MICROBIAL DISTRIBUTIONS DETECTED BY AN OLIGONUCLEOTIDE MICROARRAY ACROSS GEOCHEMICAL ZONES ASSOCIATED WITH METHANE IN MARINE SEDIMENTS FROM THE ULLEUNG BASIN

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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 (SMTZ), gas hydrate stability zone (GHSZ), and free gas zone (FGZ). These geochemical zones may harbor distinct microbial communities that are important in biogeochemical carbon cycles. The objective of this study was to describe the microbial communities in sediments from the SMTZ, GHSZ, and FGZ using molecular ecology methods (i.e., PhyloChip microarray analysis and terminal restriction fragment length polymorphism (T-RFLP)) 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 in the three geochemical zones 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 variables such as sulfate concentration, hydrate saturation of the nearest gas hydrate layer, and depth (or unmeasured variables associated with depth e.g. temperature, pressure) were correlated to differences between the three zones. The archaeal anaerobic methanotrophs typically attributed to performing anaerobic oxidation of methane were not detected in the SMTZ; however, the marine benthic group-B, which is often found in SMTZ, was detected. Within the GHSZ, 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 distributions of microbial taxa are dependent on the presence of 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 define 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 microbial distributions at depth.

Methane-bearing marine sediments can be simplistically 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 zone (SMTZ) is the uppermost geochemical zone in sediment, where seawater sulfate and underlying methane converge and approach minimum concentration (Borowski et al., 1999). Below the SMTZ, gas hydrate will form where the temperature, pressure, and methane concentration are favorable. The gas hydrate stability zone (GHSZ) 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, which is often 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) (when the methane concentration exceeds its solubility) .

The microbial communities at the SMTZ are a globally important consortium of bacteria and archaea that perform anaerobic oxidation of methane (AOM) (Knittel and 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; Knittel and Boetius, 2009; Orphan et al., 2002). The presence of this consortium has been confirmed in seep-dominant (de Beer et al., 2006; Elvert et al., 2005; Knittel et al., 2005; Losekann et al., 2007; Michaelis et al., 2002), fracture-dominant (Briggs et al., 2011), and diffusion-dominant environments (Harrison et al., 2009; Parkes et al., 2007; Thomsen et al., 2001). However, their detection in some SMTZ environments has been inconsistent and has led to the hypothesis that other microbial groups, such as Marine Benthic Group-B (MBGB), may be involved in methane oxidation (Biddle et al., 2006; Hamdan et al., 2011; Inagaki et al., 2006; Roussel et al., 2009; Sørensen and Teske, 2006).

Microbiological investigations of the GHSZ have taken place at Blake Ridge (Wellsbury et al., 2000), Nankai Trough (Reed et al., 2002), Cascadia Margin (Bidle et al., 1999; Colwell et al., 2008; Marchesi et al., 2001; Nunoura et al., 2008), the Peru Margin (Biddle et al., 2008; Fry et al., 2006; Sørensen and Teske, 2006) and the Andaman Sea (Briggs et al., 2012). 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 FGZ indicate a diverse community of bacteria and archaea (Nunoura et al., 2008; Reed et al., 2002; Wellsbury et al., 1997; 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 microbial distributions 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 and to correlate the microbial composition to abiotic features of the sediment. The Ulleung Basin is a back-arc basin in the East Sea where previous expeditions identified the presence of the SMTZ, GHSZ, and FGZ in the sediments (Bahk et al., 2009; Kim et al., 2007; Ryu et al., 2009). The second Gas Hydrate Drilling Expedition in the Ulleung Basin (UBGH2) collected samples for microbiology analysis in the SMTZ, GHSZ, and FGZ providing a unique opportunity for microbiological characterization in all three zones (Ryu et al., 2012). This characterization was accomplished 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 the UBGH2. Site UBGH2-1-1 was located in the western margin of the Ulleung Basin at $36^0$15' 04.78" N 130^0 3' 56.55" E (Figure 1). The depth of the SMTZ, determined by sulfate and methane profiles, at Site UBGH2-1-1 occurs between 6 and 8 meters below the seafloor (mbsf) and the FGZ, determined by temperature-pressure-salinity plot, occurs below 165 mbsf with the GHSZ between the SMTZ and FGZ (Ryu et al., 2012). Site UBGH2-10 was located at 36^0 55' 35.14" N 130^0 54' 00.26" E (Figure 1). The depth of the SMTZ at Site UBGH2-10 occurs between 7 and 8 mbsf and the FGZ is below 160 mbsf (Ryu et al., 2012). A total of 40 sediment samples were collected from the two sites with 10, 24, and 6 samples obtained from the SMTZ, GHSZ, and FGZ, respectively.

Core samples were retrieved using a Fugro Hydraulic Piston Corer. The Integrated Ocean Drilling program (IODP) guidelines for obtaining high quality microbiology cores were adhered to during sample recovery and processing (UBGH2 Scientists, 2010). Aboard ship, whole round cores were subcored by paring the outer two cm of sediment with an alcohol and flame sterilized chisel. The subcores were placed into sterile Whirl Pak Bags® and stored at -80°C and then express shipped at -80°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 (Briggs et al., 2011; Lipp et al., 2008). An attempt to increase DNA extraction efficiency was performed by re-extracting two samples from within the GHSZ following the Mo Bio PowerMax Soil kit protocol of heating the samples to 55°C for 10 min prior to

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.

PhyloChip. PhyloChip is a microarray-based technique that contains oligonucleotide probes for most known bacterial and archaeal 16S rRNA genes. The PhyloChip provides high-resolution detection of closely related taxa. Amplification of DNA for the PhyloChip was performed as previously described (Briggs et al., 2011). Briefly, each DNA suspension was amplified using the universal bacterial 16S rRNA gene primers 27F and 1492R or universal archaeal 16S rRNA gene 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°C followed by 30 cycles of 40 s of denaturation at 95° C, 40 s of annealing 48-58° C, 60 s at 72° C, and a final elongation step of 5 min at 72° 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 UBGH2-1-1 and UBGH2-10, except that 100 ng of internal spike DNA (control to determine background

fluorescence), 150 ng of bacterial 16S rRNA gene amplicons, and 50 ng of archaeal 16S rRNA gene amplicons were hybridized to the array. Seven samples out of the original 40 did not contain enough DNA for PhyloChip analysis.

Statistical analysis. All statistical analysis was performed using either R (R Development Core Team, 2012) or PC-ORD v. 5.0 statistical software (McCune and Mefford, 2006). Multivariate statistical analysis of PhyloChip detected taxa was conducted using R. To reduce variance, taxa that only appeared in one sample and outliers greater than two standard deviations from the mean were discarded (McCune and Mefford, 2006). Non-metric multidimensional scaling (MDS) ordination with Sørensen distance measures was used to identify relationships in community composition between samples based on detected taxa (Bray and Curtis, 1957; Kruskal, 1964). MDS is a visual tool that can depict distinct microbial communities based on samples that contain similar microbes. Such samples will cluster together in the MDS ordination (Colwell et al., 2011; Culman et al., 2008; Osborne et al., 2006; Rees et al., 2004). In addition, MDS 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. Phylotypes were ordinated with the parameters that were set as previously described (Colwell et al., 2011).

Bi-plots of the abiotic 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 (i.e. distance to the nearest sediment layer observed to contain hydrate), and hydrate saturation of closest hydrate layer (based on discrete low chloride values (Kim et al., in

review)) were superimposed on the MDS plots to identify correlations to the community structure (UBGH2 Scientists, 2010). The directions and magnitudes of the vectors indicate their correlations with sample ordinations. Only vectors that had a p-value <0.05 were considered.

Hierarchical cluster analysis was performed on all samples to identify similar groups. Sørensen distance measure with complete linkage method was used with the 'vegan' package in the R statistical software (Oksanen et al., 2011). The clustering was visualized on the MDS plot using the 'ordicluster' function.

Multi-response permutation procedure (MRPP) using a Sørensen 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 SMTZ, GHSZ, or FGZ 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 found predominantly in a single geochemical zone (Dufrene and 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.

Terminal restriction fragment length polymorphism. As an alternative method to identify the microbial community structure terminal restriction fragment length polymorphism (T-

RFLP) was performed. 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 may be represented by individual TRFs (Dunbar et al., 2001). 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^{o}C then heat-denatured for 20 minutes at 80^{o}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 electropherogram 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) using TAP-TRFLP as previously reported (Widmer et al., 2006).

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). Dissimilarity matrices were created using Sørensen distance measure for the presence and absence data from both the T-RFLP and PhyloChip using the PC-ORD v. 5.0 software (McCune and Mefford, 2006). 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.

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 2). The SMTZ contained the highest amount of extractable DNA ranging from 49-231 ng g^{-1} of sediment. Both the GHSZ and FGZ contained similar DNA quantities ranging from below detection limits to 6.2 ng g^{-1} of sediment. The additional extraction of two samples using the heating protocol did not

yield DNA above the detection limit (0.5 ng DNA g^{-1} of sediment), and the final DNA solution contained a light brown coloration.

PhyloChip analysis of microbial communities. A total of 1033 taxa were detected from 31 sediment samples that contained enough DNA for PhyloChip analysis (150 ng of bacterial 16S rRNA gene amplicons). Only the samples from the SMTZ contained enough archaeal 16S rRNA gene amplicons (50 ng) for PhyloChip analysis. The SMTZ contained 383 bacterial taxa and 33 archaeal taxa. The GHSZ contained 728 bacterial taxa and the FGZ contained 13 bacterial taxa.

Using data generated by PhyloChip hybridization arrays, MDS ordination of all samples were plotted on two axes with a final stress value of 16.82 and final instability <0.001 (Figure 3). Axis 1 explained 41.2% of the variance and axis 2 explained 36.2% for a total of 77.4% of the variance explained. Abiotic variables that significantly (p-value <0.05) correlated to the community structure were depth (r^2=0.68), pH (r^2=0.28), alkalinity (r^2=0.30), sulfate (r^2=0.38), bromide (r^2=0.30), ammonia (r^2=0.47), hydrogen sulfide (r^2=0.46), silicate (r^2=0.57), strontium (r^2=0.40), barium (r^2=0.47), iron (r^2= 0.34), and hydrate saturation of the closest hydrate layer (r^2=0.21).

MRPP analysis indicated that the different sediment groupings (SMTZ, GHSZ, 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). However, cluster analysis identified some overlap in the community structure. For example, two samples (126.29 and 130.75 mbsf) identified to be in the GHSZ clustered with the SMTZ group and one sample (95.95 mbsf) from the GHSZ clustered with the FGZ. Two separate groups were identified for the GHSZ samples (Group A and Group

B) (Figure 3). These two groups were mainly separated along the vector for hydrate saturation of the nearest hydrate layer indicating Group B samples were closer to layers that contained higher hydrate saturation. The separate GHSZ groups could not be explained by differences in site location as shown by MRPP analysis ($A=0.22$, $p>0.05$).

Indicator taxa analysis was run to identify what taxa, if any, were predominantly found in one zone. No significant indicator taxa were identified from samples in the FGZ. This method, however, found 74 taxa with a p-value of less than 0.05 in the SMTZ. The taxa associated with the Ulleung Basin SMTZ were related to *Deltaproteobacteria* already noted to occur in subseafloor hydrate and methane seep environments (Inagaki et al., 2006; Niemann et al., 2005), 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 SMTZ 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 from all GHSZ samples were less diverse with 65 taxa related to *Vibrio*-type taxa in the *Gammaproteobacteria* phylum. These *Vibrio*-type sequences have been found in marine environments such as hydrothermal vents (Brazelton et al., 2006) and sediment containing methane (Muyzer et al., 2008). Archaeal DNA was not detected within the GHSZ. An additional indicator taxa analysis was performed between

Group A and Group B samples from the GHSZ. All significant indicator taxa came from Group B and were related to the *Vibrio*-type *Gammaproteobacteria* described above.

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 SMTZ. Fifty-five bacterial TRFs were detected in the SMTZ. The most diverse zone was the GHSZ with 96 bacterial TRFs. The FGZ contained 46 bacterial TRFs. None of the TRFs that were detected could be attributed to known taxa using TAP-TRFLP available on the Ribosomal Database Project website.

T-RFLP and PhyloChip comparison. 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 matrices were significantly similar ($p = 0.001$), indicating that the community structures identified by both analyses were similar.

Discussion

Diverse microbial communities populate marine sediments where gas hydrates are present and the factors that control the distribution of microbes in these sediments are becoming more apparent (Briggs et al., 2012; Inagaki et al., 2006; Nunoura et al., 2008; Reed et al., 2002). Our report from the Ulleung Basin statistically analyzes the microbial communities in three biogeochemical zones defined by methane (SMTZ, GHSZ, and FGZ) that were detected using the PhyloChip version G3. The use of the PhyloChip to detect microbial taxa has been well documented in a variety of environments such as contaminated sites (Brodie et al., 2006), air (Brodie et al., 2007), soil (Kuramae et al., 2012), human stomach (Maldonado-Contreras et al., 2011), and shallow marine

sediments (Briggs et al., 2011). This is the first report that applied the PhyloChip to deep marine sediments; therefore, T-RFLP was performed on the same samples to test if the two methods identified the same community structure. Based on the Mantel test both techniques identified the same community structure; thus, PhyloChip served as valid technique for microbial community structure analysis in this study.

Both PhyloChip and T-RFLP did not detect archaeal 16S rRNA genes in sediment layers of the Ulleung Basin below the SMTZ. Methanogens from the archaeal domain were expected because of the presence of biogenic methane in the sediment (Ryu et al., 2012). Possible reasons for an inability to detect archaea could be the biases created during storage, DNA extraction, or PCR. Steps were taken to limit these biases. For example, the sediment samples were placed at -80^{o}C prior to DNA extraction to maintain sample integrity and to minimize storage biases (Reed et al., 2002; Rochelle et al., 1994). To verify our DNA extraction procedure two samples were re-extracted 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, which have been reported to inhibit PCR (Tebbe and Vahjen, 1993). Other possibilities for not detecting methanogens could be either the methanogenic zone was not sampled, peak methanogenesis usually occurs below the SMTZ but in the upper 50 mbsf (Colwell et al., 2008; Nunoura et al., 2008; Sivan et al., 2007), or the combination of low abundance and primer biases inhibited their detection (Newberry et al., 2004).

Despite not detecting archaea in deep samples, the bacterial community was well represented. The amount of DNA extracted from the GHSZ and FGZ was similar to that reported from other hydrate areas such as the Nankai Trough (Reed et al., 2002) and Andaman Sea (Briggs et al., 2012). In addition, the PhyloChip detected microbes that are typically found in the marine subsurface (e.g. Candidate division JS1, *Chloroflexi*, and *Planctomycetes*) (Inagaki et al., 2006; Nunoura et al., 2008). 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.

Statistical analysis of the community composition aids in the interpretation and elucidation of trends in microbial community composition. Our findings, based on MRPP, indicate that the microbial communities are significantly different in the SMTZ, GHSZ, and FGZ. The SMTZ is an important geochemical zone where a consortium of sulfate reducing bacteria (SRB) within the *Deltaproteobacteria* phylum and ANMEs has been linking to AOM (Boetius et al., 2000; Knittel and Boetius, 2009; Orphan et al., 2002). Consistent with other SMTZ environments *Deltaproteobacteria* were found at the SMTZ 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; Hamdan et al., 2011; Inagaki et al., 2006; Roussel et al., 2009; Sørensen and Teske, 2006). 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; Sørensen and Teske, 2006). Another possible explanation was that sampling missed the specific location where the ANME were most abundant. Harrison *et al.* investigated the bacterial and archaeal communities using high-resolution sampling across the SMTZ 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 SMTZ, while ANME was detected within and just below the SMTZ. The samples collected from the Ulleung Basin were within the SMTZ; however, the nearest samples to the layer of maximum sulfate reduction (modeled based on sulfate profiles, data not shown) were 57 cm and 20 cm at UBGH2-1-1 and UBGH2-10, respectively. Thus, it is possible that the sediment layer where ANME are located was missed in this study and higher resolution sampling is needed for their detection.

The GHSZ samples formed two separate groups that were separated along the hydrate saturation of the nearest hydrate layer vector (Figure 3). Group A samples clustered closer to the SMTZ samples than to the other GHSZ samples (Figure 3) and Group A did not contain any indicator taxa. This suggests that samples that are near distinct pockets that contain high hydrate saturation values have a distinct microbial community. A direct associate with microbes and gas hydrate has been shown previously in shallow gas hydrates from the Gulf of Mexico (Lanoil et al., 2001). We found that *Gammaproteobacteria* related to *Vibrio*-type species may prefer a closer association with hydrate based on the indictor taxa detected within Group B. No samples were recovered directly from hydrate containing layers. Thus, either the presence of hydrate or the

geochemical and physical properties associated with hydrate may affect microbial distributions at a distance from the hydrate and this distance may be a factor of the hydrate saturation value. 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). More samples are needed to establish whether a relationship exists between the communities, 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.

Unfortunately, only three samples produced enough DNA for PhyloChip analysis in the FGZ. Three samples is enough to calculate a median score to be used in MRPP and this showed that these samples were significantly different from the other samples in this study; however, the ability to extrapolate the significance of this difference to other sedimentary environments is difficult and should be done with caution.

Summary

In this study, PhyloChip analysis was used to determine microbial community composition in sediments from the SMTZ, GHSZ, and FGZ 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 MDS ordination and MRPP revealed that microbial communities found in the SMTZ, GHSZ, and FGZ were significantly different from each other. The

archaeal anaerobic methanotrophs typically associated with anaerobic oxidation of methane were not detected in sediment near the SMTZ; however, the MBGB, which is often found in SMTZ environments, was detected. Indicator taxa of microbial communities in the GHSZ identified *Vibrio*-types species that may prefer sediment layers closer to hydrate-containing sediment. This study provided a statistical analysis of microbial communities that allowed the identification of distribution patterns in relation to geochemical zones defined by methane.

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Figure captions

Figure 1. A map of the Ulleung Basin depicting the location of the two sites where sediment samples were obtained.

Figure 2. DNA quantities extracted from the Sites UBGH2-1-1 and UBGH2-10, 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, SMTZ: sulfate methane transition, GHSZ: gas hydrate stability zone, FGZ: free gas zone.

Figure 3. Non-metric multidimensional scaling (MDS) of data obtained from PhyloChip community analysis for samples from the SMTZ, GHSZ, 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, H_4SiO_4: silicic acid, hydrate saturation: gas hydrate saturation of the nearest hydrate layer, depth: depth from which the sample was obtained). Some vectors were removed to reduce clutter. The variance explained by each axis is given in parentheses. The insert depicts a hierarchical clustering of samples overlaid on the MDS ordination. Groups A and B are the two separate GHSZ groups.

Figure 4. A plot of the distance to the nearest hydrate layer versus the hydrate saturation of that layer. Sites UBGH2-1-1 and UBGH2-10 are represented by squares and circles, respectively. Groups A and B represented as filled symbols and open symbols, respectively,

were identified using hierarchal clustering and identified in Figure 3. Numbers indicate the depth (mbsf) from which each sample was obtained. Six samples were removed because hydrate saturation of the nearest sediment layer could not be calculated.

Figure 1 UBGH2_Coring site.eps

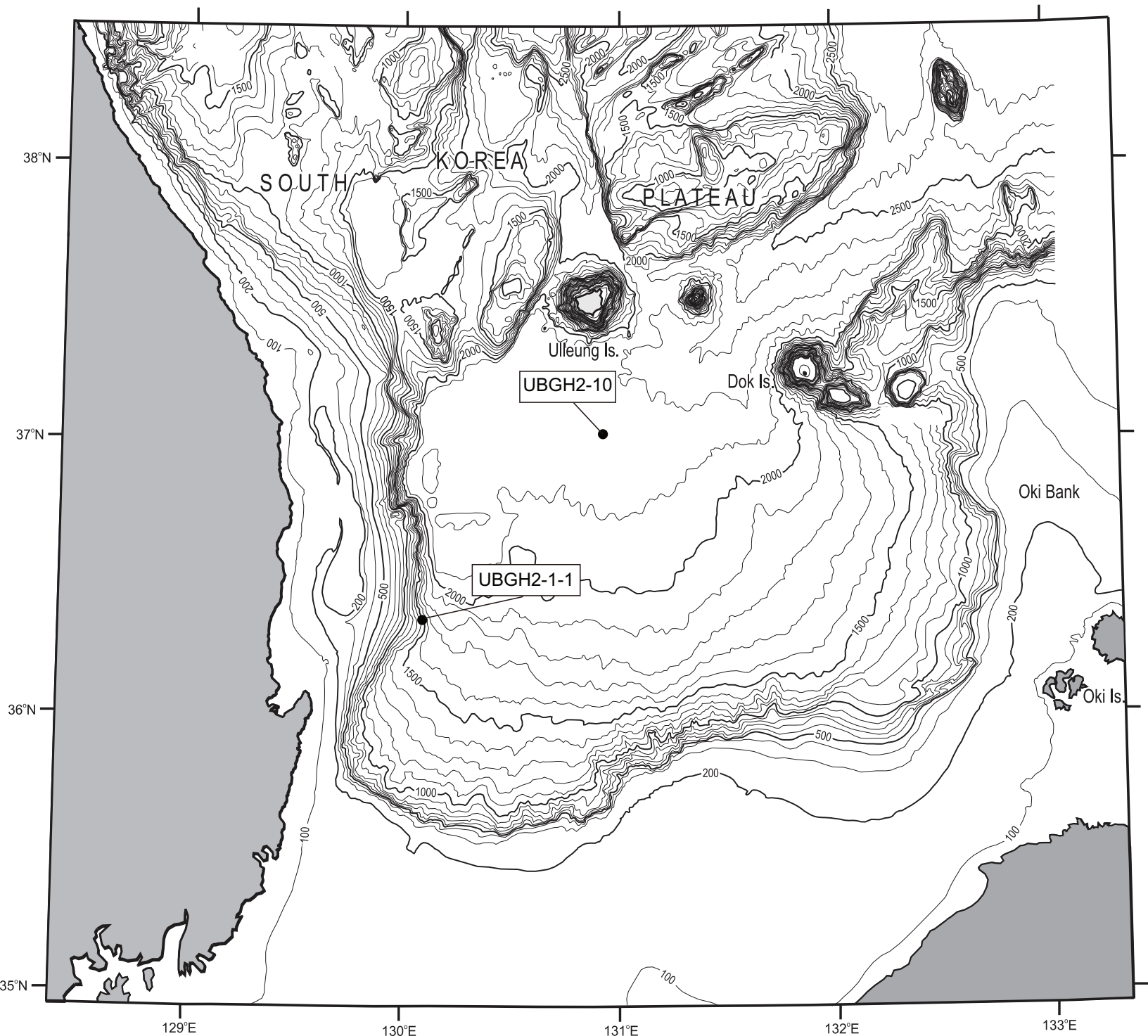

Figure 2 DNA quantities.eps

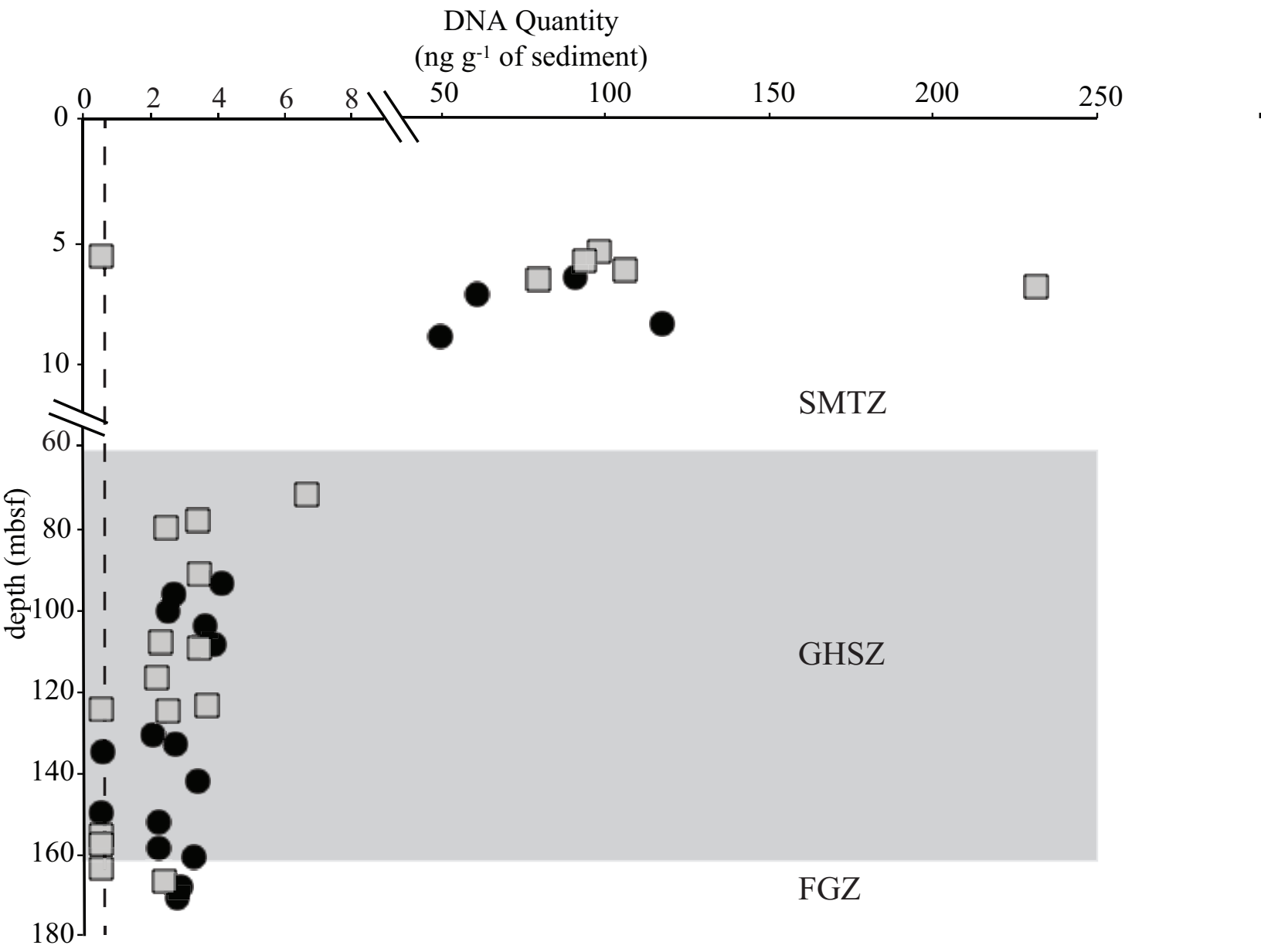

Figure 3 All samples MDS.eps

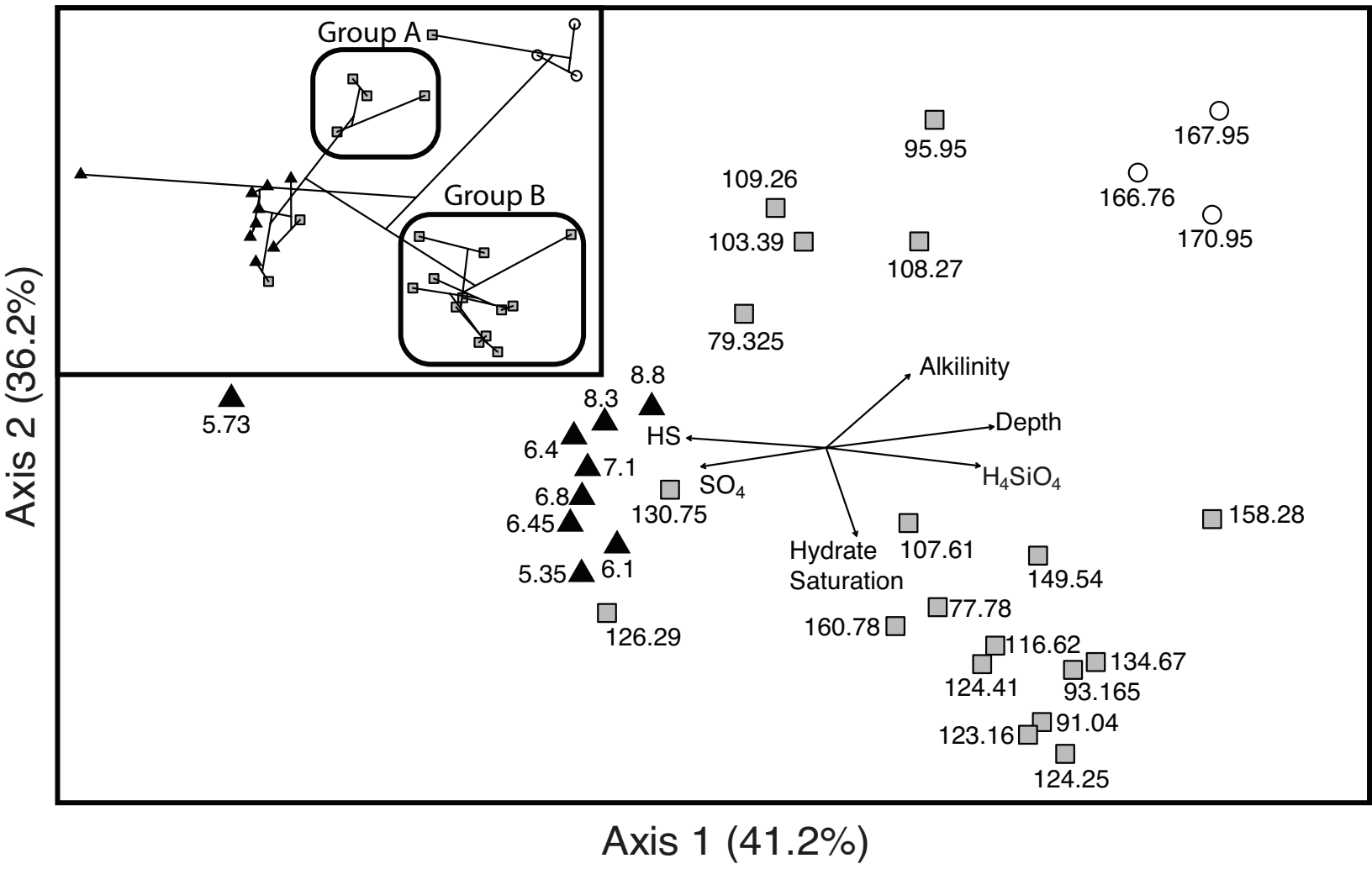

Figure 4 distance vs hydrate saturation_rev01.eps