A might be easily degraded.

Further biases can be created during PCR (Teske & Sorensen, 2008). Archaeal diversity is poorly represented in the gene databases compared to bacterial diversity; therefore, the design of primers to target archaea will be less than optimal. Some studies of seafloor sediments could not detect 16S rRNA genes from methanogens; however, primers for the *mcrA* gene were successful at detecting methanogens (Newberry*, et al.*, 2004; Colwell*, et al.*, 2008). In our study, a suite of 16S rRNA genes and functional gene primers were used to capture the greatest diversity and to overcome primer biases (Table B.1). Yet, only bacterial 16S rRNA genes and bacterial *accC* genes were detected (see supplemental text).

Detection of archaeal genes may have been hindered by an inability to detect low numbers. To increase our ability to detect archaea QPCR was performed on archaeal 16S rRNA genes. The detection limit for QPCR is 100 molecules for a 20 μl reaction, which is a much higher resolution than a EtBr stained gel (3×10^9 molecules assuming a 300 bp amplicon). Even with these low detection limits we were unable to identify the presence of archaea.

We detected in the sediments isoprenoid GDGTs derived from archaeal membrane lipids as an independent measure of archaeal presence (e.g. Damste*, et al.*, 2002; Herfort*, et al.*, 2007; Huguet*, et al.*, 2007). The calculated TEX_{86}- temperature values were consistent with surface water temperatures at the study area, which suggests a water column origin of these archaeal lipids. However, interpretation of the TEX_{86} values in deep sediments may be complicated because studies have not verified that the lipid biomarker of living sedimentary populations reflect in-situ temperature as has been demonstrated for communities that occur in the water column. We postulate that because archaeal DNA was not detected the lipids may be relicts in the deep sediments (Schouten*, et al.*, 2010; Logemann*, et al.*, 2011) and were deposited as a part of archaeal populations from the overlying water column.

Another lipid measure, the ratio of caldarchaeol to crenarchaeol is postulated to indicate the presence of methanogenic archaea when the value exceeds two (Blaga*, et al.*, 2009). The maximum value of the ratio at Site 17A was 0.7, suggesting that methanogenic archaea are not present in the sediment layers we analyzed. This was confirmed by measuring archaeol and hydroxyarchaeol, biomarkers for methanogenic archaea (Hinrichs*, et al.*, 1999), neither of which were detected at the study area. Given the various approaches used to detect archaea, biases notwithstanding, our conclusion is that archaeal cells are exceedingly rare in this environment.

Where is the methane coming from? Despite the limited quantity of organic carbon, isotopic data indicates the methane present in the gas hydrate is biologically produced. Yet, our analysis failed to identify organisms that are typically implicated in

methanogenesis. It is possible that the methanogenic sediment layer was not sampled. The sulfate methane transition zone (SMTZ) is where both sulfate and methane converge. Sediment layers below the SMTZ is usually considered the methanogenic zone because sulfate reduction is believed to outcompete methanogenesis above the SMTZ. The SMTZ at Site 17A is at 25 mbsf (Collett*, et al.*, 2008). Sediment at 20 and 40 mbsf were analyzed. Thus, methanogenesis could be occurring anywhere between 25 and 40 mbsf. Studies have indicated that the peak methanogenic zone is in the top 50 mbsf (Claypool*, et al.*, 2006; Sivan*, et al.*, 2007; Colwell*, et al.*, 2008; Nunoura*, et al.*, 2008). Other explanations could be that the methane was produced in the past and the methanogen DNA and lipids are no longer present, or that the methane could come from another location. Seismic images depict an anticline structure at Site 17A where fluids could move through permeable sediment layers and collect at the top of the anticline (Collett*, et al.*, 2008). Thus, the methane at Site17A may have collected from surrounding sediments.

In summary, Site 17A in the Andaman Sea is a unique sedimentary environment that contains biogenic methane, a thick methane hydrates layer in the deep horizon, an intermediate organic carbon content, and intermediate grain size. The low cell abundance detected can help to refine global subseafloor microbial abundance. In addition, it appears that these sediments contain a distinct microbial community that is dominated by *Firmicutes*. Several molecular analyses were unable to detect archaea. The biogenic methane present at Site 17A may have been produced in sediment layers that we did not

analyze, produced by methanogens no longer present in the system, or produced in other locations after which it migrated to Site 17A.

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Table 3.1. Distribution of major phylogenetic groups determined by primer-tagged pyrosequencing from above, within, and below the GHOZ.

Division	Subdivision or subgroup	Abundance (%) of reads[a]		
		Above	Within	Below
Acidobacteria		0.5	0.0	0.0
Actinobacteria		8.0	2.6	3.1
Bacteroidetes		6.0	2.1	5.2
Chloroflexi		0.2	0.1	0.0
Firmicutes		52.2	53.0	74.4
Planctomycetes		0.1	0.0	0.1
Proteobacteria	*Alphaproteobacteria*	7.7	2.8	3.9
	Betaproteobacteria	7.4	21.4	2.9
	Deltaproteobacteria	0.1	0.2	0.1
	Epsilonproteobacteria	0.1	0.1	0.1
	Gammaproteobacteria	1.3	16.8	1.2
	Other	3.6	0.2	2.4
TM7		0.0	0.1	0.3
Other		12.7	0.6	6.5

[a] The numbers of reads in the above, within, and below the GHOZ were 15632, 41095, and 15796 respectively.

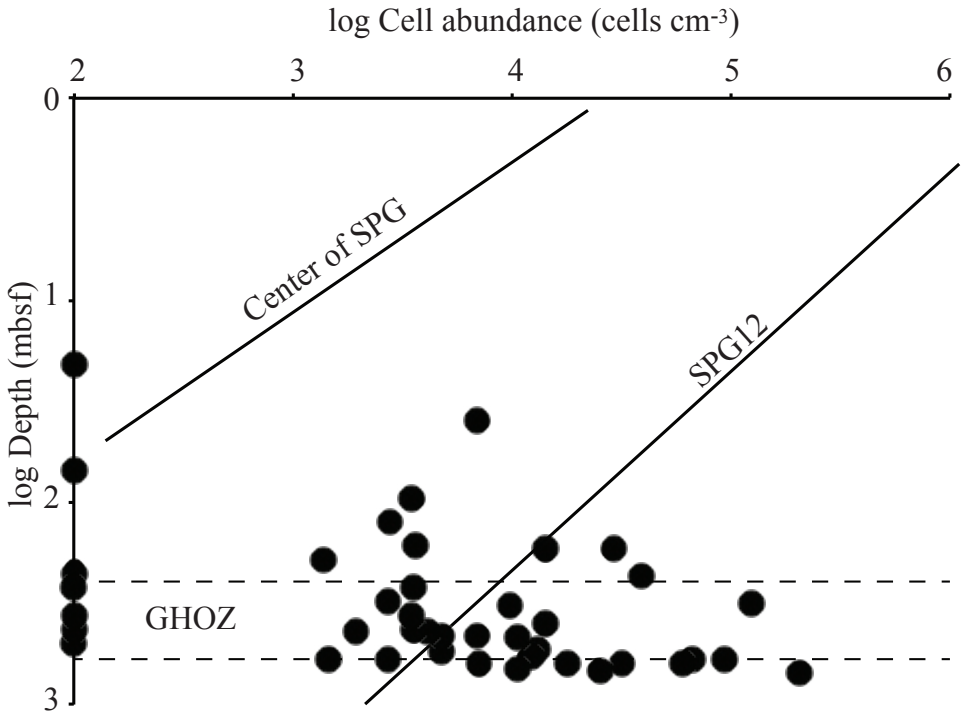

Figure 3.1. Microscopic cell numbers versus depth at site 17A in Andaman Sea sediments. Circles on the y-axis are samples that had cell abundance below the detection limit of 10^2 cells cm^{-3}. The dashed lines depict the location of the gas hydrate occurrence zone (GHOZ). The diagonal lines are downward logarithmic extrapolation of cell abundance from the top 10 m of sediment from center of the South Pacific Gyre (SPG) and site SPG12 (from the outer edge of the SPG) reported by D'Hondt *et al*. (2009).

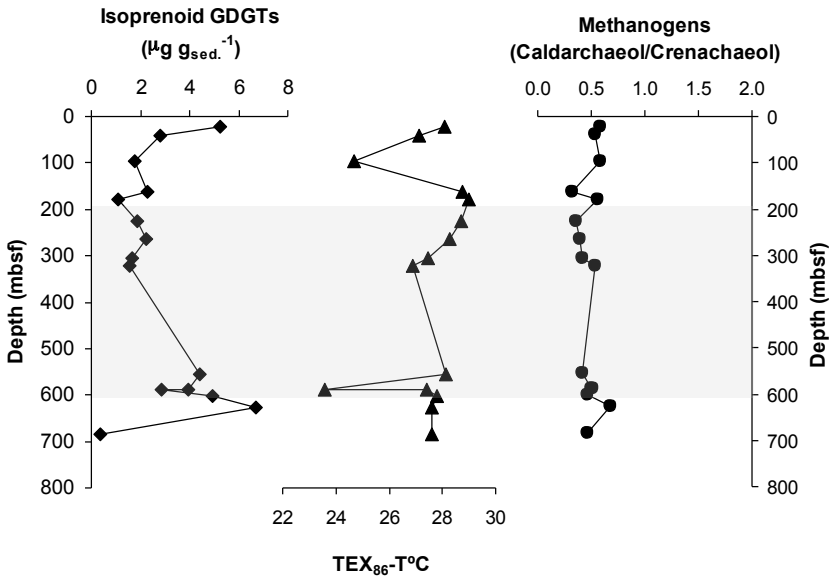

Figure 3.2. Lipid determinations for selected samples from site 17A in the Andaman Sea sediments showing downcore GDGT measurements of isoprenoid abundance (left), TEX_{86} calculated temperatures (ºC) (center), and methanogen ratio (caldarchaeol/crenarchaeol) (right). The shaded band in the center of the diagram depicts the depth range of the gas hydrate occurrence zone (GHOZ). A significant methanogen archaeal contribution is only apparent when methanogen ratio values are >2.

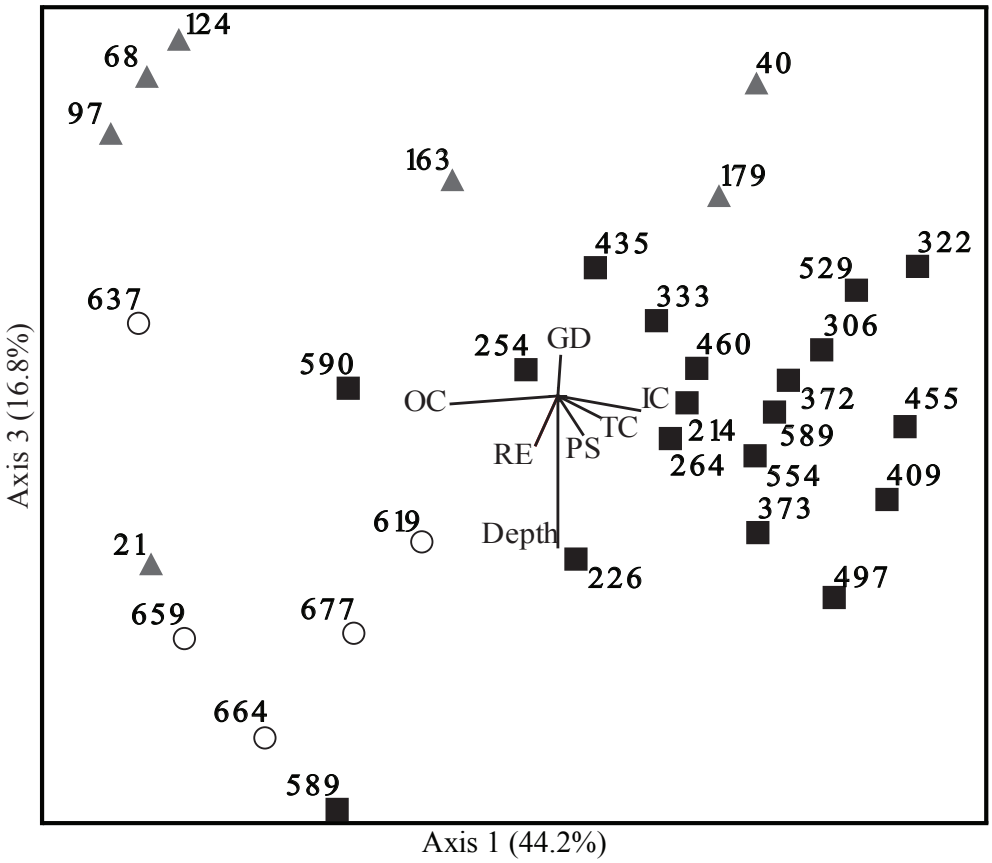

Figure 3.3. NMS ordination of the data derived from T-RFLP analysis of Andaman Sea sediments. The triangles, squares, and circles represent the central tendency of all TRFs detected in each sample from above, within, and below the GHOZ, respectively. Symbols that are closer to each other depict samples that contained similar TRFs. A bi-plot is overlaid on the ordination to identify environmental parameters that were correlated to the microbial community structure. The length of each bi-plot line corresponds to the degree of correlation with the respective axis. The values in parentheses indicate the variance explained by each axis. Together, axes 1, 2 (not shown), and 3 explained 81.5% of the variance. Numbers represent depths (mbsf) of each sample. Organic carbon: OC; resistivity: RE; grain density: GD; inorganic carbon: IC; total carbon: TC; porosity: PS

4. MICROBIAL DISTRIBUTIONS IN MARINE SEDIMENTS THAT TRANSITION GEOCHEMICAL ZONES ASSOCIATED WITH METHANE

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Abstract

The biogeochemical processes that occur in marine sediments on continental margins are complex; however, from one perspective they can be considered with respect to three geochemical zones based on the presence and form of methane: sulfate-methane transition (SMT), gas hydrate occurrence zone (GHOZ), and free gas zone (FGZ). These geochemical zones may harbor distinct microbial communities that are important in the marine sediment carbon cycle. The objective of this study was to describe the microbial communities in sediments from the SMT, GHOZ, and FGZ using molecular ecology methods (i.e., terminal restriction fragment length polymorphism or T-RFLP and PhyloChip microarray analysis) and examining the results in the context of non-biological parameters in the sediments. Non-metric multidimensional scaling and multi-response permutation procedures were used to determine whether microbial community compositions were significantly different according to whether communities were found in sediment SMT, GHOZ, or FGZ, and to correlate samples with abiotic characteristics of the sediments. This analysis indicated that microbial communities from all three zones were distinct from one another and that the abiotic variables sulfate, hydrate saturation of nearest gas hydrate layer, and unmeasured variables associated with depth were most correlated to the three zones. The archaeal anaerobic methanotrophs typically attributed to performing anaerobic oxidation of methane were not detected in the SMT; however, the marine benthic group-B, which is often found in SMTs, was detected. Within the GHOZ, samples that were typically closer to layers that contained higher hydrate saturation had indicator sequences related to *Vibrio*-type taxa. These results suggest that

the biogeographic patterns of microbial communities in marine sediments are distinct based on geochemical zones defined by methane.

Introduction

In shallow marine sediments the distribution of microbial taxa is dependent on the presence of more electrochemically positive electron acceptors. The mineralization of organic material proceeds in a continuous sequence of redox reactions that cascade from high to low free energy yield until the electron acceptor source is depleted (Konhauser, 2007). This sequence of redox reactions occurs in sediment and reflects the dominant microbial community growing at a particular depth. While the geochemical zones of electron acceptors defines the microbial distributions in shallow sediments, in deep marine sediments along continental margins geochemical zones that are defined by methane are dominant features that may control deep microbial distributions.

Methane-bearing marine sediments can be divided into three distinct geochemical zones according to the methane concentration and gas hydrate stability at particular layers in the sediment. The sulfate methane transition (SMT) is the uppermost geochemical zone in sediment, where sulfate and methane concentrations both reach minima (Borowski*, et al.*, 1999). Below the SMT, if the temperature, pressure, and methane concentration is favorable gas hydrate will form. The gas hydrate occurrence zone (GHOZ) defines depths in the sediment that both contain gas hydrate and non-gas hydrate layers depending on the lithology (Trehu*, et al.*, 2006). The geothermal gradient defines the base of gas hydrate stability and often is seismically identified as a bottom-simulating

reflector (BSR). Free-methane gas is believed to be present below the BSR (Trehu, *et al.*, 2006) in the free gas zone (FGZ).

The microbial communities at the SMT are a globally important consortium of bacteria and archaea that performs anaerobic oxidation of methane (AOM) (Knittel & Boetius, 2009), a process that is estimated to consume nearly 90% of the methane that is produced in deep sediments (Reeburgh, 2007). Distinct consortia of *Deltaproteobacteria* and anaerobic methanotrophs (ANME) have been linked to the process of AOM (Boetius, *et al.*, 2000; Orphan, *et al.*, 2002; Knittel & Boetius, 2009). The presence of this consortium has been confirmed in seep-dominant (Michaelis, *et al.*, 2002; Elvert, *et al.*, 2005; Knittel, *et al.*, 2005; de Beer, *et al.*, 2006; Losekann, *et al.*, 2007), fracture-dominant (Briggs, *et al.*, 2011), and diffusion-dominant environments (Thomsen, *et al.*, 2001; Parkes, *et al.*, 2007; Harrison, *et al.*, 2009). However, their detection in some SMT environments has been inconsistent and has led to the hypothesis that other microbial groups may be involved in methane oxidation, such as Marine Benthic Group-B (MBGB) (Biddle, *et al.*, 2006; Inagaki, *et al.*, 2006; Sorensen & Teske, 2006; Roussel, *et al.*, 2009; Hamdan, *et al.*, 2011).

Microbiological investigations of the GHOZ have taken place at Blake Ridge (Wellsbury, *et al.*, 2000), Nankai Trough (Reed, *et al.*, 2002), Cascadia Margin (Bidle, *et al.*, 1999; Marchesi, *et al.*, 2001; Colwell, *et al.*, 2008; Nunoura, *et al.*, 2008), the Peru Margin (Fry, *et al.*, 2006; Sorensen & Teske, 2006; Biddle, *et al.*, 2008) and the Andaman Sea (Briggs, *et al.*, in review). These investigations and one particular large dataset of microbial distributions along multiple Pacific Ocean margins indicate that the

microbes in locations that contain gas hydrates appear to be distinct from those in locations in which hydrate is not found (Inagaki*, et al.*, 2006). The few microbiological studies that have been performed on sediments in the BSR indicate a diverse community of bacteria and archaea (Wellsbury*, et al.*, 1997; Reed*, et al.*, 2002; Nunoura*, et al.*, 2008; Yoshioka*, et al.*, 2010). Some of these studies have suggested an increase in methanogenesis in the FGZ (Wellsbury*, et al.*, 2000).

While studies have considered the microbiology in one or two of these zones, none have considered the biogeography in all three geochemical zones at a given site. The objective of this study was to investigate microbial community composition in different geochemical zones that are defined by methane. The Ulleung Basin is a back-arc basin located offshore Korea where previous expeditions identified the presence of the SMT, GHOZ, and FGZ in the sediments (Kim*, et al.*, 2007; Bahk*, et al.*, 2009; Ryu*, et al.*, 2009). The Ulleung Basin Gas Hydrate Expedition 02 (UBGH02) collected samples for microbiology analysis in the SMT, GHOZ, and BSR providing a unique opportunity for microbial characterization in all three zones. This characterization was accomplished by using molecular techniques and subsequent statistical analysis to identify community composition trends and correlated environmental parameters. Compared to past research, this study increased the resolution of microbiological sampling in relation to hydrate-bearing sediment layers and their abiological characteristics and identified taxa that are predominantly found in the chemically and physically distinct sediment zones.

Materials and Methods

Site description and sampling. Sediment cores were collected for microbiological analysis from two sites drilled during during the Ulleung Basin Gas Hydrate Expedition 02 (UBGH02). The Ulleung Basin is a gas hydrate-bearing back-arc basin in the East Sea (Ryu*, et al.*, 2009). Site 1-1 was located in the western margin of the Ulleung Basin at $36^0$15' 04.78" N 130^0 3' 56.55" E. The depth of the SMT at site 1-1 occurs between 6 and 8 meters below the seafloor (mbsf) and the BSR occurs at 165 mbsf (Shipboard Scientific Party, 2011). Site 10 was located at 36^0 55' 35.14" N 130^0 54' 00.26" E. The depth of the SMT at site 10 occurs between 7 and 8 mbsf and the BSR is at 160 mbsf (Shipboard Scientific Party, 2011). A total of 40 sediment samples were collected from the two sites with 10, 24, and 6 samples obtained from the SMT, GHOZ, and BSR, respectively.

Core samples were retrieved using an Advanced Piston Core. The Integrated Ocean Drilling program (IODP) guidelines for obtaining high quality microbiology cores were adhered to during sample recovery and processing (Shipboard Scientific Party, 2011). Aboard ship whole round cores were subcored by paring the outer 2 cm of sediment with an alcohol and flame sterilized chisel. The subcores were placed into sterile Whirl Pak Bags® and stored at -80^{o}C and then express shipped at -80^{o}C in Doble dry shippers (MVE Biomedical Inc., Washington, PA) to Oregon State University.

DNA extraction. Total DNA was extracted from 10 g of sediment using a PowerMax Soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) as previously described (Lipp*, et al.*, 2008; Briggs*, et al.*, 2011). An attempt to increase DNA extraction

efficiency was performed by re-extracting two samples from within the GHOZ following the Mo Bio PowerMax Soil kit protocol of heating the samples to 55°C for 10 min prior to bead beating then following the protocol outlined above. The amount of DNA in each sample was measured using a Qubit (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's recommendations.

Terminal restriction fragment length polymorphism. Two μl of each DNA suspension were amplified by polymerase chain reaction (PCR) using a Veriti Thermocycler (Applied Biosystems, Inc., Foster City, CA). Samples were amplified using either universal bacterial 16S rRNA gene 27F labeled with FAM (27F-FAM) (Edlund*, et al.*, 2008) and 926R primers (Lipp*, et al.*, 2008) or universal archaeal 16S rRNA gene 21F labeled with FAM (21F-FAM) (DeLong, 1992) and 958R primers (Lipp*, et al.*, 2008). Each 20 μl PCR reaction contained 0.5 μM of each primer, 800 μM dNTPs, 1.25 units ExTaq polymerase, and 1x PCR buffer (Takara Bio, Inc., Otsu, Japan). PCR conditions were as previously described, using 34 cycles (Takai*, et al.*, 2001). PCR products were combined from three reactions for each sample. Water blanks treated the same as extracted samples were used as controls. Amplified DNA products were purified via alcohol precipitation and resuspended in 20 μl of water.

Amplified DNA products were incubated with the restriction enzyme *mspI* for 3 h at 37°C then heat-denatured for 20 minutes at 80°C (Reed*, et al.*, 2002). Restricted DNA products were submitted to Oregon State University Center for Genome Research and Biocomputing for size separation by capillary gel electrophoresis. Fragment sizes were

detected using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Inc.), yielding electropherograms with peaks representing terminal restriction fragments (TRFs).

The size and area under each peak of the TRFs were determined using Peak Scanner v. 1.0 software (Applied Biosystems, Inc.). Only peaks representing fragment lengths between 50 and 500 base pairs were retained. Peak lengths and areas for each sample were relativized and recalculated to reduce within-sample variance in peak area (Colwell*, et al.*, 2011). TRF sizes were compared to the Ribosomal Database Project-II (RDP-II) as previously reported (Widmer*, et al.*, 2006).

PhyloChip. Amplification of DNA for the PhyloChip was performed as previously described (Briggs*, et al.*, 2011). Briefly, each DNA suspension was amplified using the general bacterial primers 27F and 1492R or general archaeal primers 4F and 1492R (Hazen*, et al.*, 2010) in a Veriti thermocycler. Each 20 μl PCR mixture contained 1.25 units of ExTaq polymerase (Takara, Tokyo, Japan), 1x PCR buffer, 800 μM of each dNTP, and 0.5 μM of each primer. PCR conditions consisted of an initial denaturation step of 5 min at 95^{o}C followed by 30 cycles of 40 s of denaturation at 95º C, 40 s of annealing 48-58º C, 60 s at 72º C, and a final elongation step of 5 min at 72º C. Sample DNA products from different annealing temperatures were combined from six PCR runs. The combined products were purified using an Amicon Ultra 30K clean up column (Millipore, Billerica, MA.). The cleaned DNA products were stained and visualized on an E-gel containing EtBr (Invitrogen, Inc.). Amplified DNA quantities were determined by comparing the fluorescent intensity of the bands on the E-gel to a DNA ladder with known quantities of DNA.

PhyloChip version G3 sample preparation and data analysis were performed as previously described (Hazen*, et al.*, 2010) on 33 samples from sites 1-1 and 10, except that 100 ng of internal spike DNA, 150 ng of bacterial 16S rRNA gene amplicons, and 50 ng of archaeal 16S rRNA gene amplicons were hybridized to the array.

Mantel test. A Mantel test was used to test the similarity between microbial community compositions as determined by T-RFLP and PhyloChip analyses (Mantel, 1967). Dissimilarity matrixes were created using Sorenson distance measure for the presence and absence data of both the T-RFLP and PhyloChip. Only bacterial TRFs and phylotypes were included in the ordination because of the lack of archaeal TRFs. A Monte Carlo test with 999 randomizations was used to determine the significance of the similarity between T-RFLP and PhyloChip distance matrices.

Ordinations. Multivariate statistical analysis of PhyloChip detected taxa was conducted using PC-ORD v. 5.0 (McCune & Mefford, 2006). To reduce variance, taxa that only appeared in one sample, and outliers greater than two standard deviations from the mean were discarded (McCune & Mefford, 2006). Non-metric multidimensional scaling (NMS) ordination with Sorenson distance measures was used to identify relationships in community composition between samples (Bray & Curtis, 1957; Kruskal, 1964). Phylotypes were ordinated with the parameters that were set as previously described (Colwell*, et al.*, 2011). Two separate ordinations were created for all samples and samples only in the GHOZ.

Multi-response permutation procedure (MRPP) using a Sorenson distance measure was used to determine the statistical significance of group membership of

samples with *a priori* groups (determined according to whether the DNA was derived from SMT, GHOZ, or BSR sediment samples). MRPP is a nonparametric statistical analysis that provides an agreement statistic (A), which equals one when all samples are identical and zero when all samples are different, and a p-value, which indicates the likelihood that the A-value is due to chance. For this analysis, p-values less than or equal to 0.05 were considered to be significant.

Indicator taxa analysis using PhyloChip-detected taxa in each zone was performed to determine which, if any, taxa were indicative of a single geochemical zone (Dufrene & Legendre, 1997). A Monte Carlo test with 4999 randomizations was used to determine the significance of each phylotype as an indicator of a particular geochemical zone.

Bi-plots of the measured geochemical variables: pH, alkalinity, salinity, chloride, sulfate, bromide, phosphate, ammonium, hydrogen sulfide, silicic acid, sodium, potassium, magnesium, calcium, strontium, lithium, boron, barium, iron, manganese, distance to closest hydrate, and hydrate saturation of closest hydrate layer were superimposed on the NMS plots to identify correlations to the community structure (Shipboard Scientific Party, 2011). The directions and magnitudes of the vectors indicate their correlations with sample ordinations. Only vectors that had a higher correlation than 0.2 were considered.

Cluster analysis. Hierarchical cluster analysis was performed on samples within the GHOZ to identify group composition. Euclidean distance measure with Ward's linkage method was used (McCune & Mefford, 2006).

Results

DNA Extraction. DNA extraction yield ranged from below detection limit (0.5 ng DNA g^{-1} of sediment) to 256.22 ng g^{-1} of sediment (Figure 4.1). The SMT contained the highest amount of extractable DNA ranging from 49-231 ng g^{-1} of sediment. Both the GHOZ and FGZ contained similar DNA quantities ranging from below detection limits to 6.2 ng g^{-1} of sediment. The re-extraction of two samples using the heating protocol did not yield DNA above the detection limit (0.5 ng DNA g^{-1} of sediment). In addition, the final DNA solution contained a light brown coloration suggestive for the presence of humic acids.

T-RFLP analysis of microbial communities. A total of 116 bacterial and 46 archaeal TRFs were detected in all of the samples analyzed. After amplification only three samples contained enough archaeal DNA (100 ng) to proceed with T-RFLP analysis. All three samples were from the SMT. Fifty-five bacterial TRFs were detected in the SMT. The most diverse zone was the GHOZ with 96 bacterial TRFs. The BSR contained 46 bacterial TRFs. None of the TRFs that were detected could be attributed to known taxa by comparison to the RDP-II website.

PhyloChip analysis of microbial communities. A total of 1033 taxa were detected from 33 sediment samples that contained enough DNA for PhyloChip analysis (150 ng of bacterial 16S rRNA gene amplicons). Only the samples from the SMT contained enough archaeal 16S rRNA gene amplicons (50 ng) for PhyloChip analysis. The SMT contained 383 bacterial taxa and 33 archaeal taxa. The GHOZ contained 728 bacterial taxa and the BSR had 13 bacterial taxa.

T-RFLP and PhyloChip comparison. To test if T-RFLP and PhyloChip identified the same community similarities, a Mantel test was performed on the Sorenson distance matrixes created for the T-RFLP and PhyloChip data. There was a lack of archaeal TRFs; therefore, only bacterial TRFs and taxa for 25 samples were used in the Mantel test. Based on this test both distance matrixes were significantly similar ($p = 0.001$) indicating that the community structures identified by both analyses were similar. PhyloChip analyses were used for all further community comparisons because unlike T-RFLP the PhyloChip analysis can identify specific taxa.

Statistical analysis of community structure. Using data generated by PhyloChip hybridization arrays, NMS ordination of all samples was plotted on two axes with a final stress value of 16.82 and final instability <0.001 (Figure 4.2). Axis 1 explained 41.2% of the variance and axis 2 explained 36.2% for a total of 77.4% of the variance explained. MRPP analysis indicated that the different sediment groupings (SMT, GHOZ, and FGZ) identified *a priori* as three geochemically distinct zones contained microbial communities that were significantly different from each other ($A=0.12$, $p <0.05$). The groups were mainly separated along axis 1; however, the GHOZ samples were separated into two groups along axis 2. In order to examine whether environmental parameters of the sediments in which the microbial communities occur are related to the distribution of communities in NMS space, bi-plots of the abiotic data were superimposed on the NMS plots. Abiotic variables that had a higher correlation to axis 1 than 0.2 were depth ($r^2=0.592$), alkalinity ($r^2=0.275$), sulfate ($r^2=0.440$), bromide ($r^2=0.264$), ammonia ($r^2=0.327$), hydrogen sulfide ($r^2=0.347$), silicate ($r^2=0.441$), strontium ($r^2=0.220$), and

barium (r^2=0.305). The only abiotic variable correlated to axis 2 was the hydrate saturation of the closest hydrate layer (r^2=0.279). Thus, depth and hydrate saturation of the closest hydrate layer had the highest correlation of the measured environmental variables to the axes that separated each microbial group.

Indicator taxa analysis was run to identify what taxa, if any, were predominantly found in one zone or the other. This method found 74 and 65 taxa with a p-value of less than 0.05 in the SMT and GHOZ, respectively. No significant indicator taxa were identified from samples in the BSR. The taxa associated with the Ulleung Basin SMT were related to *Deltaproteobacteria* already noted to occur in subseafloor hydrate and methane seep environments (Niemann*, et al.*, 2005; Inagaki*, et al.*, 2006), candidate division WPS-1 observed in the south China Sea and subseafloor methane hydrate on the Peru Margin (Inagaki*, et al.*, 2006; Li*, et al.*, 2008), and candidate division JS1 from a mud volcano (Heijs*, et al.*, 2005). Archaeal taxa detected in the Ulleung Basin SMT were related to *Thermoplasmata* from marine sediments in Guaymas Basin (Teske*, et al.*, 2002), Baja California (Orphan*, et al.*, 2008), and Skan Bay (Kendall*, et al.*, 2007), marine benthic group-B (MBGB) from deep sea sediments (Vetriani*, et al.*, 1999), marine benthic group-D (MBGD) from a mud volcano (Omoregie*, et al.*, 2008), and Thaumarchaeota from a hydrothermal vent (Schrenk*, et al.*, 2004). The indicator taxa for the GHOZ were less diverse with all 65 taxa related to *Vibrio*-type species in the *Gammaproteobacteria* phylum. These *Vibrio*-type sequences have been found in marine environments such as hydrothermal vents (Brazelton*, et al.*, 2006) and methane-

containing sediment (Muyzer*, et al.*, 2008). Archaeal DNA was not detected within the GHOZ.

Statistical analysis of GHOZ samples. To further explore the community structure within the GHOZ an additional ordination was conducted using PhyloChip data acquired from only the GHOZ samples (Figure 4.3). This ordination had a stress value of 8.61 and a final instability <0.001. Axis 1 explained 58.1% of the variance and axis 2 explained 33.7% for a total of 91.8% of the variance explained. Cluster analysis was performed to identify group membership and these groups were applied to the NMS plot identified as group A and B. The two groups identified by the cluster analysis could not be explained by differences in site location as shown by MRPP analysis (A=0.22, p>0.05). Axis 1 was the main axis that separated the two groups. The measured abiotic variables that had a higher correlation to axis 1 than 0.2 were hydrate saturation of the nearest hydrate layer (r^2=0.274), potassium (r^2=0.227), and chloride concentrations (r^2=0.201). No measured abiotic factors were significantly correlated with axis 2. All significant indicator taxa came from group B (Figure 4.3) and were related to the *Vibrio*-type *Gammaproteobacteria* described above.

Discussion

Biogenic methane is a significant fraction of the methane that occurs in marine sediments where gas hydrates are present. Diverse communities populate these formations and the factors that control the distribution of microbes in marine sediments are becoming more apparent (Reed*, et al.*, 2002; Inagaki*, et al.*, 2006; Nunoura*, et al.*,

2008; Briggs*, et al.*, in review). Our report from the Ulleung Basin statistically analyzes the microbial communities in three biogeochemical zones defined by methane (SMT, GHOZ, and FGZ).

Archaeal 16S rRNA genes were not detected in sediment layers of the Ulleung Basin below the SMT. One study from sediments in the Andaman Sea was not able to detect archaeal 16S rRNA and functional genes (Briggs*, et al.*, in review). However, the Andaman Sea environment is different than most methane-containing continental margins because it has lower organic carbon. Archaeal taxa have been detected in most studies from methane-containing margins (Reed*, et al.*, 2002; Inagaki*, et al.*, 2006; Colwell*, et al.*, 2008; Nunoura*, et al.*, 2008). Based on these previous studies archaeal taxa were expected to be present in the Ulleung Basin sediments.

Possible reasons for an inability to detect archaea could be the biases created during storage, DNA extraction, or PCR. The sediment samples were placed at -80^{o}C prior to DNA extraction to ensure a representative microbial population and to minimize storage biases (Rochelle*, et al.*, 1994; Reed*, et al.*, 2002). Inefficient cell lysis has been reported to cause biases during DNA extraction; more vigorous lysing steps such as a freezer mill homogenization and proteinase K digestion extract more archaeal DNA from marine subsurface sediments (Lipp*, et al.*, 2008). An attempt to increase DNA extraction efficiency was performed by re-extracting two samples with an additional heating step prior to bead beating. This additional step either decreased the amount of DNA or increased co-extracted PCR inhibitors because bacterial and archaeal 16S rRNA genes could not be detected after this additional DNA extraction step. There was also a brown

coloration in the extracted DNA solution suggesting the co-extraction of humic substances. Humic substances have been reported to inhibit PCR (Tebbe & Vahjen, 1993). Also, the primers used during PCR could cause amplification bias. Primers for archaeal functional genes have been reported to detect archaea when 16S rRNA gene primers fail (Newberry*, et al.*, 2004). One or more of these biases could account for not detecting archaeal 16S rRNA genes below the SMT.

Despite not detecting archaea in deep samples, the bacterial community was well represented. The amount of DNA extracted from the GHOZ and BSR was similar to that reported from other hydrate areas such as Nankai Trough (Reed*, et al.*, 2002) and Andaman Sea (Briggs*, et al.*, in review). In addition, the bacterial diversity from all samples was large and comparable to other studies using clone libraries, which suggests that our DNA extraction and amplification procedures were adequate to assess a range of bacterial cell types.

Both PhyloChip and T-RFLP analyses was performed from the same DNA extraction on all sediment samples, which allows for a comparison of the two methods. T-RFLP analysis of microbial communities is based on the concept that the same microorganisms in different communities will yield TRFs of the same length (Liu*, et al.*, 1997). T-RFLP is a rapid and relatively inexpensive technique but is not sensitive to species level differences and multiple taxa can be represented by individual TRFs (Dunbar*, et al.*, 2001). On the other hand, PhyloChip is a microarray-based technique that contains oligonucleotide probes for most known bacterial and archaeal 16S rRNA genes. The PhyloChip is more labor intensive but provides higher resolution of closely

related taxa. In addition, the array is more sensitive to rare microbes than Sanger sequencing approaches (DeSantis*, et al.*, 2007).

A Mantel test offers a means of determining whether the microbial community structures identified by T-RFLP and PhyloChip analyses are statistically similar. The Mantel test uses a permutation method to compare two distance matrices, circumventing the problem of interdependence of values within the distance matrices, and the test yields a p-value that indicates whether the two matrices are significantly similar (Mantel, 1967). This test indicated that for the Ulleung Basin sediments both T-RFLP and PhyloChip analyses identified the same microbial community structure. T-RFLP and PhyloChip both served as valid techniques for microbial community structure analysis in this study. However, if specific details related to the community components are desired then the PhyloChip can provide these data and is a superior measure of community characteristics.

Statistical analysis of the community composition aids in the interpretation and elucidation of trends in microbial community composition. NMS is a visual tool that can depict distinct microbial communities based on samples that contain similar microbes. Such samples will cluster together in the NMS ordination (Rees*, et al.*, 2004; Osborne*, et al.*, 2006; Culman*, et al.*, 2008; Colwell*, et al.*, 2011). MRPP is used to test if *a priori* groups are significantly different in the NMS ordination. In addition, NMS allows ordination of environmental parameters associated with samples on the same plot as the sample ordination, and can be used to determine correlations between microbial community distributions and environmental factors. Our findings indicate that the microbial communities are significantly different in the SMT, GHOZ, and FGZ and that

sulfate concentration, gas hydrate saturation of the nearest hydrate layer, and factors associated with depth have the highest correlation to the different communities.

The SMT is an important geochemical zone where AOM is performed by a consortium of sulfate reducing bacteria (SRB) within the *Deltaproteobacteria* phylum and ANMEs (Boetius*, et al.*, 2000; Orphan*, et al.*, 2002; Knittel & Boetius, 2009). Consistent with other SMT environments *Deltaproteobacteria* were found at the SMT from both Ulleung Basin sites. However, PhyloChip analysis from the Ulleung Basin did not identify ANME despite the capacity for the PhyloChip to detect these microbes as previously reported from similar marine sediments (Briggs*, et al.*, 2011). Instead PhyloChip analysis detected MBGB in the Ulleung Basin sediments. The absence of ANME and the enrichment of other lineages (e.g., MBGB) have been reported in other areas where geochemical evidence indicates AOM (Biddle*, et al.*, 2006; Inagaki*, et al.*, 2006; Sorensen & Teske, 2006; Roussel*, et al.*, 2009; Hamdan*, et al.*, 2011). This has led to the hypothesis that other microbial groups may be involved in methane oxidation and is one possible explanation for these results (Biddle*, et al.*, 2006; Inagaki*, et al.*, 2006; Sorensen & Teske, 2006). Another possible explanation was that sampling missed the specific location where the ANME were most abundant. Harrison *et al.* looked at the bacterial and archaeal communities above, within, and below the SMT in the Santa Barbara Basin (Harrison*, et al.*, 2009). They found that MBGB sequences were more frequently detected in sediment just above and below the SMT, while ANME was detected within and just below the SMT (Harrison*, et al.*, 2009). The nearest samples to

the SMT were 57 cm and 20 cm at site 1-1 and site 10, respectively. Thus, higher resolution sampling may have detected ANME in the Ulleung Basin.

Microbial communities in samples from within the GHOZ were distributed in a way that could be explained according to distance of the samples to a hydrate layer and gas hydrate saturation of that layer. NMS ordinations and cluster analysis of the GHOZ identified two distinct communities and the highest correlation to the axis that separated the two groups (among the abiotic parameters that were available for measurement) was hydrate saturation of the nearest hydrate layer (Figure 4.3). Group A samples typically had lower hydrate saturation and were more distant from the closest hydrate; while, group B samples typically had higher hydrate saturation and were nearer to the hydrate. This relationship is depicted using data from the two Ulleung Basin sites plotted according to distance to the nearest hydrate and hydrate saturation (Figure 4.4). More samples are needed to establish whether a relationship exists between the community, distance, and hydrate saturation. Analysis of samples that occur very close to hydrate saturation values of 5-20% and 60-90% would be especially illuminating. Despite the need for more studies our results suggest that hydrate occurrence or geochemical and physical factors concurrent with hydrate occurrence affect the community composition.

Studies that have used clone libraries to study community diversity in hydrate and non-hydrate sites found that candidate division JS1 and to a lesser extent *Planctomycetes* are dominant in hydrate-containing areas compared to hydrate-free areas (Inagaki*, et al.*, 2006; Nunoura*, et al.*, 2008). Consistent with their findings both bacterial candidate division JS1 and *Planctomycetes* were detected frequently in the sediments from the two

boreholes that we studied in the Ulleung Basin. The frequent observation of candidate division JS1 and *Planctomycetes* in many of the samples that we examined (both near and far from hydrate) precluded them from being candidate indicator taxa. These results infer that candidate division JS1 are not affected by hydrate, but may prefer sedimentary habitats that contain geochemical and geophysical factors conducive for hydrate formation.

While candidate division JS1 and *Planctomycetes* may not be indicative of distinct hydrate-containing layers, *Gammaproteobacteria* related to *Vibrio*-type species may prefer a closer association with hydrate based on the indictor taxa detected within group B. Both cultivation (D'Hondt*, et al.*, 2004) and molecular studies (Inagaki*, et al.*, 2006; Nunoura*, et al.*, 2008) have detected *Vibrio*-type species in hydrate-bearing sediments.

Summary

In this study, T-RFLP and PhyloChip analyses were used to determine microbial community composition in sediments from the SMT, GHOZ, and BSR obtained from the Ulleung Basin, a model system where deep drilling collected samples from all three geochemical zones. Both T-RFLP and PhyloChip analyses identified the same fundamental community structures in the respective sample types. Comparison of these microbial communities using NMS ordination revealed that microbial communities found in the SMT, GHOZ, and BSR were significantly different from each other and the key abiotic variables that were most correlated to the community structure were sulfate,

hydrate saturation of nearest hydrate layer, and depth, the latter likely being representative of factors such as age of the sediments, quality of the organic matter, or sediment compaction. The archaeal anaerobic methanotrophs typically associated with anaerobic oxidation of methane were not detected in sediment near the SMT; however, the MBGB, which is often found in SMT environments, was detected. Indicator taxa of microbial communities in the GHOZ identified *Vibrio*-types species that may prefer sediment layers closer to hydrate-containing sediment. This study provided a robust statistical analysis of microbial communities that allowed the identification of biogeographic patterns in relation to geochemical zones defined by methane.

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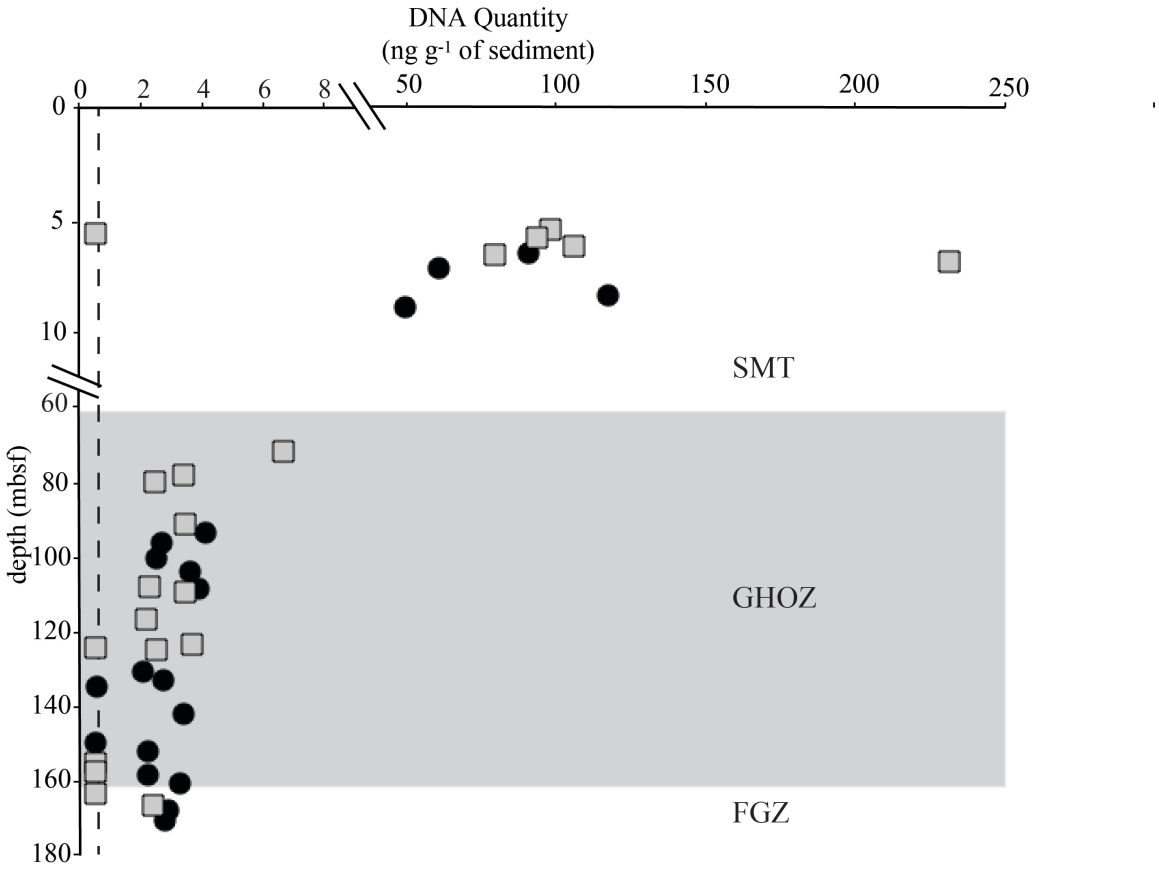

Figure 4.1. DNA quantities extracted from sites 1-1 and 10, black circles and grey squares, respectively. Both axes have been modified to depict the broad range of DNA quantity and depth. The dashed line is the detection limit of 0.5 ng of DNA g^{-1} of sediment and symbols on that line were below the detection limit. mbsf: meters below seafloor, SMT: sulfate methane transition, GHOZ: gas hydrate occurrence zone, FGZ: free gas zone.

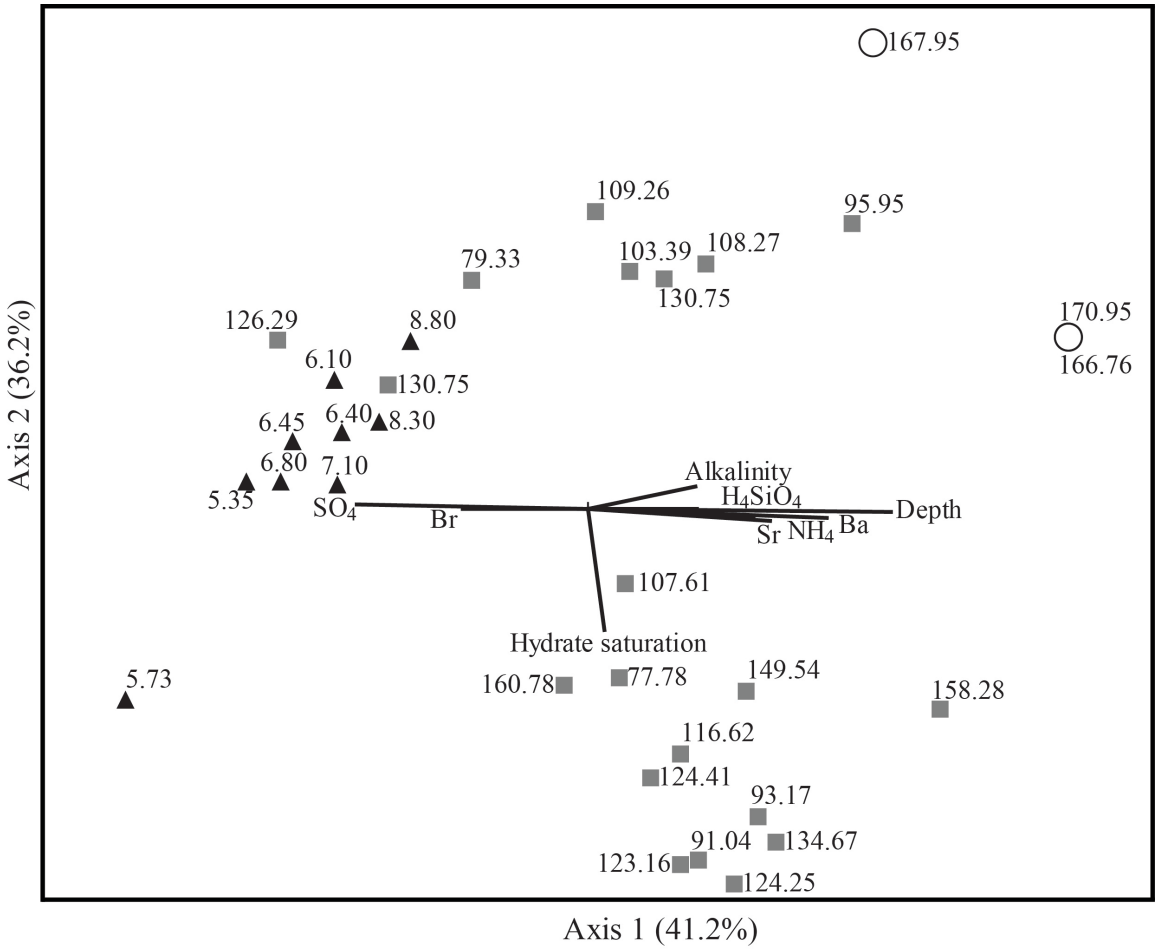

Figure 4.2. Non-metric multidimensional scaling (NMS) of data obtained from PhyloChip community analysis for samples from the SMT, GHOZ, and FGZ represented as black triangles, grey squares, and open circles, respectively. Numbers indicate the depth (mbsf) from which each sample was obtained. The vectors superimposed on the plot represent the direction and magnitude of the correlations of the indicated environmental parameters that were obtained on sediment samples from comparable depths as the DNA for community analysis (SO_4: sulfate, Br: bromide, Sr: strontium, NH_4: ammonium, Ba: barium, H_4SiO_4: silicic acid, hydrate saturation: gas hydrate saturation of the nearest hydrate layer, depth = depth from which the sample was obtained). The variance explained by each axis is given in parentheses.

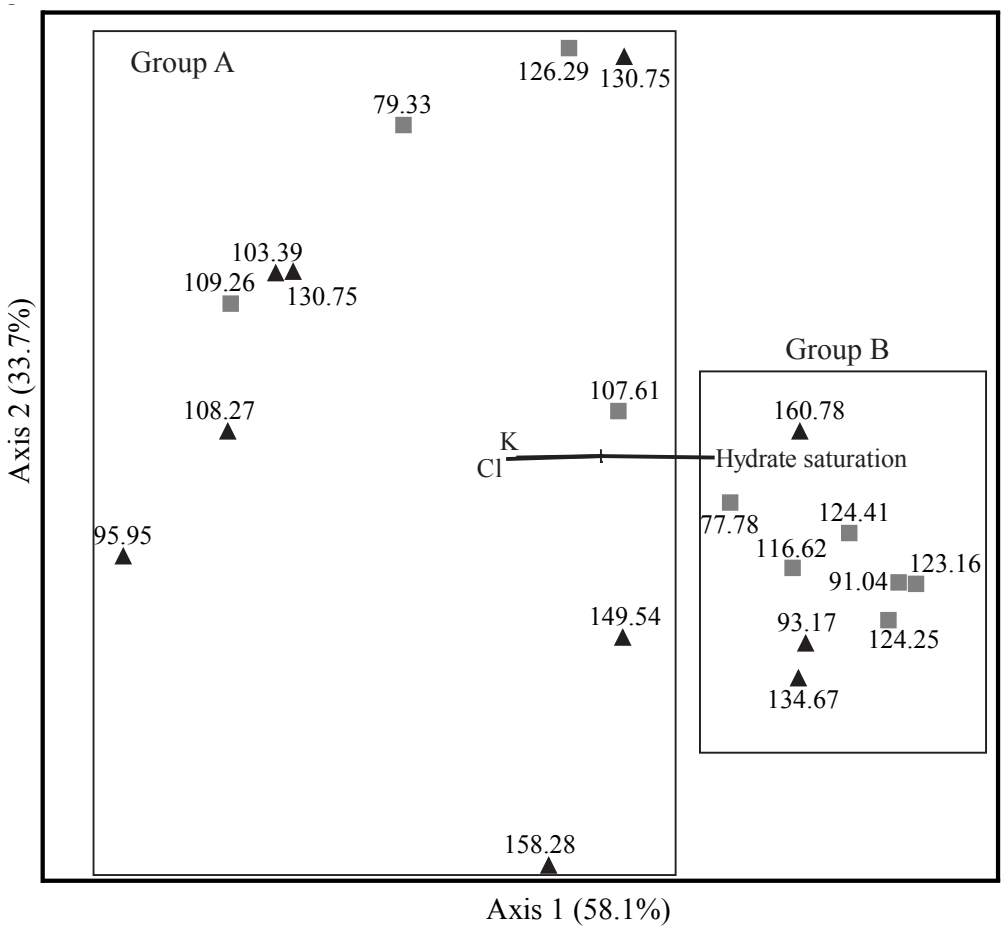

Figure 4.3. NMS of data obtained from PhyloChip community analysis for samples from the GHOZ of site 1-1 and site 10 represented as black triangles and grey squares, respectively. Numbers indicate the depth (mbsf) from which each sample was obtained. The vectors superimposed on the plot represent the direction and magnitude of the indicated environmental parameters (K = potassium; Cl = chloride; hydrate saturation = hydrate saturation of the nearest hydrate layer) that were obtained on sediment samples from comparable depths as the DNA for community analysis. The total variance explained by each axis is given in parentheses. The two groups of similar microbial communities determined by cluster analysis are identified as group A and B.

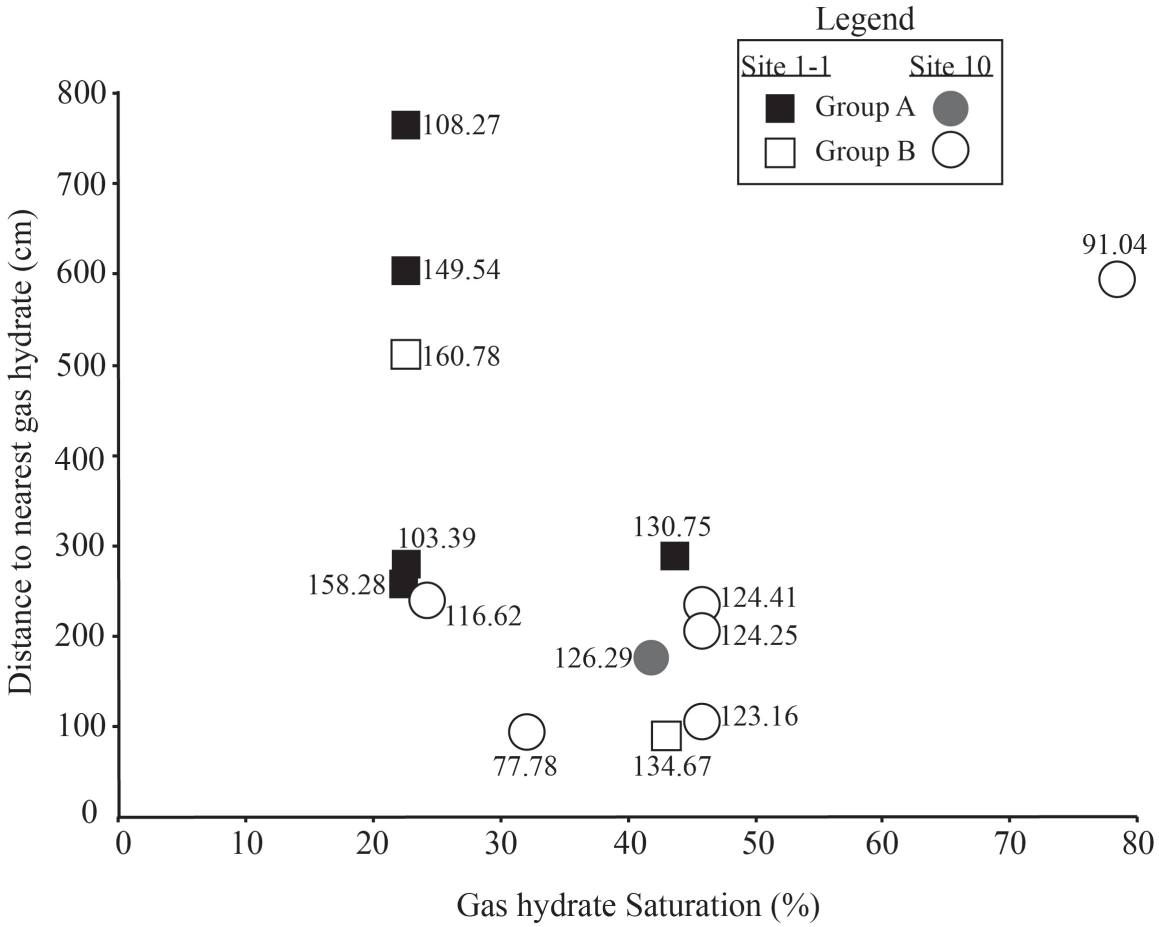

Figure 4.4. A plot of the distance to the nearest hydrate layer versus the hydrate saturation of that layer. Site 1-1 is represented by squares; site 10 is represented by circles. Groups A and B represented as filled symbols and open symbols, respectively, were identified using hierarchal clustering and identified in Figure 4.3. Numbers indicate the depth (mbsf) from which each sample was obtained. Samples without hydrate saturation values were identified to have hydrate by IR imaging; however, chloride values used to calculate hydrate saturation were not available.

5. CONCLUDING REMARKS

This dissertation expands our knowledge of microbial biogeography in sediments that contain methane. The objective of this research was to describe the cell abundance, biogeographic patterns, and the factors that control distributions of microbes in the biogeochemical zones that are defined by methane in marine sediments, namely, sulfate-methane transition (SMT), gas hydrate occurrence zone (GHOZ), and the free gas zone (FGZ). Samples used for this analysis came from northern Cascadia margin (NCM), Indian Ocean, Andaman Sea, and the Ulleung Basin. While many of the samples that I acquired for this dissertation were samples of opportuntiy and are from widely distributed geographic locations, they all came from subseafloor areas containing methane. Not all of the samples were collected by trained microbiologists. As such, contamination issues, while always present, could be a confounding factor when analyzing the results. To limit these issues flourescent microbeads were used to identify drill fluid contamination from both the NGHP01 and UBGH02 expeditions. In addition, spiked and negative controls were used in all DNA extractions and PCR amplifications. And finally, DNA sequences that were suspect because they were highly similar to human associated microbes or seawater clones were excluded from analysis. The overall objective was only possible by pairing microbiological samples with samples collected by geochemists and geologists for separate analysis of abiological parameters. The abiotic data obtained in close proximity to the microbiology data allowed for a fuller understanding of the conditions under which these cells survive.

Chapter 2 reports on a new SMT setting, where the AOM consortium occurs as macroscopic pink to orange biofilms within subseafloor fractures. These biofilm samples

recovered from the Indian and northeast Pacific Oceans had a cellular abundance of 10^7 to 10^8 cells cm^{-3}. Sequencing of 16S rRNA genes indicated the presence of microbes related to previously identified anaerobic oxidation of methane (AOM) consortia (*Deltaproteobacteria* and ANME-1) and stable carbon isotope composition of the biofilm (-35 to -43‰) suggests that the production of biomass is associated with AOM. These biofilms are a novel, but apparently widespread, aggregation of cells represented by the ANME-1 clade that occur in fracture-dominant SMT environments. Their presence may be associated with characteristics typical of fractures in the seafloor and as such may represent an alternative condition where AOM may occur in addition to the well-documented diffusive and seep-dominant environments.

Chapter 3 reports on the microbiology at a unique sedimentary environment in the Andaman Sea that contains biogenic methane, yet low concentrations of organic carbon. The methane at this site exists as gas hydrate between the depths of 250 to 600 meters below seafloor (mbsf) and as such represents the deepest gas hydrate samples yet recovered from the seafloor. Microscopic cell enumeration revealed that sediment layers harbored relatively low concentrations of microbes (below detection limit of 10^2 cells cm^{-3} to 10^5 cells cm^{-3}). Sequences of 16S rRNA genes detected using clone libraries and primer-tagged pyrosequencing were dominated by members of Firmicutes, such as *Bacillus* species. Archaeal genes were never detected despite the use of several DNA extraction methods, multiple displacement amplification, nested PCR, and multiple primers for 16S rRNA and functional genes. This is consistent with the inability to detect lipid biomarkers for methanogenic archaea and the water column origin of detected

archaeal lipids. Statistical analysis of terminal restriction fragment length polymorphism (T-RFLP) performed on the amplified DNA found that the microbial communities from the GHOZ were distinct from those obtained from sediments above or below this zone. Compared to other seafloor settings that contain biogenic methane, this Bacteria-dominated deep subseafloor habitat has unique phylogenetic diversity and low cell abundance. The biogenic methane present at site 17A may have been produced in sediment layers that we did not analyze, produced by methanogens no longer present in the system, or produced in other locations after which it migrated to site 17A.

In Chapter 4, T-RFLP and PhyloChip were used to determine microbial community composition in sediments from the SMT, GHOZ, and the FGZ obtained from the Ulleung Basin, in the East Sea offshore Korea. Comparison of these microbial communities using NMS ordination revealed that microbial communities found in the SMT, GHOZ, and FGZ were significantly different from each other and the key abiotic variables that were most correlated to the community structure were sulfate, gas hydrate saturation of nearest gas hydrate layer, and unmeasured variables associated with depth. The archaeal anaerobic methanotrophs typically attributed to performing AOM were not detected in the SMT; however, representatives of the marine benthic group-B (MBGB), which are often found in SMTs, were detected. Two distinct communities were found within the GHOZ, which could be explained by the distance to the nearest hydrate layer and the hydrate saturation of that layer. Samples that were typically closer to layers that contained higher hydrate saturation had indicator taxa related to *Vibrio*-type species.

These results suggest that the microbial communities in the GHOZ of the Ulleung Basin sediments may be determined by proximity to hydrate-containing sediment layers.

The cell abundance reported in this dissertation do not conform to the assumptions made when Whitman *et al*. calculated total oceanic subsurface cellular abundance (Whitman*, et al.*, 1998). Whitman *et al.* assumed that all marine sedimentary enviornments followed the trend described by Parkes *et al.*, which was based on five locations from highly-productive (high organic carbon burial) continental margins (Parkes*, et al.*, 1994). Studies from the Andaman Sea (described herein) and South Pacific Gyre (SPG) sediments suggest a correlation of cell counts to organic carbon content in that lower organic carbon content supports lower cell abundance. Sites in the SPG with the lowest organic carbon (<0.25%) contains 3-4 orders of magnitude fewer cells cm^{-3} than assumed by Whitman *et al.* (D'Hondt*, et al.*, 2009). These recent studies suggest that estimates of the total oceanic subsurface cell abundance will decrease as much as 3-4 orders of magnitude when low organic carbon regions are considered. However, further research that estimates cell abundance from transects across sites that contain various levels of organic carbon are needed to verify the relationship of cell abundance to organic carbon.

The macroscopic biofilms found in fracture-dominant SMT environments contain an estimated 1-2 orders of magnitude more cells cm^{-3} than assumed by Whitman *et al*. (1998). Further refinement of cell abundance in the subsurface will have to include the extrapolation of the abundance of fracture-dominant environments. Several studies have considered microbial communites from fractured rock in terrestrial enviroments such as

the South African Gold mines (Wagner*, et al.*, 2006), limestone formations (Castegnier*, et al.*, 2006), and simulated basalt aquifers (Lehman*, et al.*, 2001); however, marine sediment fractures have not been extensively examined partially because of the difficulty in differentiating natural fractures from drilling induced fractures. Briggs *et al*. (2011) inferred the presence of natural fractures because macroscopic biomass were discovered inhabiting the fractures when the cores were opened. The extent of such fractures is potentally widespread in gas-rich regions because sediment fracturing is a proposed mechanism for methane venting through hydrate stability zones (Riedel*, et al.*, 2006) and methane vents occur worldwide (Judd, 2004). However, quantitative data on the presence of fracture-dominant SMT environments is needed before these cell abundances can be extrapolated. Perhaps the use of three-dimensional seismic tomography (Plaza-Faverola*, et al.*, 2010) can help to target locations where fractures are abundant thereby allowing a more systematic investigation of cell abundances in these settings.

A major objective of many descriptive studies on microbial distributions is to identify the biogeographic patterns of microbes and abiotic parameters that may be related to the patterns. The studies reported here have used a higher sampling resolution in methane-bearing sediments than previously reported and describe the effect that geochemical zones defined by methane has on the microbial communities. SMT environments are found globally in marine sediments and characteristically contain both sulfate and methane. The AOM consortium is thought to be dependent on both sulfate and methane, which would narrow their distribution to the SMT, where both methane and sulfate are available. Biogeographic studies of ANME have found that ANME-1

typically prefer environments higher in methane and lower in sulfate concentrations whereas ANME-2 prefer environments low in methane and high in sulfate (Girguis*, et al.*, 2005). Diffusive-dominant SMT environments typically contain low methane and high sulfate and are composed of ANME-2. The fracture-dominant sediment described in Chapter 2 is composed of ANME-1 suggesting high methane and low sulfate concentrations occur despite the geochemical evidence that suggests a diffusive-dominant environment (e.g., deep SMT) (Briggs*, et al.*, 2011). Fractures in the sediment are potentially paths for fluid movement thereby, allowing the advection of methane through the fractures. Future research using a pore-fluid array sampler (Lapham*, et al.*, 2008) could be used to determine if methane advection is actually occurring through the fractures in sediments where the biofilm is found and this would assist the interpretation of microbial biogeography and distribution in these important sediments.

AOM rates are affected by the flux of methane. In diffusion-dominant environments the AOM rate can be 0.001-1 nmol cm^{-3} day^{-1}, while seep-dominant environments have AOM rates of 100-10,000 nmol cm^{-3} day^{-1} (Knittel & Boetius, 2009 and references therein). If methane is advected through the fractures then AOM rates would be expected to be higher than typically seen in diffusion-dominant environments. Seafloor sediments containing biofilm can be incubated with radioactive tracers, i.e., $^{14}CH_4$ and $^{35}SO_4^{2-}$ (Treude*, et al.*, 2003), to verify that AOM rates are higher in the biofilm.

Statistical analysis of sediment microbial communities from both the Andaman Sea and Ulleung Basin indicated that the GHOZ contains distinctive microbial

communities. The Ulleung Basin sediment contains microbial taxa previously reported in gas hydrate-bearing sediments (Inagaki*, et al.*, 2006; Nunoura*, et al.*, 2008). The higher-resolution sampling done in the Ulleung Basin allowed for the first reporting that *Vibrio*-type species may be enriched in sediment layers that occur in close proximity to gas hydrate. In addition, and consistent with other reports, representatives of the candidate division JS1 were often found in gas hydrate containing areas even though the results of this research do not indicate that the presence of this group was influenced by specific gas hydrate-containing sediment layers. Additional studies should consider the microbial communities in distinct hydrate layers that vary in hydrate saturation.

The Andaman Sea sediments contained a unique microbial community that was not consistent with other gas hydrate-containing areas. Firmicutes have been found in several gas hydrate-containing areas, but are rarely a dominant taxa (Newberry*, et al.*, 2004; Inagaki*, et al.*, 2006; Nunoura*, et al.*, 2008). There were few environmental clues that would explain the dominance of Firmicutes; however, one possible explanation is that these Firmicutes have formed spores, a trait of *Bacillus* species, in these austere sediments. The oldest samples collected in the Andaman Sea were 12.5 Ma and *Bacillus* species have been reported to survive in 250 Ma salt crystals as spores (Vreeland*, et al.*, 2000). On the basis of age only, it seems possible that these seafloor microbes could survive in the marine sediments of the Andaman Sea if they are present as spores.

In addition, archaea were not detected in the Andaman Sea sediments despite the presence of biogenic methane. One hypothesis based on seismic images was that the methane was produced elsewhere and migrated to site 17A. Additional cores should be

drilled near site 17A at the likely site of origin for fluids that would migrate into the sediments bearing methane to verify if methanogens are present or if methanogenesis is occurring in these sediments. This hypothesis has potential applications for using gas hydrate deposits as an energy resource and could be useful for determining the optimal places to collect methane.

Subsurface marine sediments were once thought to be biologically devoid regions of Earth because of the austere conditions. Advances in technology in the last two-decades have provided an opportunity to probe these sediments and despite the austere conditions a deep biosphere exists. We have started to ask fundamental questions about the types and distributions of microbial taxa in these locations and have found that marine sediments contain a diverse assemblage of bacterial and archaeal taxa and their processes may be intimately tied to global biogeochemical cycles. However, questions remain about the viability and activity of the microbes. Technological advances for in-situ sampling such as circulation obviation retrofit kits (CORKs) may provide such answers by allowing repeated sampling and time course experiments. Furthermore, the limit of life in the deep biosphere has not been reached. The ultimate controlling factor on the limit of life may be temperature, available pore space, or electron donors and acceptors. Regardless of the controlling factors, the mysteries of the deep biosphere will only unfold with future drilling expeditions.

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6. APPENDICES

Appendix A: Chapter 2 supplemental figures.

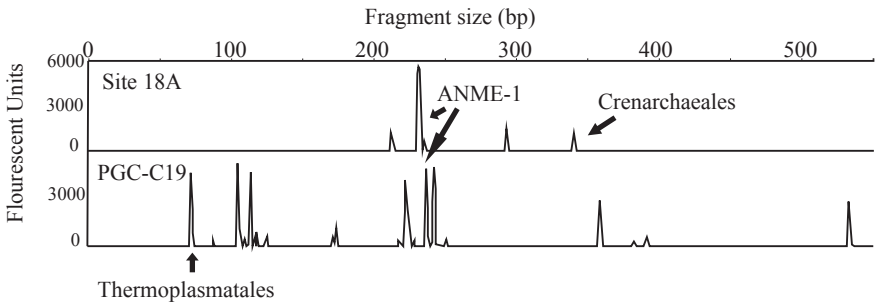

Figure A.1. Representative electropherograms obtained from archaeal terminal restriction fragment length polymorphism analysis from the biofilms at Site 18A (India) and PGC0807 (NCM). In silico digestion was performed on clone sequences and compared to peak sizes to determine the probable identity of archaeal peaks.

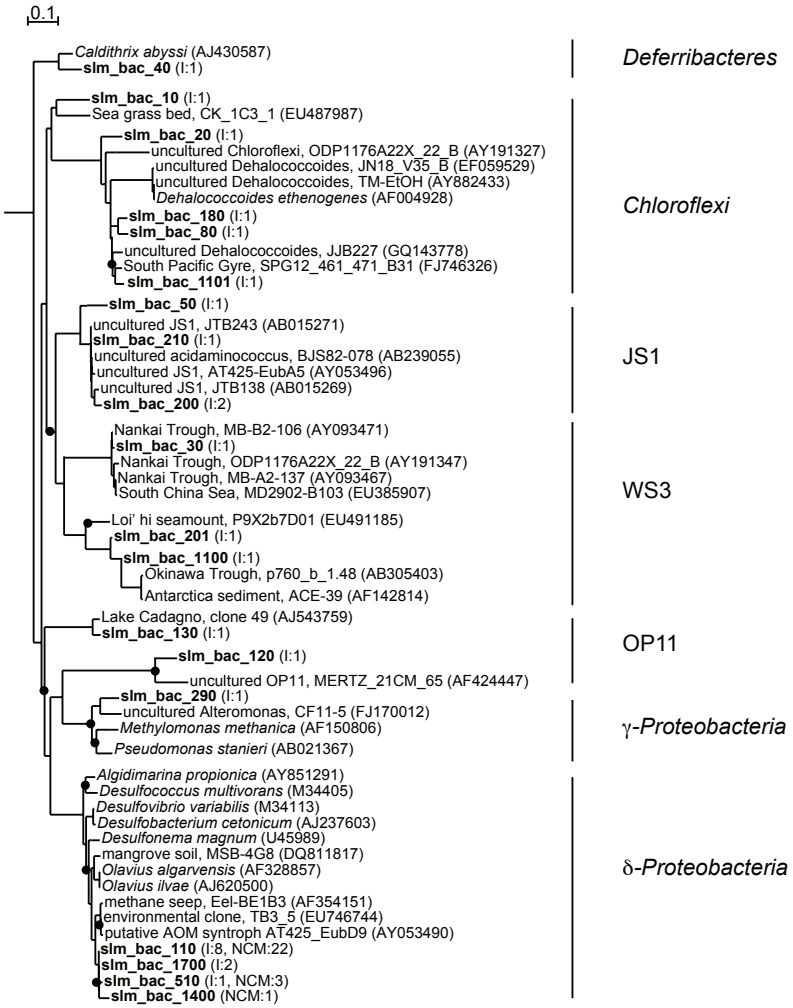

Figure A.2. Neighbor-joining distance tree (created in FastTree (Price*, et al.*, 2009)) showing representative bacterial phylotypes derived from DNA extracted from subseafloor biofilms and then amplified (using 16S rRNA gene primers) and cloned. Representative biofilm phylotypes from this study are indicated in bold type. The solid circles at nodes indicate bootstrap values <40%. The designations in parentheses after the biofilm phylotypes indicate the number of clones obtained for a particular phylotype from either sediments from offshore India (I) or the northern Cascadia Margin (NCM).

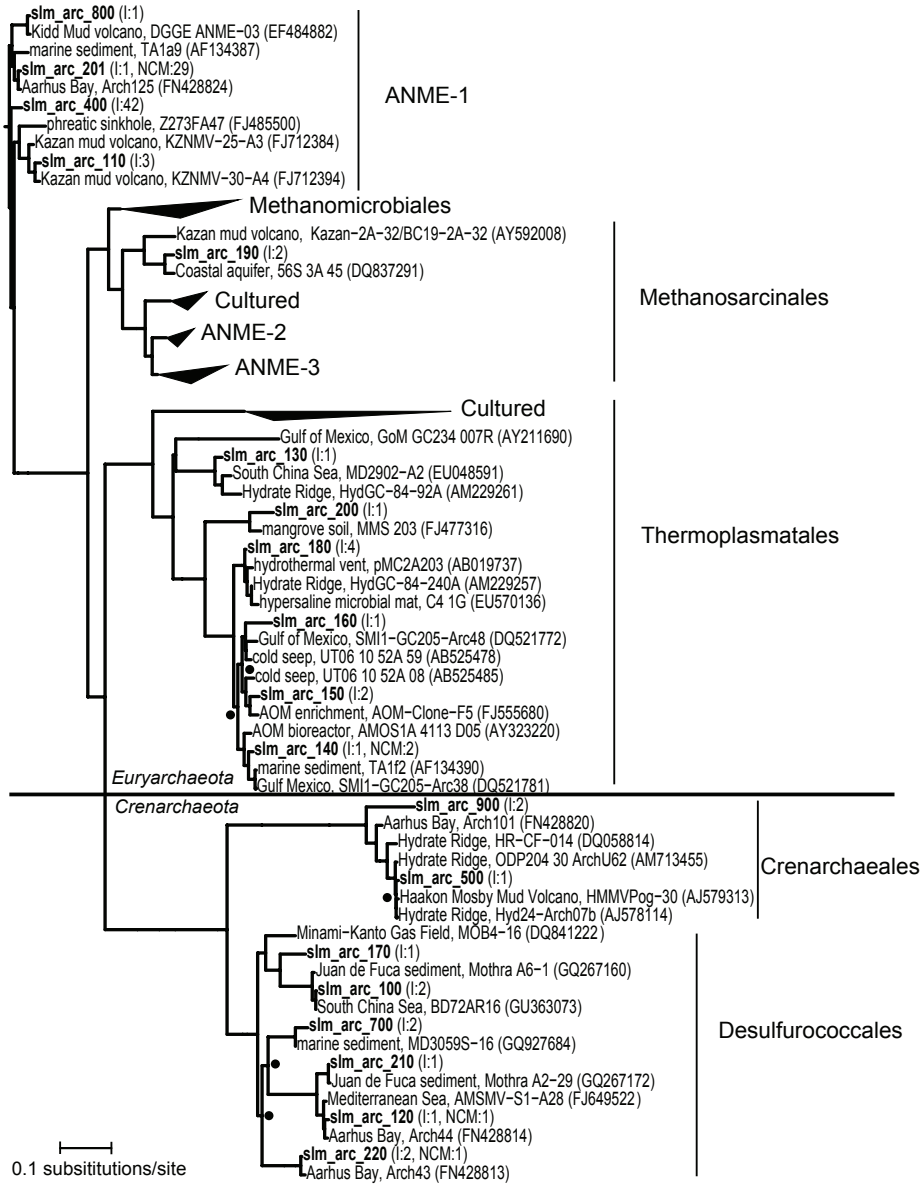

Figure A.3. Neighbor-joining distance tree (created in FastTree (Price, et al., 2009)) showing representative archaeal phylotypes derived from DNA extracted from subseafloor biofilms and then amplified (using 16S rRNA gene primers) and cloned. Representative biofilm phylotypes from this study are indicated in bold type. The solid circles at nodes indicate bootstrap values <40%. The designations in parentheses after the biofilm phylotypes indicate the number of clones obtained for a particular phylotype from either sediments from offshore India (I) or the northern Cascadia Margin (NCM).

Appendix B. Chapter 3 supplemental materials

Materials and methods

Functional gene amplification. Amplification of functional genes involved in cycling single carbon compounds was attempted for 13 samples within the GHOZ. The functional genes targeted were acetyl-CoA carboxylase (*accC*), citrate lyase (*aclB*), pyruvate oxidoreductase (*porA*), oxoglutarate oxidoreductase (*oorA*), 1,5-bisphosphate carboxylase (RubisCO: *cbbL*), particulate methane monooxygenase (*pmoA*), methanol dehydrogenase (*mxaF*), and methyl co-enzyme M reductase (*mcrA*). PCR conditions consisted of an initial denaturation step of 5 min at 95º C followed by 30 cycles of 40 s at 95º C, 40 s at optimal annealing temperature (Table B.1), 60 s at 72º C, and a final elongation step of 5 min at 72º C.

Clone library construction and phylogenetic analysis. Bacterial 16S rRNA gene clone libraries were constructed using bacterial specific forward 27F and universal reverse 1492R primers (Table B.1) from sample depths 435.3, 554.3, 588.5, and 588.7 mbsf. PCR amplification of the 16S rRNA gene used the same protocol as stated above. In addition, a clone library of the *accC* functional gene from sample depths 372.7, 408.6, and 496.7 mbsf was created. PCR products of either 16S rRNA gene or *accC* functional gene were cloned using pCR® 2.1 TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones from each library were sequenced at the CGRB using an ABI Genetic analyzer 3100.

Sequence analysis was performed using Geneious (Drummond*, et al.*, 2009) and the Greengenes NAST server (DeSantis*, et al.*, 2006). Bacterial 16S rRNA gene sequences with >97% similarities were assigned to the same phylotype. Representative phylotypes were then sequenced in the reverse direction. Greengenes was used to align and classify the full 1400 bp 16S rRNA genes. In addition, phylotypes were digested in-silico with *mspI* and the end fragment sizes were compared to terminal restriction fragments (TRFs) obtained from T-RFLP analysis. The functional gene *accC* DNA sequences were translated in Geneious. The 165 amino acid sequence was aligned using ClustalW.

Nucleotide accession numbers. The nucleotide sequences of the 16S rRNA and *accC* gene clones were deposited in the GenBank database under the following accession numbers: JN580273-JN580275 and JN603236-JN603237, respectively.

Results

Clone libraries and phylogenetic analysis. Clone libraries of 16S rRNA genes and functional genes indicated that members of Firmicutes dominate sediment layers within the GHOZ. A total of 174 bacterial 16S rRNA gene clones were sequenced. With a similarity value of 97% only 3 operational taxonomic units (OTUs) were identified. Each clone library contained all three OTUs. Firmicutes related to *Bacillus simplex* (EU427329, 99.7% similarity) and *Anaerococcus octavius* (Y07841, 95.2% smililarity) comprised 88% of the total clones and Bacteroidetes related to *Sediminibacterium* sp. (AB470450, 96.8% similarity) made up the remaining 12% of clones.

Within the GHOZ, we attempted to detect functional genes that are used in the cycling of single carbon compounds. The *accC* functional gene was detected in 9 of the 13 samples tested in the GHOZ. However, the other functional genes, *mcrA*, *aclB, oorA*, *cbbL, pmoA*, *mxaF,* and *porA,* were not detected in any samples. A total of 81 *accC* genes were sequenced, translated, and aligned. Two unidentified *accC* homologous genes were detected.

Table B.1. List of primers and optimal annealing temperatures used to detect and amplify functional genes in DNA extracted from sediments in the Andaman Sea.

Primer	Oligonucleotide sequence (5'-->3')	Anneal temp.	Reference
Methyl Coenzyme M reductase (*mcrA*)			
me2R	TCATBGCRTAGTTDGGRTAGT		(Nunoura, *et al.*, 2008)
me3mF	ATGTCNGGTGGHGTMGGSTTYAC	52°C	(Nunoura, *et al.*, 2008)
AOMF	GCTGTGTAGCAGGAGAGTCA		(Inagaki, *et al.*, 2004)
AOMR	GATTATCAGGTCACGCTCAC		(Inagaki, *et al.*, 2004)
Acetyl CoA carboxylase (*accC*)			
acacF	GCTGATGCTATACATCCWGGWTAYG	49°C	(Auguet, *et al.*, 2008)
acacR	GCTGGAGATGGAGCYTCYTCWATTA		(Auguet, *et al.*, 2008)
Citrate lyase (*aclB*)			
aclF	TAGAGGATGCRGCTAAWTGGATTGAT	54°C	(Takai, *et al.*, 2005)
aclR	GTTGGGGCCRCCWCKKCKNAC		(Takai, *et al.*, 2005)
Pyruvate oxidoreductase (*porA*)			
porF	GCTGCAGARGAAGANTGGCATT	49°C	(Takai, *et al.*, 2005)
porR	TTTTCRAANCCRATRTGSATCCAAG		(Takai, *et al.*, 2005)
Oxoglutarate oxidoreductase (*oorA*)			
oorF	TTCTTCGCTGGGTAYCCNATHACNCC	52°C	(Takai, *et al.*, 2005)
oorR	CATACCAGCTATYTCRTCYTCCATYTG		(Takai, *et al.*, 2005)
RubisCo (*cbbL*)			
cbbLF	GACCAGTCGGYAAYGTNTTYGGNTTY	47°C	(Takai, *et al.*, 2005)
cbbLR	CTGACCAGTCTNAYRTTYTCRTCRTCY		(Takai, *et al.*, 2005)
Particulate methane monoxygenase (*pmoA*)			
A189F	GGNGACTGGGACTTCTGG	47°C	(Inagaki, *et al.*, 2004)
A682R	GAASGCNGAGAAGAASGC		(Inagaki, *et al.*, 2004)
Methanol dehydrogenase (*mxaF*)			
mxaF	GCGGCACCAACTGGGGCTGGT	52°C	(Inagaki, *et al.*, 2004)
mxaR	GGGCAGCATGAAGGGCTCCC		(Inagaki, *et al.*, 2004)

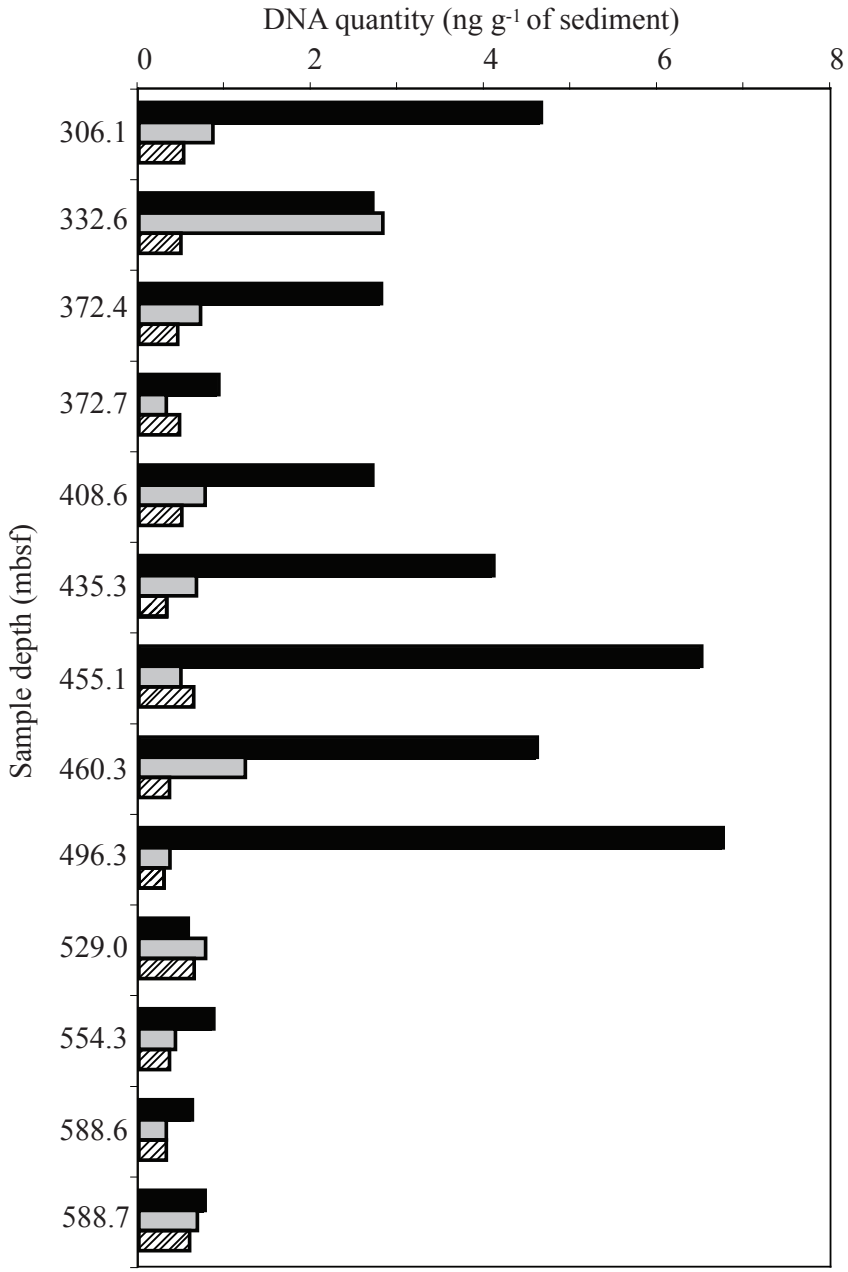

Figure B.1. The amount of DNA extracted for 13 samples within the GHOZ using the three methods of DNA extraction. The solid black, grey, and striped bars represent the three different extraction methods attempted, MoBio, MoBio FM, MoBio FM PK, respectively.

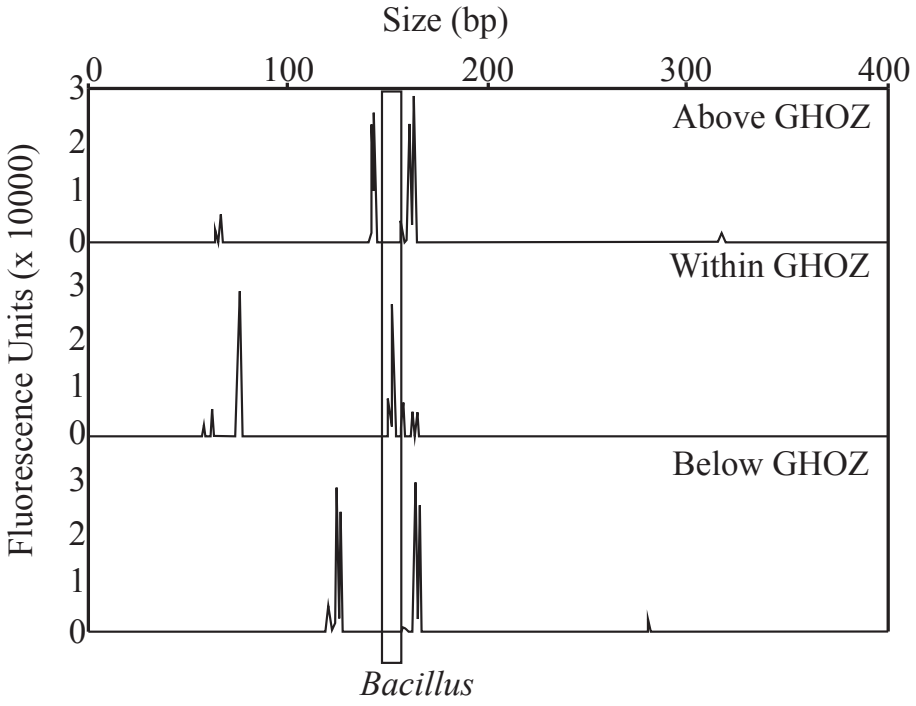

Figure B.2. Three representative T-RFLP electropherograms digested with *mspI* from above, within, and below the GHOZ are depicted. The in silico digestion of clone sequences identified the TRF at 154 bp to be related to *Bacillus*.

Appendix C. List of sediment samples used for molecular analysis

See Table 2.1 for biofilm samples described in Chapter 2.

Table C.1. The core, section, and depth in meters below seafloor (mbsf) obtained from the Andaman Sea described in Chapter 3 (Collet, *et al*. 2008).

NGHP01 Site 17A

Core	Section	Depth (mbsf)	Core	Section	Depth (mbsf)
3H	5	20.95	52X	1	435.3
5H	5	39.95	52X	6	440.87
8H	5	68.4	53X	4	447.48
11H	5	96.9	54X	2	455.08
14X	4	123.85	54X	3	456.04
18X	5	162.66	54X	6	460.26
19X	5	171.7	58X	4	496.73
21X	2	178.8	63X	7	529.04
22X	5	191.69	66X	4	554.33
25X	1	214.3	72X	1	588.58
26X	3	226.76	72X	1	588.73
29X	3	254.84	72X	3	589.86
30X	3	264.4	73X	3	600.7
34X	5	306.05	74X	2	609
36X	4	322.57	75X	2	618.57
39X	2	332.6	76X	2	626.25
43X	6	372.38	77X	3	637.08
43X	6	372.68	78X	3	647.51
47X	3	408.6	79X	4	659.1
47X	5	410.63	80X	2	663.76
50X	4	419	82X	4	676.97
51X	5	430.72	83X	2	684.12

Table C.2. Samples obtained from the Ulleung Basin described in Chapter 4 (Shipboard Scientific Party, 2011).

UBGH2 Site01				UBGH2 Site10			
Hole	**Core**	**Section**	**Depth (mbsf)**	**Hole**	**Core**	**Section**	**Depth (mbsf)**
B	1H	5g	6.4	B	1H	4d	5.35
B	1H	6a	7.1	B	1H	4f	5.73
D	1H	5b	8.3	C	1H	4c	5.75
D	1H	5e	8.8	B	1H	5a	6.10
D	12H	2	93.165	B	1H	5c	6.45
D	12H	4	95.945	B	1H	5e	6.80
D	13H	2	100.16	C	5H	5b	71.305
D	13H	5	103.39	C	7H	3	77.78
D	14H	2	108.27	C	7H	4	79.325
D	21C	1b	130.75	C	11C	1c	91.04
D	23H	2	132.815	C	14H	3	107.61
D	23H	3	134.67	C	14H	5	109.26
D	24H	3	141.85	C	15H	3	116.62
D	25H	3	149.54	D	1H	1c	123.16
D	25H	4	151.955	D	1H	2	124.25
D	26H	3	158.28	C	16H	3a	124.405
D	26H	4	160.78	C	16H	4b	126.285
D	27H	2b	167.95	D	9H	3	155.96
D	27H	4b	170.95	D	9H	5	158.305
				D	10H	2	162.35
				D	10H	5b	166.76

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