

RESEARCH ARTICLE

Bacterial dominance in subseafloor sediments characterized by methane hydrates

Brandon R. Briggs¹, Fumio Inagaki^{2,3}, Yuki Morono^{2,3}, Taiki Futagami², Carme Huguet⁴, Antoni Rosell-Mele^{4,5}, Thomas D. Lorenson⁶ & Frederick S. Colwell¹

¹Oregon State University, Corvallis, OR, USA; ²Geomicrobiology Group, Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Nankoku, Kochi, Japan; ³Geobio-Technology and Engineering Laboratory, Submarine Resources Research Project, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Nankoku, Kochi, Japan; ⁴Institut de Ciència i Tecnologia Ambientals (ICTA), Edifici de Ciències, Universitat Autònoma de Barcelona, Cerdanyola del Valles, Barcelona, Spain; ⁵Institució Catalana de Recerca i Estudis Avançats (ICREA), Passeig Lluís Companys 23, Barcelona, Spain; and ⁶United States Geological Survey, Menlo Park, CA, USA

Correspondence: Frederick S. Colwell, Oregon State University, 104 COAS Administration Building, Corvallis, OR 97331, USA. Tel.: 541 737 5220; fax: 541 737 2064; e-mail: rcolwell@coas.oregonstate.edu

Received 1 October 2011; revised 23 December 2011; accepted 12 January 2012. Final version published online 16 February 2012.

DOI: 10.1111/j.1574-6941.2012.01311.x

Editor: Tillmann Lueders

Keywords

pyrosequencing; Andaman Sea; geologic sediments/chemistry/microbiology; molecular sequence data; lipids.

Abstract

The degradation of organic carbon in subseafloor sediments on continental margins contributes to the largest reservoir of methane on Earth. Sediments in the Andaman Sea are composed of ~ 1% marine-derived organic carbon and biogenic methane is present. Our objective was to determine 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 – 10^5 cells cm⁻³). Archaea were never detected despite the use of both DNA- and lipid-based methods. Statistical analysis of terminal restriction fragment length polymorphisms revealed distinct microbial communities from above, within, and below the GHOZ, and GHOZ samples were correlated with a decrease in organic carbon. Primer-tagged pyrosequences of bacterial 16S rRNA genes showed that members of the phylum *Firmicutes* are predominant in all zones. Compared with other seafloor settings that contain biogenic methane, this deep subseafloor habitat has a unique microbial community and the low cell abundance detected can help to refine global subseafloor microbial abundance.

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 5000 gigatons of methane carbon (Boswell & Collett, 2011), and this methane is important to consider in computational models of the global carbon cycle and in estimates of accessible energy resources.

Gas hydrate systems are often composed of biologically produced methane (Kvenvolden, 1995), yet we are still trying to understand fundamental aspects about the microbial processes that cycle the carbon in hydrate-rich sediments. Microbes responsible for the creation of the methane that exists in hydrates and the processes that they carry out need to be characterized to fully under-

stand the ecological and biogeochemical role of the largest methane reservoir on Earth (c.f. Reece, 2007). Furthermore, a more complete accounting of the bacterial and archaeal diversity that occurs in and near sediments where 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 m below seafloor (mbsf) hole in the Andaman Sea (Collett *et al.*, 2008). The low geothermal gradient (21.0 °C km⁻¹) allows methane hydrate to exist deep (600 mbsf) 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 have less than 1% by weight total organic carbon (TOC) (Johnson *et al.*, 2009), while most hydrate-containing sediments along continental

margins that have been microbiologically investigated contain 1–11% TOC (Meister *et al.*, 2005). The combination of a thick hydrate zone extending deep into the sediments and 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 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 only created a single-clone library from a single sample from each layer (Reed *et al.*, 2002). Inagaki *et al.* (2006) used statistical analysis to compare the microbial communities in areas characterized by hydrate and nonhydrate areas 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. Nunoura *et al.* (2008) used clone libraries to describe the microbial communities at several sites along the hydrate-bearing Cascadia margin and found a dominance of the bacterial candidate division JS1 and the Deep-Sea Archaeal Group (DSAG). In addition, methanogens have been detected by both culture-dependent and culture-independent methods in methane hydrate-containing sediment (Mikucki *et al.*, 2003; Colwell *et al.*, 2008). 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 seafloor 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 hydrates. 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 (10°45.1912' N, 93°6.7365' E) during the NGHP Expedition 01. This site exists in a forearc basin

that contains mainly marine-deposited nanofossil ooze that accumulated at a rate of 5.6 cm ka⁻¹ (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 seismically observed bottom-simulating reflector, where methane is believed to exist as a free gas, is estimated at approximately 600 mbsf (Collett *et al.*, 2008).

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 –80 °C before being shipped frozen overnight in dry shippers to Oregon State University for molecular microbiological analyses. In the laboratory, the frozen cores were subsampled by paring the outer layer of sediment core with an alcohol and flame-sterilized chisel. The outer 2–3 cm of the core was discarded, and the inner core (subcore) was used for the subsequent experiments such as DNA and lipid extractions. Fluorescent microsphere beads were used during drilling to identify possible contamination of the cored sediment samples, which were not detected in the subcore material.

Geochemistry

Stable carbon isotope ratio determinations of methane were made on a continuous flow–isotope ratio mass spectrometer (Finnigan MAT 252, GC-CF-IRMS) and referenced to the conventional Pee Dee Belemnite standard through a known CO₂ isotope standard as previously described (Lorenson *et al.*, 2008).

Cell counts

To determine the abundance of cells with intact DNA in the sediment, 1 g of frozen subcore samples was 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/ethanol (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 sub-cored material from 13 samples within the GHZO as described previously (Lipp *et al.*, 2008). The additional extractions included a freezer mill homogenization for six 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 h 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). 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).

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 described earlier. 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 aforementioned PCR and nested PCR for archaeal 16S rRNA genes were then performed again using the MDA product. DNA extracted from pure cultures of *Escherichia coli* or *Methanosarcina mazei* was used as positive controls for bacterial or archaeal amplifications, respectively.

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 enzymes *HhaI* and *MspI* were 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. 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 multiresponse 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 GHZO. 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 taxonomic hierarchy 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 to 4 g of homogenized freeze-dried sediment from six samples above the GHZ, six samples within the GHZ, and three samples below the GHZ 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- μ m 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 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 hydroxyarchaeol 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 μ L of sample, in split/splitless mode, compounds were separated through an Agilent DB-5MS capillary column (30 m \times 0.25 mm internal diameter \times 25 μ m film thickness) with a 5-m precolumn. Oven temperature was held at 80 $^{\circ}$ C for 1 min, increased to 120 $^{\circ}$ C at a rate of 20 $^{\circ}$ C min $^{-1}$, then to 320 $^{\circ}$ C at 6 $^{\circ}$ C 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 $^{\circ}$ C, source 250 $^{\circ}$ C, Ms Quad 150 $^{\circ}$ C, and ionization energy 70 eV, scan range from 50 to 650 *m/z*. The glycerol compounds were derivatized online with *N,O*-bis(trimethylsilyl)trifluoroacetamide.

Results

Geochemistry

The methane carbon isotopic composition was the most ^{13}C depleted near the seafloor with a $\delta^{13}\text{C}$ value of -80‰ . The value gradually became more ^{13}C enriched with depth until about 300 mbsf after which the $\delta^{13}\text{C}$ value remained about -67‰ . Propane, considered to be of thermogenic origin, occurs below 550 mbsf (Collett *et al.*, 2008). These values indicate that 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 – 10^5 cells cm $^{-3}$) as compared to those previously reported from the organic-rich subseafloor sediments (Fig. 1). Seven samples contained cell abundance below the detection limit of 10^2 cells cm $^{-3}$.

Nucleic acid extractions and amplification

The Mo Bio 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 g $^{-1}$ of sediment (Supporting Information,

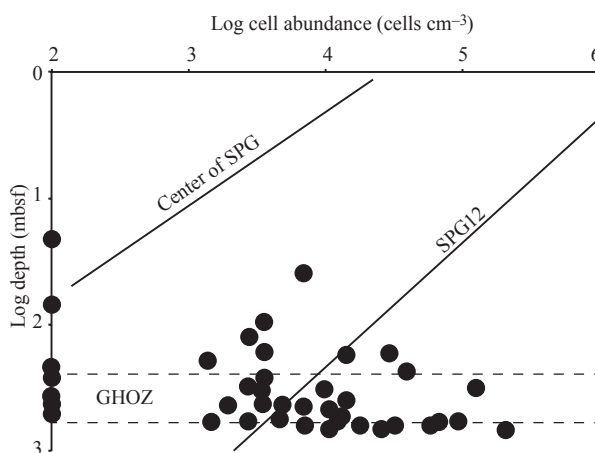


Fig. 1. Microscopic cell numbers vs. depth at site 17A in Andaman Sea sediments. Circles on the y-axis are samples that had cell abundance below the detection limit of 10^2 cells cm $^{-3}$. The dashed lines depict the location of the GHZ. The diagonal lines are downward logarithmic extrapolation of cell abundance from the top 10 m of sediment from center of the SPG and site SPG12 (from the outer edge of the SPG) reported by D'Hondt *et al.* (2009).

Fig. S1). Furthermore, neither bacterial nor archaeal 16S rRNA genes were detected in the MoBio FM or MoBio FM PK DNA extractions. Bacterial 16S rRNA genes were detected from the Mo Bio 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 (Tables S1 and S2), 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. Digestion of the 16S rRNA gene amplicons with *HhaI* resulted in only one identifiable TRF; whereas, a total of 111 TRFs were identified when the 16S rRNA gene amplicons were digested with *MspI*. Of the total 111 TRFs, 58, 62, and 57 TRFs were found above, within, and below the GHZO, respectively. 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 GHZO, respectively. The in-silico digestion of cloned sequences compared with TRFs identified the peak at 154 bp as most likely being related to the genus *Bacillus*. In all samples within the GHZO, the TRF at 154 bp was the most prominent peak. This TRF was present, although peak height was much smaller, in samples above the GHZO. It was not detected below the GHZO (Fig. S2).

A total of 72 523 primer-tagged pyrosequencing reads were classified using GAST after the poor-quality sequences and suspected contaminants (e.g., human associated enterics) (Knights *et al.*, 2011) were discarded. Firmicutes were the dominant phylum with 52%, 53%, and 74% of reads from above, within, and below the GHZO, respectively (Fig. 2). Other significant phyla detected were *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and other bacterial phyla that could not be assigned to a phylum. The *Gamma* and *Betaproteobacteria* seemed predominant in the GHZO consisting of ~21% and ~17% of the reads, respectively (Fig. 2), while *Bacteroidetes* decreased in frequency in the GHZO to ~2%. *Actinobacteria* and *Alphaproteobacteria* had the highest frequency above the GHZO (Fig. 2).

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 ratio remained below 0.7, suggesting little contribution from methanogenic archaea (Fig. 3). Given the absence of detectable archaeal DNA in the sedi-

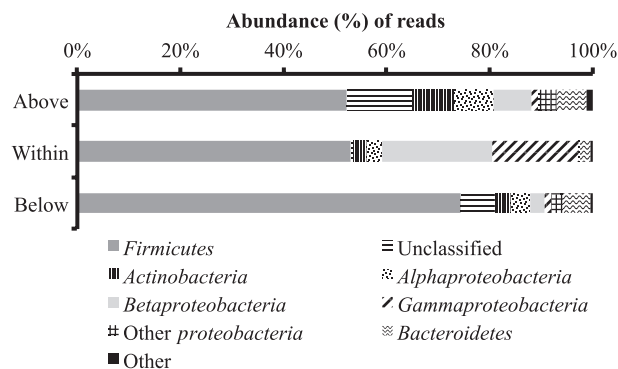


Fig. 2. Distribution of major phylogenetic groups determined by primer-tagged pyrosequencing from above, within, and below the GHZO. The numbers of reads from above, within, and below the GHZO were 15632, 41095, and 15796, respectively.

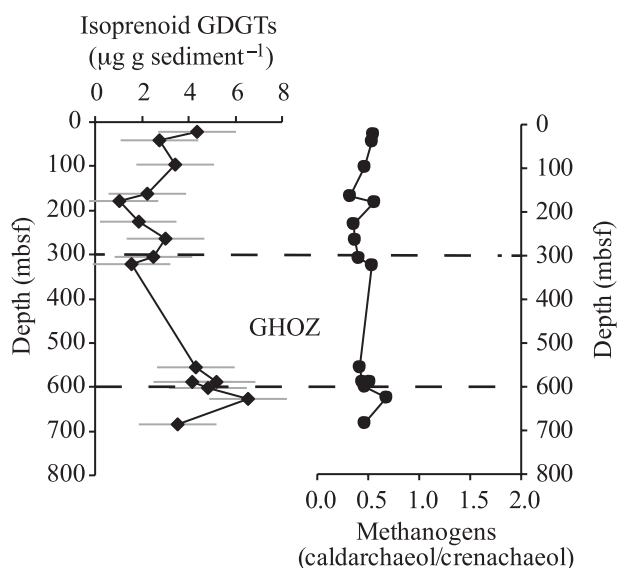


Fig. 3. Lipid determinations for selected samples from site 17A in the Andaman Sea sediments showing downcore GDGT measurements of isoprenoid abundance with one standard deviation error bars (left) and methanogen ratio (caldarchaeol/crenarchaeol) (right). The dashed lines in the center of the diagram depict the depth range of the GHZO. A significant methanogen archaeal contribution is only apparent when methanogen ratio values are > 2 (Blaga *et al.*, 2009).

ment examined, both DNA- and lipid-based analyses consistently suggest that most GDGTs were relict biomarkers derived from past activity.

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 GHZO (Fig. 4). The samples from within and below the GHZO separated along axis 1 on the NMS plot. A bi-plot overlaid on the ordination identified environmental parameters that correlate with 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 Fig. 4). Samples from above and within the GHZO 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 with the axes in the NMS plot.

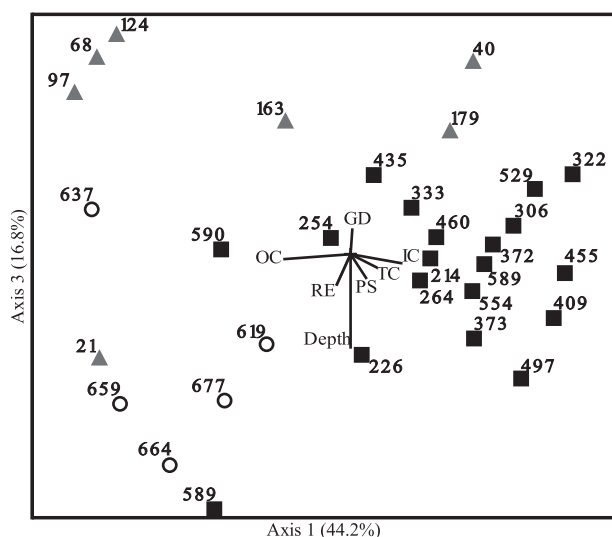


Fig. 4. NMS ordination of the data derived from T-RFLP analysis of Andaman Sea sediments. The triangles, squares, and circles represent the central tendency of all TRFs detected in each sample from above, within, and below the GHZO, respectively. Symbols that are closer to each other depict samples that contained similar TRFs. A bi-plot is overlaid on the ordination to identify environmental parameters that were correlated with 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. OC, organic carbon; RE, resistivity; GD, grain density; IC, inorganic carbon; TC, total carbon; PS, porosity.

Discussion

Biogenic methane is a significant fraction of the methane that occurs in marine sediments where 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 (Inagaki *et al.*, 2006; Nunoura *et al.*, 2008). This study in the Andaman Sea, where methane hydrates extend deep into the sediments and where the TOC concentrations are low to intermediate for methane hydrate-bearing sediments, complements existing studies in hydrate-containing marine sediments 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 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; Morono *et al.*, 2009). The sediment layers at Site 17A are unique for a 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.* data have proposed carbon availability and/or lithology (i.e., resistivity, grain size, and pore connectivity) to be key drivers in cell densities and microbial community structures (Inagaki *et al.*, 2003; D'Hondt *et al.*, 2004; Hoshino *et al.*, 2011).

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 < 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 (Fig. 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).

Statistical analysis of T-RFLP data

Statistical analysis of the community composition identified by molecular analysis aids in the interpretation of trends in microbial community composition 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 (Osborne *et al.*, 2006; Colwell *et al.*, 2011). Based on T-RFLP data at Site 17A, a distinct microbial community existed within the GHZO when compared with microbes obtained from sediment layers above and below this zone. However, it is difficult to determine whether the presence of hydrate significantly affects the community or whether 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 GHZO at Site 17A were correlated with an increase in inorganic carbon and a decrease in organic carbon (Fig. 4). 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 can be represented by 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 Data S1). 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 nonhydrate sediments obtained along the Pacific Ocean margins (Inagaki *et al.*, 2006). Using clone libraries to study community diversity, Inagaki *et al.* (2006) found that hydrate-saturated sediment is typically dominated by the bacterial candidate division JS1 and the DSAG. The bacterial phylum *Chloroflexi* and the Marine Crenarchaeotic Group I dominate nonhydrate 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 subsurface environments has been difficult to determine. Culture-independent methods such as QPCR, catalyzed reporter deposition fluorescence *in situ* hybridization, intact polar lipids, and slot-blot hybridization assays have been performed, and each technique produced a different result (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 culture-independent methods (Reed *et al.*, 2002; Mikucki *et al.*, 2003; Colwell *et al.*, 2008). While the question of archaeal vs. bacterial dominance remains 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 seafloor sediments (Lipp *et al.*, 2008). Lipp *et al.* (2008) 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 sub-seafloor sedimentary biosphere. We used the three methods developed by Lipp *et al.* on the samples from within the GH0Z at Site 17A and found that the more vigorous cell-lysing steps reduced DNA yield, whereas Lipp *et al.* 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 with 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 (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 (Tables S1 and S2). Yet, only bacterial 16S rRNA genes and bacterial *accC* genes were detected (see Data S1).

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- μ L 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., Huguet *et al.*, 2007). However, it is difficult to ascertain the source (e.g., sediment or water column), and 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).

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 was 0.7 at Site 17A, 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 composition indicates that 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. Sediment layers below the sulfate methane transition zone (SMTZ), where both sulfate and methane converge, are 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 was 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 (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 Site 17A 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.

Acknowledgements

This research was supported by the U.S. Department of Energy, Office of Fossil Energy, National Energy Technology Laboratory, and a National Science Foundation – Japanese Society for the Promotion of Science, East Asia Pacific Summer Institute (EASPI) fellowship. Support for

lipid analyses was provided to C.H. with a Juan de la Cierva grant from the MICINN, a fellowship to A.R.M. from the European Commission (Marie Curie-IOF), and the analytical assistance of Núria Moraleda. Pyrosequencing for this research was conducted as a part of the Census of Deep Life sponsored by the Deep Carbon Observatory and the Alfred P. Sloan Foundation. We gratefully acknowledge the support provided by Hilary Morrison and Mitch Sogin at the Marine Biological Laboratory, Woods Hole MA for assistance with the pyrosequencing and analysis. We thank the scientific parties and vessel crews of the National Gas Hydrate Program Expedition 01 (NGHP01) for the collection of samples. We appreciate Hiu Yip who was supported by an undergraduate research fellowship through the Subsurface Biosphere Initiative at Oregon State University for assistance with DNA extraction. Additional samples from Site 17A were provided by Art Spivak to complete the analysis.

References

- Biddle JF, Lipp JS, Lever MA *et al.* (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *P Natl Acad Sci USA* **103**: 3846–3851.
- Biddle JF, Fitz-Gibbon S, Schuster SC, Branchley JE & House CH (2008) Metagenomic signatures of the Peru Margin seafloor biosphere show a genetically distinct environment. *P Natl Acad Sci USA* **105**: 10583–10588.
- Bidle KA, Kastner M & Bartlett DH (1999) A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the Cascadia margin (ODP site 892B). *FEMS Microbiol Lett* **177**: 101–108.
- Blaga CI, Reichart GJ, Heiri O & Damste JSS (2009) Tetraether membrane lipid distributions in water-column particulate matter and sediments: a study of 47 European lakes along a north-south transect. *J Paleolimnol* **41**: 523–540.
- Boswell R & Collett TS (2011) Current perspectives on gas hydrate resources. *Energy Environ Sci* **4**: 1206–1215.
- Bray JR & Curtis JT (1957) An ordination of the upland forest communities in southern Wisconsin. *Ecol Monogr* **27**: 325–349.
- Briggs BR, Pohlman J, Torres ME, Riedel M, Brodie E & Colwell FS (2011) Macroscopic biofilms in fracture-dominated sediment that anaerobically oxidize methane. *Appl Environ Microbiol* **77**: 6780–6787.
- Collett TS, Riedel M & Cochran J, Boswell R, Presley J, Kumar P, Sathe A, Sethi A, Lall M & Sibal V (2008) *National Gas Hydrate Program (NGHP) Expedition 01 Initial Reports*. Ministry of Petroleum and Natural Gas, Directorate General of Hydrocarbons, New Delhi, India.
- Colwell FS, Boyd S, Delwiche ME, Reed DW, Phelps TJ & Newby DT (2008) Estimates of biogenic methane production rates in deep marine sediments at Hydrate Ridge, Cascadia margin. *Appl Environ Microbiol* **74**: 3444–3452.
- Colwell F, Schwartz A & Briggs B (2011) Microbial community distribution in sediments from the Mount Elbert Gas Hydrate Stratigraphic Test Well, Alaska North Slope. *Mar Petrol Geol* **28**: 404–410.
- Culman SW, Gauch HG, Blackwood CB & Thies JE (2008) Analysis of T-RFLP data using analysis of variance and ordination methods: a comparative study. *J Microbiol Meth* **75**: 55–63.
- DeLong EF (1992) Archaea in coastal marine environments. *P Natl Acad Sci USA* **89**: 5685–5689.
- D'Hondt S, Jorgensen BB, Miller DJ, *et al.* (2004) Distributions of microbial activities in deep seafloor sediments. *Science* **306**: 2216–2221.
- D'Hondt S, Spivack AJ, Pockalny R, *et al.* (2009) Seafloor sedimentary life in the South Pacific Gyre. *P Natl Acad Sci USA* **106**: 11651–11656.
- Escala M, Rosell-Mele A & Masque P (2007) Rapid screening of glycerol dialkyl glycerol tetraethers in continental Eurasia samples using HPLC/APCI-ion trap mass spectrometry. *Org Geochem* **38**: 161–164.
- Fry JC, Webster G, Cragg BA, Weightman AJ & Parkes RJ (2006) Analysis of DGGE profiles to explore the relationship between prokaryotic community composition and biogeochemical processes in deep seafloor sediments from the Peru Margin. *FEMS Microbiol Ecol* **58**: 86–98.
- German CR, Bowen A, Coleman ML, *et al.* (2010) Diverse styles of submarine venting on the ultraslow spreading Mid-Cayman Rise. *P Natl Acad Sci USA* **107**: 14020–14025.
- Hazen TC, Dubinsky EA, DeSantis TZ, *et al.* (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* **330**: 204–208.
- Hinrichs KU, Hayes JM, Sylva SP, Brewer PG & DeLong EF (1999) Methane-consuming archaeobacteria in marine sediments. *Nature* **398**: 802–805.
- Hoshino T, Morono Y, Terada T, Imachi H, Ferdman TG & Inagaki F (2011) Comparative study of seafloor microbial community structures in deeply buried coral fossils and sediment matrices from the challenger mound in the porcupine seamount. *Front Microbiol* **2**: 231.
- Huguet C, Hopmans EC, Febo-Ayala W, Thompson DH, Damste JSS & Schouten S (2006) An improved method to determine the absolute abundance of glycerol dibiphytanyl glycerol tetraether lipids. *Org Geochem* **37**: 1036–1041.
- Huguet C, Schimmelmann A, Thunell R, Lourens LJ, Damste JSS & Schouten S (2007) A study of the TEX₈₆ paleothermometer in the water column and sediments of the Santa Barbara Basin, California. *Paleoceanography* **22**: PA3203.
- Huse SM, Dethlefsen L, Huber JA, Mark Welch D, Relman DA & Sogin ML (2008) Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS Genet* **4**: e1000255.
- Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki K, Nealson KH & Horikoshi K (2003) Microbial communities

- associated with geological horizons in coastal seafloor sediments from the Sea of Okhotsk. *Appl Environ Microbiol* **69**: 7224–7235.
- Inagaki F, Nunoura T, Nakagawa S, *et al.* (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *P Natl Acad Sci USA* **103**: 2815–2820.
- Johnson JE, Phillips SC, Miranda E, Giosan L & Rose K (2009) Long-term variability of carbon and nitrogen in the Bay of Bengal and Arabian Sea: results from the NGHP Expedition 1. *EOS Trans AGU* **90**: Abstract OS44A-01.
- Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, Bushman FD, Knight R & Kelley ST (2011) Bayesian community-wide culture-independent microbial source tracking. *Nat Methods* **8**: 761–763.
- Kornilova O & Rosell-Mele A (2003) Application of microwave-assisted extraction to the analysis of biomarker climate proxies in marine sediments. *Org Geochem* **34**: 1517–1523.
- Kruskal JB (1964) Nonmetric multidimensional scaling: a numerical method. *Psychometrika* **29**: 115–129.
- Kvenvolden KA (1995) A review of the geochemistry of methane in natural gas hydrate. *Org Geochem* **23**: 997–1008.
- Lipp JS, Morono Y, Inagaki F & Hinrichs KU (2008) Significant contribution of Archaea to extant biomass in marine subsurface sediments. *Nature* **454**: 991–994.
- Logemann J, Graue J, Koster J, Engelen B, Rullkötter J & Cypionka H (2011) A laboratory experiment of intact polar lipid degradation in sandy sediments. *Biogeosci Discuss* **8**: 3289–3321.
- Lorenson T, Claypool G & Dougherty J (2008) Natural gas geochemistry of sediments drilled on the 2005 Gulf of Mexico JIP cruise. *Mar Petrol Geol* **25**: 873–883.
- Marchesi JR, Weightman AJ, Cragg BA, Parkes RJ & Fry JC (2001) Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. *FEMS Microbiol Ecol* **34**: 221–228.
- McCune B & Mefford MJ (2006) *Multivariate Analysis of Ecological Data*. MjM Software, Gleneden Beach, OR, USA.
- Meister P, Prokopenko M, Skilbeck CG, Watson M & McKenzie JA (2005) Data report: compilation of total organic and inorganic carbon data from Peru Margin and Eastern Equatorial Pacific drill sites (ODP Legs 112, 138, and 201). *Proc ODP Sci Results* **201**: 1–20.
- Mielke PW Jr & Berry KJ (2001) *Permutation Methods: A Distance Function Approach*. Springer, New York.
- Mikucki JA, Liu Y, Delwiche M, Colwell FS & Boone DR (2003) Isolation of a methanogen from seep marine sediments that contain methane hydrates, and description of *Methanoculleus submarinus* sp. nov. *Appl Environ Microbiol* **69**: 3311–3316.
- Morono Y & Inagaki F (2010) Automatic slide-loader fluorescence microscope for discriminative enumeration of seafloor life. *Sci. Drilling* **9**: 32–36.
- Morono Y, Terada T, Masui N & Inagaki F (2009) Discriminative detection and enumeration of microbial life in marine subsurface sediments. *ISME J* **3**: 503–511.
- Nunoura T, Inagaki F, Delwiche M, Colwell FS & Takai K (2008) Seafloor microbial communities in methane hydrate-bearing sediment at two distinct locations (ODP Leg204) in the Cascadia Margin. *Microbes Environ* **23**: 317–325.
- Opatkiewicz AD, Butterfield DA & Baross JA (2009) Individual hydrothermal vents at Axial Seamount harbor distinct seafloor microbial communities. *FEMS Microbiol Ecol* **70**: 413–424.
- Osborne CA, Rees GN, Bernstein Y & Janssen PH (2006) New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. *Appl Environ Microbiol* **72**: 1270–1278.
- Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ & Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediments. *Nature* **371**: 410–413.
- Reeburgh WS (2007) Oceanic methane biogeochemistry. *Chem Rev* **107**: 486–513.
- Reed DW, Fujita Y, Delwiche ME, Blackwelder DB, Sheridan PP, Uchida T & Colwell FS (2002) Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. *Appl Environ Microbiol* **68**: 3759–3770.
- Rethore G, Montier T, Le Gall T, Delepine P, Cammas-Marion S, Lemiegre L, Lehn P & Benvenugu T (2007) Archaeosomes based on synthetic tetraether-like lipids as novel versatile gene delivery systems. *Chem Commun* **20**: 2054–2056.
- Roussel EG, Sauvadet AL, Chaduteau C, Fouquet Y, Charlou JL, Prieur D & Cambon Bonavita MA (2009) Archaeal communities associated with shallow to deep seafloor sediments of the New Caledonia Basin. *Environ Microbiol* **11**: 2446–2462.
- Sapp M, Parker ER, Teal LR & Schratzberger M (2010) Advancing the understanding of biogeography-diversity relationships of benthic microorganisms in the North Sea. *FEMS Microbiol Ecol* **74**: 410–429.
- Sass AM, McKew BA, Sass H, Fichtel J, Timmis KN & McGenity TJ (2008) Diversity of *Bacillus*-like organisms isolated from deep-sea hypersaline anoxic sediments. *Saline Syst* **4**: 8.
- Schippers A & Neretin LN (2006) Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. *Environ Microbiol* **8**: 1251–1260.
- Schouten S, Middelburg JJ, Hopmans EC & Damste JSS (2010) Fossilization and degradation of intact polar lipids in deep subsurface sediments: a theoretical approach. *Geochim Cosmochim Acta* **74**: 3806–3814.
- Sivan O, Schrag DP & Murray RW (2007) Rates of methanogenesis and methanotrophy in deep-sea sediments. *Geobiology* **5**: 141–151.
- Sorensen KB & Teske A (2006) Stratified communities of active Archaea in deep marine subsurface sediments. *Appl Environ Microbiol* **72**: 4596–4603.

- Teske A & Sorensen KB (2008) Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J* **2**: 3–18.
- Vreeland RH, Rosenzweig WD & Powers DW (2000) Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* **407**: 897–900.
- Wellsbury P, Goodman K, Cragg BA & Parkes RJ (2000) The geomicrobiology of deep marine sediments from Blake Ridge containing methane hydrate (Sites 994, 995 and 997). *Proc Ocean Drill Prog Sci Results* **164**: 379–391.
- Whiticar MJ (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem Geol* **161**: 291–314.
- Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes: the unseen majority. *P Natl Acad Sci USA* **95**: 6578–6583.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The amount of DNA extracted for 13 samples within the GHZO using three methods of DNA extraction.

Fig. S2. Three representative T-RFLP electropherograms from above, within, and below the GHZO in the Andaman Sea sediments are depicted.

Table S1. List of primers and optimal annealing temperatures used to detect and amplify 16S rRNA genes in DNA extracted from sediments in the Andaman Sea.

Table S2. List of primers and optimal annealing temperatures used to detect and amplify functional genes in DNA extracted from sediments in the Andaman Sea.

Data S1. Functional gene and 16S rRNA gene clone library methods.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.