A p p e n d i x O n e

IN SITU DEPLOYABLE REACTOR ENABLES EXAMINATION OF MICROBIAL COMMUNITIES FROM HIGH PRESSURE ENVIRONMENTS

David H. Case[1]

in collaboration with,

Yuki Morono[2], Akira Ijiri[2], Patricia Tavormina[1], Makoto Nagasawa[3], Masamitsu Matsumoto[3], Victoria Orphan[1], and Fumio Inagaki[2]

[1]Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA, 91125, USA

[2]Geomicrobiology Group, Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science and Technology, Monobe, Nankoku, Kochi, Japan

[3]Syn Corporation, Inc., Kyotanabe City, Japan

This chapter is in preparation for publication.

A1.0 ABSTRACT

High-pressure environments represent the volumetric majority of habitat space for microorganisms on the planet, including the pelagic deep-sea, deep-sea sediments, and both the terrestrial and marine deep subsurface biospheres. However, the importance of pressure as an environmental variable affecting, and possibly constraining, microbial life remains poorly constrained. This is due in part to the difficulty of accessing, sampling, and transporting samples from high-pressure environments back to the laboratory. In order to address this obstacle, we designed a new high-pressure corer which is deployable on the payload of a piloted or remotely operated deep-sea vehicle, can retrieve sediment samples *in situ*, can be recovered shipboard and transported back to onshore laboratories, and which can maintain high pressure conditions throughout multi-month incubations including daily amendments with liquid media and gases and daily effluent sampling for geochemical or microbiological analysis.

A 45-day incubation at 10 MPa and 4˚C of sediments from the seafloor of the Joetsu Knoll, Japan, indicated periods of both aerobic and anaerobic methanotrophy. These rates were generally in agreement with previously reported rates of methane oxidation in aqueous environments, with the exception that our calculated aerobic rates outpaced anaerobic rates. Whether, how, and to what extent pressure impacts the physiology of microorganisms in the deep-sea remains an open area of research, one hopefully made more accessible to researchers through the emergence of new technologies for high-pressure sampling and incubations.

A1.1 INTRODUCTION

High-pressure (HP) environments, including the deep sea, seafloor, and deep subsurface, represent the most volumetrically abundant habitats on the planet for microorganisms. However, our understanding of the diversity, physiology, and adaptability of HP-tolerant ("piezotolerant"), HP-preferring ("piezophilic"), and HP-requiring ("hyperpiezophilic" or "obligately piezophilic") microorganisms remains in early stages of research. The first active microbial communities from deep-sea sediments were described in 1957 from >10,000 meters below sea level (mbsl) in the Philippine Trench (Zobell and Morita 1957). The first isolation of an obligate microbial piezophile species from deep-sea sediments did not occur until over 20 years later, of a gammaproteobacterial *Colwellia* species (Yayanos et al. 1981). Since then, driven by the widespread use of molecular techniques, the diversity of piezophilic and hyperpiezophilic microorganisms has been extended to many clades of *Bacteria* (Yanagibayashi et al. 1999; Kato et al. 2008; Nagata et al. 2010; Zhang et al. 2015) and *Archaea* (Zeng et al. 2009; Birrien et al. 2011; Zhang et al. 2015), and probably varies significantly depending on particular environment.

Preliminary experiments have shown sediment-hosted microbial communities from the deep sea to be sensitive to changes in pressure. Diversity, as measured by 16S rRNA genes, diverges over time if sediments from the deep sea are maintained at atmospheric vs at representative deep-sea pressures (Yanagibayashi et al. 1999). Unsurprisingly, then, metabolic activity also differs whether experiments on deep-sea sediments are conducted at low or high pressures (Picard and Ferdelman 2012). Similar tests of diversity and metabolic activity as a function of pressure from deep biosphere and hydrothermal vent samples would increase our understanding of microbial life in extreme environments.

A significant obstacle in the study of piezophilic microorganisms has been sample recovery. High-pressure (e.g., deep-sea) environments are generally difficult and expensive to access, and even once accessed it is challenging to maintain samples at HP during transport back to a

research vessel and/or the home laboratory. Because of these logistical challenges, development of new sampling technology has been identified as a top priority in the field of HP microbiology (Kim and Kato 2010; Kato 2011; Zhang et al. 2015). Recent development of deep-subsurface coring technology which can maintain *in situ* pressure through recovery represents good progress (Kubo et al. 2014), but can only be deployed on large drill ships such as the D/V *Chikyu* at great expense and time commitment. Development of an affordable HP sampling device that could be deployed on the payload of a piloted or remotely operated vehicle (e.g., DSV *Shinkai 6500* or ROV *Hyperdolphin*) and which could retain *in situ* pressure through sample retrieval and shipment back to onshore laboratories would enable many members of the scientific community to pursue environmental microbiology research at high pressures. A device meeting many of these criteria was developed at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) in the 1990s, and successfully deployed, but was limited to small volumes of surface sediments and was specifically designed to perform dilution-to-extinction experiments rather than stable isotope or amendment incubations (Kyo et al. 1991; Kato et al. 2008).

In order to address the technical considerations of working in deep-sea HP environments, we (specifically co-authors Inagaki, Morono, and Ijiri) worked with engineers at Syn Corporation Ltd. (co-authors Nagasawa and Matsumoto) to develop and test a new HP corer. The goal for this device was to be (i) deployable on the payload of an ROV, (ii) to have a "push core-like" structure enabling sampling down to >10 cm below seafloor, (iii) to maintain HP through recovery onboard ship and shipment to onshore laboratories, (iv) to have inlet ports for adding liquid media and/or gas phase (including stable isotope) amendments to the incubation chamber, and (v) to have an outlet port to enable time-course tracking of an experiment without sacrificing pressure on the entire vessel. After fabrication, deployment of the device was tested on deep-sea sediments at the Joetsu Knoll, Japan, during R/V *Natsushima* cruise NT13-15 in July 2013 at a depth of 985 meters below sea level (mbsl; 9.9 MPa). Subsequent onshore incubation of the deep-sea sediments within the HP corer chamber, including liquid media and gaseous amendments, was performed for 45

days at the Kochi Core Center branch of JAMSTEC in Kochi, Japan. This thesis chapter reports microbiological and geochemical results indicating a successful deployment and onshore use of the HP corer. Technical details of the HP corer are in preparation for publication at a later date.

The well-characterized geology, geochemistry, and microbiology of the Joetsu Knoll made it a favorable control site for testing deployment of the HP corer. Massive gas hydrates outcrop on the seafloor at the Joetsu Knoll, sourced from thermogenically produced methane (Matsumoto et al. 2005). In addition to this rich source of reduced carbon, the Joetsu Knoll is bathed in oxygen-rich bottom water (>210 μmol/kg; Gamo and Horibe 1983), fueling diverse chemosynthetic microbial consortia. Previous 16S rRNA clone libraries from sediments at the Joetsu Knoll have revealed the presence of anaerobic methane oxidizing archaea (e.g., ANME-1 and ANME-2) in addition to a diversity of other *Archaea* and *Bacteria* (Yanagawa et al. 2011). Despite the high concentration of oxygen in overlying bottom waters, the presence, distribution, and/or activity of aerobic methanotrophs has not been specifically investigated at the Joetsu Knoll.

A1.2 METHODS

A1.2.1 EXPERIMENTAL SETUP

Sample collection was performed during cruise NT13-15 aboard the R/V *Natsushima* during July 2013. The study site was the Joetsu Knoll, a well-characterized location of methane seepage offshore Joetsu, Japan (Fig. 1; 37˚31.1'N, 137˚58.0'E, 985 meters below sea level, 9.9 MPa pressure, Yanagawa et al. 2011). Two sediment cores were collected during Dive 1555 of the ROV *Hyperdolphin*: firstly, sediment was collected into the HP corer (hereafter, "HP Core") by abrading the internal core cylinder against an exposed vertical wall of sediment interlaced with white methane hydrates and bacterial films (Fig. 1). The HP Core's internal cylinder was then immediately placed into the external cylinder and secured by tightening. In this manner, sediment

was collected at environmentally relevant pressure and sealed into the HP Core *in situ*, in order to maintain pressure throughout core recovery and onshore experimentation. Unfortunately the Teflon seal on the HP Core was compromised, most likely by sediment grains lodged against the Teflon core liner, resulting in a loss of pressure during transit of the ROV *Hyperdolphin* from seafloor to the R/V *Natsushima*. The core was quickly re-pressurized to 10 MPa onboard ship by injection of filtered artificial seawater; in total, the HP Core experienced a loss of pressure for <3.5 hours. The HP Core was stored at 4˚C and 10 MPa onboard, during shipment, and upon arrival at the Kochi Core Center. Besides the HP Core, a second core was collected from adjacent sediment into a traditional M-type corer (hereafter, "M core"). The material collected into the M core contained a mixture of sediment and bottom water, which by the time of recovery onboard ship had separated by density. Immediately onboard ship, subsamples of the "M core water" and "M core sediment" were frozen at -80˚C for later DNA extraction and sequencing.

The HP Core was kept for the duration of experimentation (total 45 days) in a walk-in 4˚C refrigerator in the laboratory. Twelve days after collection from the seafloor, the HP Core was amended with $^{13}CH_4$ (50 mL of 50% $^{13}CH_4$) and $^{15}N_2$ (50 mL of 50% $^{15}N_2$) and daily tracking of pressure, temperature, dissolved inorganic carbon concentration (DIC), and $\delta^{13}C_{DIC}$ began for the course of a 45-day experiment in high pressure incubation of seafloor microbial assemblages (Fig. 3-4). Temperature and pressure were continuously monitored (Δt=1 sec), with daily samples taken for $\delta^{13}C_{DIC}$. During daily sampling, pressure was reset to 10 MPa by injection of sterile artificial seawater that contained no carbon sources (Supplemental Text). Samples for DNA extraction were taken at 11, 25, and 45 days; the T11 days and T25 time points were by necessity of design captured from the effluent outflow (the only outflow port) at the top of the HP Core. This involved bleeding 6 mL of effluent from the top port, following by filtration onto a polycarbonate membrane and freezing. The T45 time point, however, was taken from the sediment at the bottom of the HP Core, which was only possible because the vessel could exclusively be opened to the atmosphere during takedown of the experiment. Besides the addition

of $^{13}CH_4$ and $^{15}N_2$ at the beginning of the experiment, the only other injections of exogenous gas were addition of 100% O_2 on days 29, 30, 35, 37, 39, and 44. In all cases 10 mL of O_2 was injected, excepted for the first injection on day 29, which was 5 mL of O_2.

A1.2.2 DISSOLVED INORGANIC CARBON (DIC) AND $\delta^{13}C$ MEASUREMENT

Carbon concentration and isotopic measurements were conducted on 0.2 μm-filtered effluent water samples <24 hours after collection. Measurements were performed on an isotope-monitoring gas chromatography/mass spectrometry (irm-GC/MS) ThermoFinnigan Delta Plus XP isotope-ratio mass spectrometer connected to TRACE GC as previously described (Ijiri et al. 2012).

A1.2.3 DNA EXTRACTION AND SEQUENCING

The M-core-sediment and M-core-water samples were extracted with the MoBio PowerMax soil DNA isolation kit according to manufacturer protocols (~5 g/extraction). The T11, T25, and T45 (duplicate samples of T45 were extracted and sequenced) time points were extracted with the MoBio PowerSoil DNA isolation kit according to manufacturer protocols (~0.5 g/extraction). In addition, duplicate T45 sediments were separately subjected to a simplified hot alkaline extraction (Morono et al. 2014), in which sequential cell lysis is performed in heated 1M sodium hydroxide solution (Supplemental Text).

Samples were prepared for deep sequencing of the V4 region of the 16S rRNA gene according to a slightly modified version of the Earth Microbiome Project's recommended protocol (Mason et al. 2015). New England Biolabs Q5 polymerase enzyme was substituted for 5-PRIME HotMasterMix. Sequencing was performed on an Illumina MiSeq platform at Laragen, Inc., and data processing (joining paired ends, trimming sequences, chimera checking, 97% OTU

picking, and taxonomic assignment) were performed as previously described (Case et al. 2015). Nonmetric multidimensional scaling (NMDS) analyses were performed in the R environment using the 'vegan' package on square-root-transformed tables of relative sequence abundance (Oksanen et al. 2013; R Core Team 2014).

In addition to sequencing of the 16S rRNA gene, an assay of the monooxygenase intergenic spacer region ("MISA") between pmoC and pmoA was performed following previously described protocols (Tavormina et al. 2010; see Supplemental Text for primer sequences, which have been modified since the initial publication) on two samples: the M-core-sediment and T45 sediment. Transformation of the MISA fragment into E. coli was performed with the 10G Elite Solo kit from Lucigen Corporation. Inserts were amplified with the Lucigen Corporation GC Vector Amplification pSMART kit and separately digested with HaeIII and RsaI restriction enzymes in order to generate restriction fragment length polymorphism (RFLP) patterns. Unique inserts were sequenced at Laragen, Inc. The resulting traces were manually checked for quality, translated to amino acid sequences, aligned against pure culture and previously published pmoA fragments in MUSCLE (Edgar 2004), and trimmed to the pmoA amino acids positions 5-49 of *M. capsulatas* Bath (an approach employed in Tavormina et al. 2010). These pmoA fragments, both experimental and from known organisms, were used to generate a 100-bootstrap, maximum likelihood tree in RAxML (Stamatakis 2014).

A1.3 RESULTS

The HP-Core was successfully deployed on the payload of the ROV *Hyperdolphin* during Dive 1555, capturing sediment in a challenging deep-sea environment and retaining sediment within the reaction chamber through recovery onboard ship. The loss of pressure during recovery was unfortunate, but with increased deployment experience and technical improvements, the HP-Core has been successfully recovered without pressure loss (F. Inagaki, personal communication).

Further, the HP-Core successfully maintained high pressure through shipping and during the course of 45 days of experimentation. There is no indication that the HP-Core would not have continued to retain pressure for a significantly longer experimental duration.

Over the course of 45 days, $\delta^{13}C_{DIC}$ was observed to increase, albeit relatively slowly for the first ~30 days (Fig. 4). A model of exponential increase in $\delta^{13}C_{DIC}$, fit from the data between T29 and T32, fits the data from T33-T45 very well (R^2=0.97; Fig. 4); this increase appears to be linked to the addition of O_2 beginning at T29. Stepwise rates of methane oxidation were calculated by subtracting the moles of ^{13}C observed between time points ($t_n - t_{n-1}$), on the assumption that new ^{13}C in the DIC pool represented newly oxidized $^{13}CH_4$ (Eq. 1; a factor of 2 was added because the methane amendment was only 50% $^{13}CH_4$). The trend in methane oxidation rate, by definition, mirrors the increase in $\delta^{13}C_{DIC}$ and shows increasing rates of methane oxidation late in the experiment.

[Eq.1] CH_4 oxidation rate (nM/day) =
$$2 \bullet 10^6 \bullet [([DIC]_n \bullet V_{HP\text{-}Core} - ([DIC]_n/(1+R_{std}((\delta^{13}C_n/1000)+1)) - ([DIC]_{n-1}/(1+R_{std}((\delta^{13}C_{n-1}/1000)+1))] / (n - n_{-1})$$

Within the period of the experiment prior to rapid increase in methane oxidation rate (T0-T29), slow but measurable methane oxidation is observed (c.f. inset of Fig. 4). Within the period T0-T29, more significant increase in $\delta^{13}C$ is apparent from T0-10 than T11-29.

iTag sequencing reveals three categories of microbial communities with our HP-Core and M-core dataset: the M-core water and sediment samples, the HP-Core effluent samples from T11 and T25, and the HP-Core sediment samples from T45 (Fig. 5-6). The M-core samples, both sediment and water, are characterized by high relative abundances of OTUs associated with Candidate Division JS1 bacteria (20-30%), Desulfobacteraceae (6-10%), Methylococcales (2-12%), and various ANME archaea (1-4%; Fig. 5; Table S1). The effluent samples from T11 and T25 lack OTUs associated with AMNE or Methylococcales and instead demonstrate high relative abundances of OTUs associated with Bacteroidetes, delta-, epsilon-, and gamma-proteobacteria;

they are the most abundant in the a Helicobacteraceae-associated OTU (9-17%), which is observed in moderate relative abundance throughout the iTag dataset (Fig. 5). In contrast the sediment samples from T45, similar to the M-core samples, are rich in OTUs associated with Methylococcales (31-30%) and moderately rich in Candidate Division JS1 OTUs (2-4%). However, the T45 sediment samples also share much of the diversity of delta-, epsilon-, and gamma-proteobacteria observed in the HP-Core effluent samples. Furthermore, the T45 sediment samples appear to be characteristically rich in a Pisirickettsiaceae-associated OTU (16-20%) which is relatively poor in the M-core and HP-core effluent samples (Fig. 5). The extraction method (MoBio vs Hot Alkaline) appears to make a measurable but overall small difference in the overall microbial 16S rRNA signature recovered from T45 sediments. When extracted with the MoBio kit, a Methylococcales-associated OTU is recovered at about two-thirds the relative abundance as recovered in samples extracted with the Hot Alkaline method. In contrast, a BD1-5-associated OTU is twice as abundant in MoBio-extracted samples as compared to Hot Alkaline-extracted samples (Fig. 4). These differences are apparent in multidimensional ordination, where the T45 samples overall plot closely together, but are distinctly separate according to extraction method (Fig. 5).

The MISA assay, applied to our M-core-water and T45.1-MoBio sediment from the HP-Core, is intentionally broad enough to recover sequences from both pmoA and amoA genes (Tavormina et al. 2010). Our dataset revealed both gene types, with pmoA more abundance than amoA. Furthermore, the pmoA genes recovered indicate both a diversity of pmoA genes present, as well as a difference in the pmoA composition between the M-core-water and T45.1-MoBio HP-Core sediment. Each of the two samples was particularly rich in one pmoA gene (Patterns 1 and 16 in Fig. 7), although overall these pmoA contigs were more similar to one another than to other pmoA gene from cultured or genome-sequenced organisms in the dataset (Fig. 7).

A1.4 DISCUSSION

A1.4.1 EVALUATION OF SUCCESS OF TECHNICAL GOALS

Of the five goals described for the first iteration of the HP corer, three were successfully achieved, one was partially achieved, and one was unachieved. The first goal, to develop a HP corer deployable on the payload of a piloted or remotely operated deep-sea vehicle, was achieved by deployment with the ROV *Hyperdolphin*. Furthermore, the HP corer was accompanied on the dive by a variety of other sampling devices: 6 push cores, a plastic tote for recovery of push cores previously deployed on the seafloor, a temperature probe and deep-sea high-pressure CO_2 injection system (Ohtomo et al. 2015), and an "M-type" corer. These other instruments and sampling devices were successfully employed on the same dive as the HP corer, lasting exactly two hours on the seafloor. Therefore, deployment of the HP corer does not impede other research aims during valuable at-sea time, and individual dives do not need to be dedicated to exclusive deployment of the HP corer.

The second goal, to sample >10 cm beneath the seafloor, was partially achieved. Due to the nature of the environment on the seafloor at the Joetsu Knoll, rather than choose to employ the HP corer like a traditional push core (straight down into sediment), we instead abraded the HP corer against a wall of mixed sediment, methane hydrate, and bacterial mats. This sampling action, while not strictly the equivalent to a push-core-like method, nonetheless captured a significant amount of sediment that might have been similar to the amount captured in a push-core-like maneuver. Abrading the HP corer against a wall of sediment allowed us to visualize the corer at all times, preferable for a first deployment of the new technology. There was no indication that pushing the HP corer vertically into a flat sediment bed would not have produced good sampling results as well.

Unfortunately, the third technical goal, to maintain HP conditions through recovery onboard ship, was not achieved. The HP corer arrived at the sea surface having lost pressure, most likely as

a result of sediment grains compromising the Teflon seal where the core interfaces with the core liner. Pressure was quickly restored with filtered seawater, and held stably over the next 24 hours, which encompassed the remainder of the time at sea. It is possible that the sediment grains compromising the vessel's seal were washed out during repressurization, enabling the maintenance of HP onboard. The HP corer successfully maintained pressure during shipment to the Kochi Core Center, demonstrating its resilience to shipping and handling. In subsequent deployments, the HP corer has been successfully employed and recovered onboard without pressure loss. Therefore, the system is capable of achieving the third goal of maintaining pressure from deep-sea sample collection to onshore incubation, but caution must be taken when choosing a sampling location and type.

Goals four and five, to be able to add liquid and gas amendments to the incubator during an experiment, and to be capable of extracting time-resolved output samples from the incubator, were successful. Sampling from the outflow port was performed daily. Sampling generated minor loss of internal pressure (generally less than 1 MPa, depending on user technique; Fig. 3). Pressure was restored daily by pumping in of fresh, sterile liquid media. Additionally, gas-phase amendments were injected in-line with the liquid media throughout the incubation.

Based on our experience with this first deployment, when deploying this first generation of the HP corer we recommend choosing carefully the sampling site and being extra cautious to avoid unnecessary sediment disturbance – excess sediment clouding bottom waters increase the likelihood of a compromised Teflon seal. Depending on time constraints, it is advisable to choose a seafloor location, hold the vehicle steady for enough time to let particles settle out, and only then to perform sampling. Additionally, it is good practice to perform HP corer sampling as the last function of a deep-sea dive. This minimizes both the amount of jostling on the HP corer and the time duration between sampling and recovery onboard ship.

Some recommendations can be made for future iterations of the technical design for the HP corer. The first generation had one outflow sampling port, located at the top of the vessel. This

port worked well, but daily outflow samples only represented the pelagic microbial community at the top of the incubation. Based on our 16S rRNA sequencing data (Fig. 5-6), we suspect this resulted in observing a different microbial community from that which resided deeper in the incubation column, in particular in the sediments settled at the bottom. Future iterations of the HP corer would be improved by having multiple outflow ports located at various heights along the incubation column. Similarly, the inflow port for adding liquid media and gas amendments only existed at the bottom of the incubation column. Although this worked for our experimental design, it is conceivable that future experiments would benefit from an ability to add amendments from either the top or bottom of the chamber – requiring engineering of additional inflow ports in future designs.

A1.4.2 MICROBIALLY MEDIATED METHANE OXIDATION DURING HP INCUBATION WITH $^{13}CH_4$

During the 45-day incubation, DIC and $\delta^{13}C_{DIC}$ data (collected and analyzed daily) suggested methane oxidation (Fig. 4). In the first 11 days (T0-T11) of the experiment methane oxidation, as determined by incorporation of ^{13}C into the DIC pool, appeared to be accelerating. However, it then plateaued and between T11 and T28 little methane oxidation was observed. We suspect the initial methane oxidation occurred by aerobic processes, and ceased when O_2 was fully consumed. If so, the theoretical amount of O_2 consumed by aerobic methanotrophy can be calculated by stoichiometric conversion using the following equation and ^{13}C data to track the number of moles of methane consumed between T0 and T11:

$$CH_4 + 2O_2 \rightleftharpoons CO_2 + 2H_2O \qquad \text{Eq. 2}$$

With 4.25 μmol of CH_4 consumed between T0 and T11 (calculated from data in Fig. 4), complementary oxidation of 8.50 μmol of O_2 is required. This is a relatively small amount compared to known bottom water O_2 concentrations in the Japan Sea (>220 μmol/kg), but it is likely that through the course of shipment of the HP corer to Kochi and static storage for twelve days at 4˚C prior to stable isotope amendment, a significant amount of aerobic respiration could have consumed most of the available O_2 in the incubation chamber. Respiratory processes could have continued during T0-T11, all independent of ^{13}C label and thus undetected by our geochemical measurements.

Between T11 and T28, the rate of methane oxidation was slower, with 7 μmol of CH_4 oxidized during the seventeen days. If we assume that during this period aerobic methanotrophy was replaced by sulfate-coupled anaerobic methanotrophy (AOM; Eq. 3), then only 7 μmol of sulfate are stoichiometrically required:

$$CH_4 + SO_4^{2-} \rightleftharpoons HS^- + HCO_3^- + H_2O \qquad \text{Eq. 3}$$

This is well within the bounds of seawater chemistry, where sulfate is generally present at ~28 mmol/kg. Anaerobic conditions during this period were supported by oxidation-reduction potential (ORP) measurements. When the first ORP measurement was taken, at T29, it was at the very reduced value of -300 mV. Although we do not have ORP data to help define exactly when anaerobic conditions began, it is clear than by T29 anaerobic conditions prevailed.

Further evidence for aerobic methanotrophy was the nearly immediate onset of an exponential rise in $\delta^{13}C_{DIC}$ upon additions of O2 beginning at T29. Over the course of six O_2 injections between T29 and T45, 55 mL of 100% were added to the incubation chamber, corresponding to 242 μmol of O_2. Conversion of $\delta^{13}C_{DIC}$ into consumption of methane molecules, we find aerobic methanotrophy would have consumed 143 μmol of O_2 between T29 and T45, consistent with our known amount of injected O_2. Regular ORP measurements between T29 and T45 reflected the addition of oxygen but also its rapid consumption: after T30, ORP averaged -33 mV (max=67 mV, min=-120 mV).

The hypothesis of sequential aerobic, anaerobic, and aerobic phases in the HP incubation chamber are additionally supported by sequencing data of the 16S rRNA and pmoA genes (Fig. 5-7). M-core samples, taken from sediments nearby the sampling location for the HP corer at the Joetsu Knoll, revealed the presence of anaerobic methanotrophs spanning the ANME-1, -2, and -3 clades as well as likely aerobic methanotrophs of the gammaproteobacterial *Methylococcales* order (Fig. 5). Thus, the requisite microorganisms for aerobic and anaerobic methane oxidation metabolisms were most likely present, at least at the time of sampling, in the sediments in the HP incubation chamber. The presence of aerobic methanotrophs in the M-core, and therefore also likely the HP corer, is further supported by abundant recovery of pmoA genes related to gammaproteobacterial methane oxidizers (Fig. 7). In addition, the microbial diversity data collected from the HP incubation chamber at T45 provides an excellent portrait of the microorganisms inhabiting the HP-incubated sediments at the end of experimentation. These sediments abundantly contained a *Methylococcales*-associated OTU but lacked ANME OTUs (Fig. 5). Sequences from the pmoA gene also revealed an abundance of gammaproteobacterial methanotrophs in the T45 sediment.

On the bulk scale, the sediments from the HP incubation at T45 differed in 16S rRNA gene diversity from the M-core samples (Fig. 6). This difference was likely influenced by several factors. First, because the M-core and HP core were not sampled at the exact same position on the seafloor, we cannot rule out spatial heterogeneity of microbial diversity and geochemistry. Indeed, short-range spatial diversity has been recorded previously at methane seep locations (Orphan et al. 2004). Second, it is likely that during the course of the 45-day incubation microbial successional processes resulted in a change in microbial diversity. Some of the major differences in 16S rRNA gene diversity between the M-core samples and the HP core T45 samples were the loss of ANME-associated OTUs and the retention of only one of two major *Methylococcales*-associated OTUs (Fig. 5).

In addition, the T45 sediments are richer in other gammaproteobacterial (e.g., *Colwelliaceae*) and epsilonproteobacterial OTUs (e.g., *Campylobacteraceae*) than the M-core sediments. These OTUs were observed at high relative abundance in the T11 and T25 effluent samples, suggesting they grew up during the course of incubation (Fig. 5). A *Colwelliaceae*-associated OTUs was observed to colonize sterile carbonate substrates in 13-month *in situ* methane seep incubations, suggesting the clade is adaptable to changing environmental parameters (Case et al. 2015). Similarly, many members of the *Epsilonproteobacteria*, including *Campylobacteraceae*, are sulfur-oxidizers (Campbell et al. 2006). As oxygen was depleted in the HP incubation chamber, sulfate would have been used as an electron acceptor, producing sulfide (both in AOM and anaerobic respiratory processes). This might explain the increased abundance of epsilonproteobacterial-associated OTUs at T11 onward.

The T45 sediments demonstrated slightly different 16S rRNA gene diversity whether extracted using a MoBio kit or with the hot alkaline lysis method (Fig. 6). This is likely due to differential lysis of cell walls (Morono et al. 2014), with the hot alkaline method yielding significantly higher relative abundances of *Methylococcales*-associated OTUs than the MoBio kit. Overall, however, the similarity among all T45 sediment samples (regardless of extraction method) is higher than between T45 samples and either T11 and T25 effluent samples or the M-core samples (Fig. 6).

A1.4.3 COMPARISON OF METHANE OXIDATION RATES MEASURED IN THE HP INCUBATION TO PREVIOUSLY PUBLISHED RATES

The rates of methane oxidation calculated from our high pressure experiment are similar to, but not higher than, previously published rates of aerobic and anaerobic methane oxidation (Fig. 8). Methane oxidation rates are often observed to vary by many orders of magnitude, depending on both methane and electron acceptor concentrations (Rudd and Hamilton 1974;

Harrits and Hanson 1980; Devol 1983; Iversen et al. 1987; Reeburgh et al. 1991; De Angelis and Lilley 1993; Ward and Kilpatrick 1993; Hoehler et al. 1994; Joye et al. 1999; Valentine et al. 2001; Nauhaus et al. 2002; Girguis et al. 2003; Carini et al. 2005). Our rates of methane oxidation between T11 and T29 (presumed to be AOM) are most similar to rates observed in Hoehler et al. (1994), in which sediment samples from shallow (10 mbsl) waters were incubated with $^{14}CH_4$. However, our relative rates of aerobic vs anaerobic methanotrophy are in contrast to multiple studies which examined both aerobic and anaerobic methane oxidation rates (Reeburgh et al. 1991; Hoehler et al. 1994; Joye et al. 1999). We note that of these studies, ours is the only one to be performed at high pressure. Our observation of higher aerobic than anaerobic methane oxidation rates may reveal a difference in sensitivity of aerobic vs anaerobic microorganisms to pressure, although we cannot definitely conclude this without more detailed physiological studies of pure cultures and environmental samples at a variety of pressure conditions.

A1.5 CONCLUSIONS

We have presented microbiological and geochemical data representing the first successful *in situ* deployment of a new high pressure chamber. The chamber fulfills most of the engineering goals, including: (i) deployable on the payload of a piloted or remotely operated deep-sea vehicle, (iv) containing input ports for liquid media and gas phase amendments, and (v) including an outlet port for time-resolved sampling without loss of pressure. Other goals, including (ii) the ability to sample >10 cm below the seafloor and (iii) ability to maintain HP conditions through recovery onboard and shipment to onshore laboratories, were partially successful or have been successful subsequent to our experiments. Future iterations of the HP corer design will incorporate improvements for sampling (e.g., multiple effluent outflow ports), and *in situ* deployment of the HP corer will hopefully increase over time as multiple laboratory groups gain access to the technology.

We demonstrated the usefulness of the HP corer by recovering sediments from the Joetsu Knoll, a methane-rich outcrop at ~1,000 mbsl in the Japan Sea. Incubation of these sediments at 10 MPa for 45 days in the presence of $^{13}CH_4$ demonstrated likely periods of aerobic and anaerobic methane oxidation, supported by multiple geochemical and microbiological measurements. The high rates of aerobic methane oxidation as compared to anaerobic methane oxidation differed from previous studies, further demonstrating the usefulness of the HP corer and importance of including pressure as a variable in future studies.

A1.6 ACKNOWLEDGEMENTS

We thank the crew of the R/V *Natsushima*, cruise 13-15, for their help at sea, without which this work would not have been possible. Masazumi Tsutsumi, Yoko Ohtomo, Go-Ichiro Uramoto, Tatsuhiko Hoshino, Takeshi Terada, Nan Xiao, and other members of the Kochi Core Center's Geomicrobiology Group helped with field and laboratory work. David Case was supported by a National Science Foundation (NSF) Graduate Research Fellowship as well as the East Asian and Pacific Summer Institutes (EAPSI) Summer Fellowship, co-funded by the NSF and the Japan Society for the Promotion of Science (JSPS).

A1.7 FIGURES

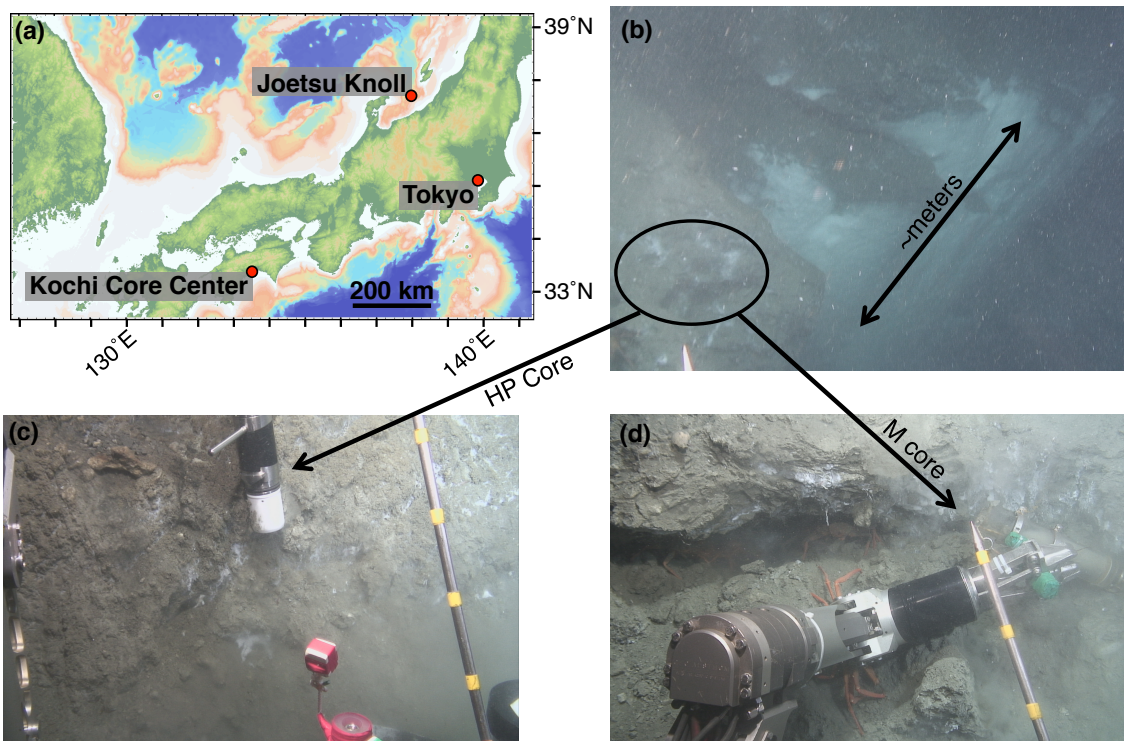

Figure 1. Contextualization of study site. (a) Map of central Japan, including the Joetsu Knoll study site. (b) Image capture from the ROV *Hyperdolphin* dive 1555, demonstrating the sampling location along a several meter-sized wall of methane clathrate. Samples were taken from roughly in the area of the black circle, near methane clathrates but with clear access to sediments for capture. (c) Sediment captured using the HP Core. (d) Sediment captured using the M-core.

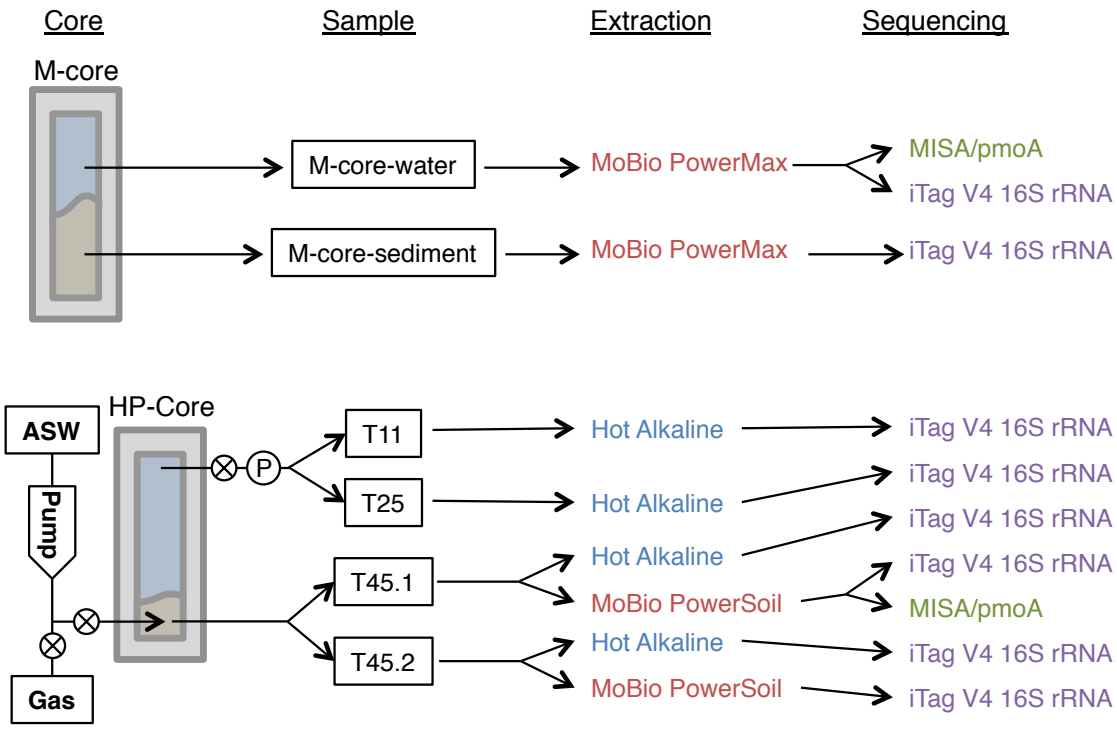

Figure 2. Schematic of samples and analyses. The M-core provided contextual evidence for the microbial diversity present in sediments at the time of sampling. The HP-Core, due to its technical aspects, was only able to be sampled from the effluent port during the course of experimentation, and from the sediment settled at the bottom during experimental takedown. All samples were subjected to 16S rRNA iTag sequencing. Two sediment samples, one each from the M-core and HP-Core T45, were subjected to the MISA/pmoA assay which specifically targets methane- and ammonia-oxidizing microorganisms.

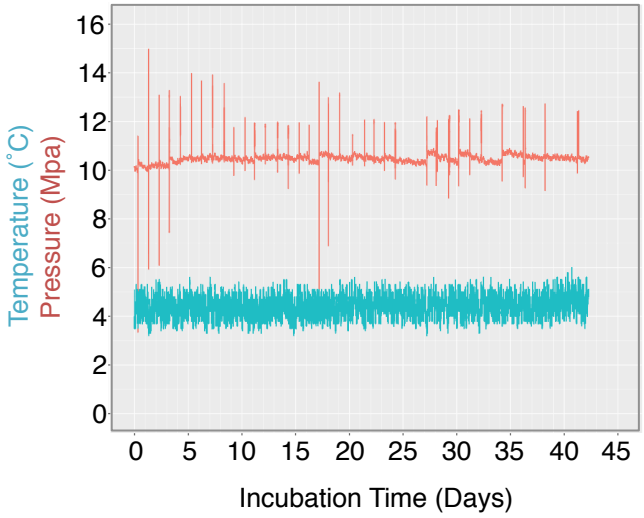

Figure 3. Log of HP-Core temperature and pressure over the duration of incubation. Temperature was maintained at ~4.5˚C by storing the HP-Core in a walk-in refridgerator throughout the experiment. Pressure was maintained at ~10 MPa (chosen to match the environmental pressure at the sampling depth of 985 mbsl) by injection of sterile artificial seawater via modified HPLC pump (c.f. Methods). Spikes in the pressure log record the daily effluent sampling for $\delta^{13}C_{DIC}$, during which time pressure fluctuated as the effluent port was opened. Over the course of >40 days, user technique improved and the fluctuations in pressure decreased in frequency and magnitude.

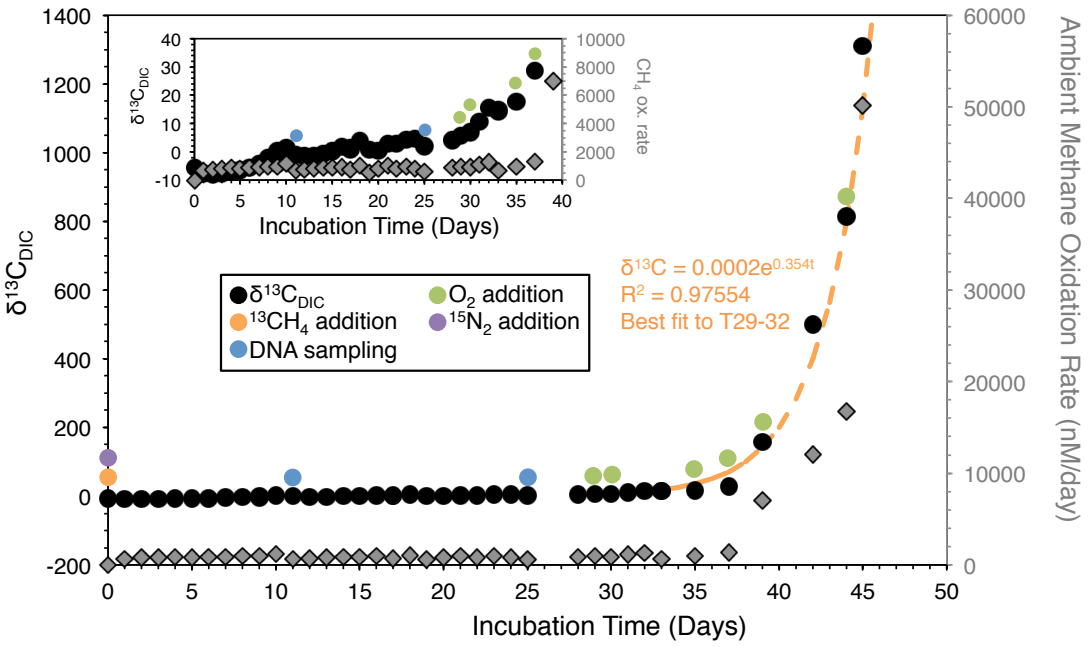

Figure 4. Time-resolved record of HP-Core incubation. Daily $\delta^{13}C_{DIC}$ measurements are given in black circles. Colored circles represent sampling or amendments (see legend). Gray diamonds are the calculated methane oxidation rate between each day and the day prior. Inset shows the same data on a smaller y-axis in order to better resolve trends within the first 40 days of the experiment.

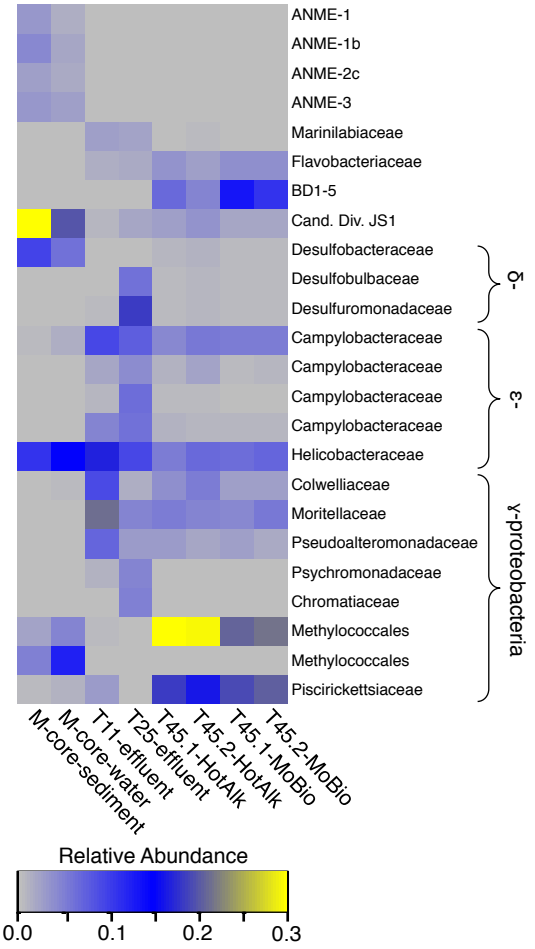

Figure 5. Heat map of major OTUs identified in the 16S rRNA iTag dataset. OTUs were only selected for presentation if they were present at >2% relative abundance in the M-core, HP-Core-effluent (T11 & T25), or HP-Core-sediment (T45) samples. M-core samples are characterized by their richness in Candidate Division JS1 bacteria. T11 and T25 effluent samples host a wide diversity of δ-, ε-, and ɣ-proteobacteria, but notably differ from the T45 samples which are rich in a Methylococcales-associated OTU. The full table of 16S rRNA data is provided in the Supplementary Information.

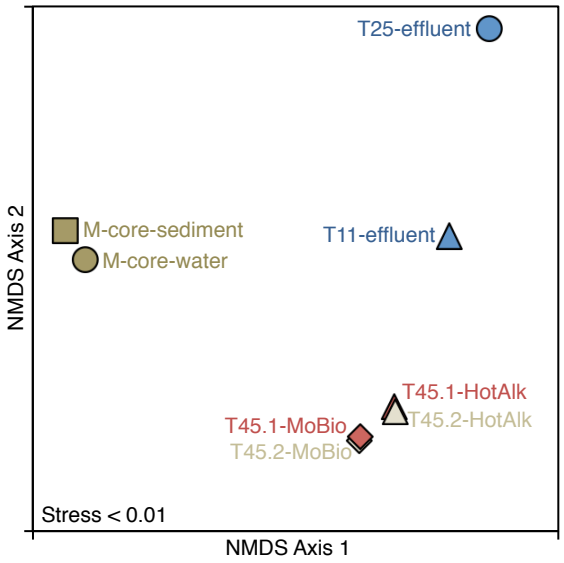

Figure 6. Nonmetric multidimensional scaling (NMDS) plot of 16S rRNA iTag data from this study. The microbial communities in the samples naturally break into three categories: M-core sediments, HP-Core effluent, and HP-Core sediments. Among the HP-Core sediments, DNA extraction method accounts for a measurable but small difference in recovered microbial community composition.

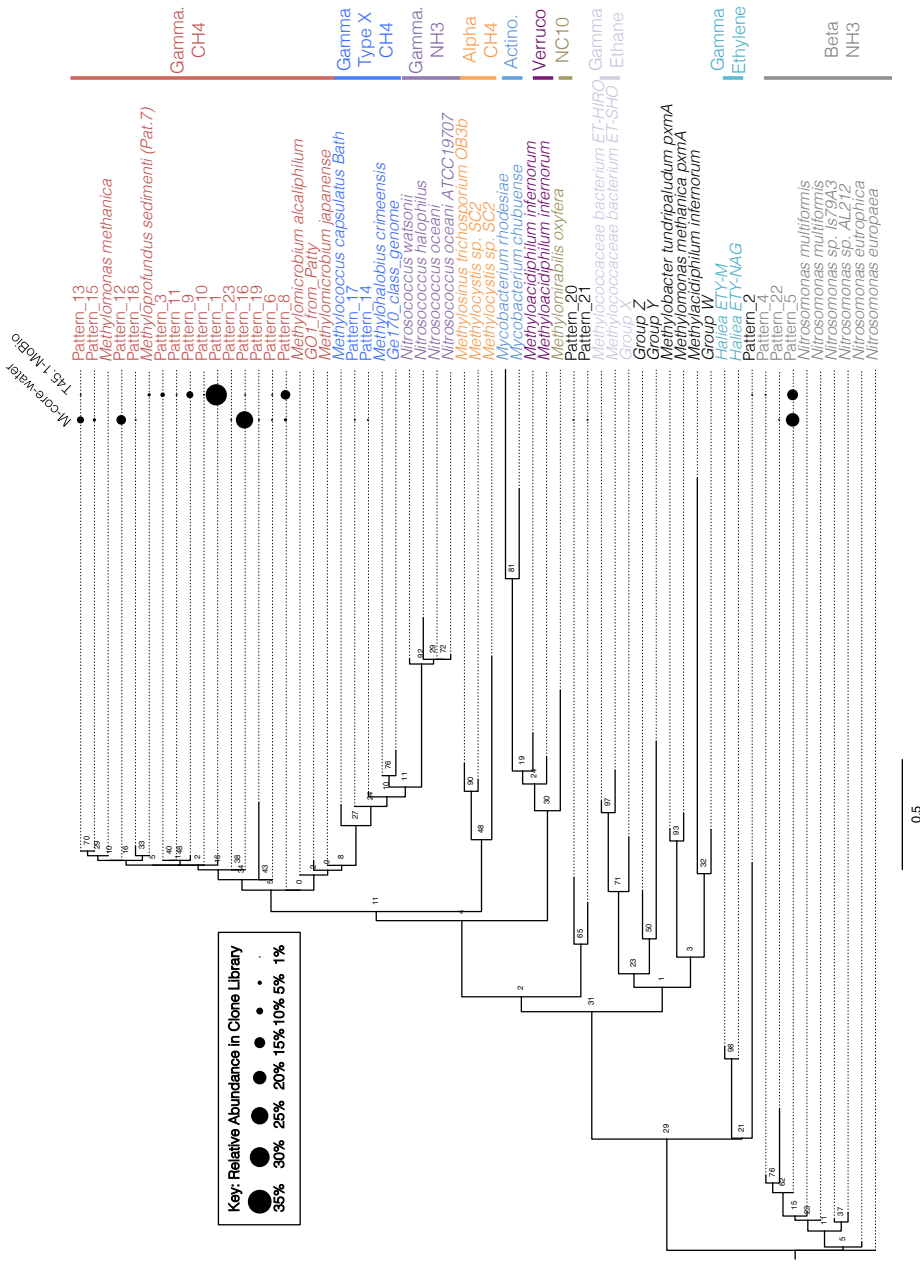

Figure 7. Maximum likelihood tree of pmoA (trimmed to amino acids 5-49 of *M. capsulatas Bath*) sequences generated in the MISA assay. Sequences from this study are defined as "Pattern_*x*" according to unique HaeIII and RsaI RFLP profiles. Sequences from cultured organisms and sequenced genomes of methane- and ammonia-oxidizers are given with their species name as appropriate. Multiple sequences alignments were generated in MUSCLE and the tree was generated in RAxML with 100 bootstraps. Black circles represent relative abundance of pmoA sequences in the M-core-water and T45.1-MoBio samples. The largest contrast between the two samples is seen in the abundance of different methane-oxidizing organisms.

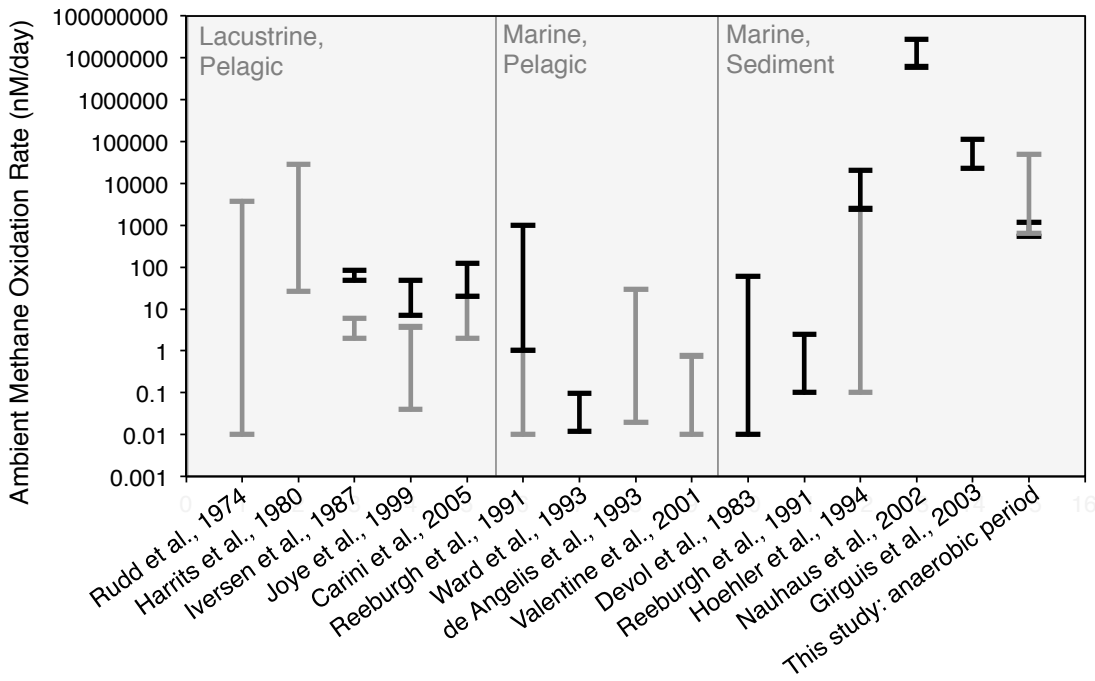

Figure 8. Comparison of ambient methane oxidation rate measurements between this study and previous studies. Two values are given for this study: calculated methane oxidation rates for the period of putative aerobic conditions (T0-T10 & T29-T45) and putative anaerobic conditions (T11-T28). Methane oxidation rates derived from aerobic methanotrophy are given in gray, whereas rates from anaerobic methane oxidation are given in black.

A1.8 SUPPLEMENTAL MATERIAL: TEXT

Composition of artificial seawater used to pressurize HP Core daily.
The artificial seawater (ASW) was made by reference to composition of methanosarchina sp. Strain BT-MS1, without the addition of yeast extract or any carbon source. Trace element solution used was not for methanosarchina but methanothermococcus okinawensis, because it was hypothesized that a trace element solution including $CuSO_4$ may be better to stimulate methanotrophy in our incubation. After all components were dissolved, the ASW was autoclaved.

Specific protocol for hot alkaline DNA extraction.
The protocol is modified after (Morono et al. 2014). Recipes for lysis and neutralization buffer can be found in the original publication.

1. Prewarm water baths to 50˚C and 70˚C.
2. Add 50 µL of lysis solution to ~50 mg sediment in PCR tube.
3. Heat for 20 min at 50˚C.
4. Centrifuge in mini-fuge for 30 sec at 25˚C.
5. Transfer supernatant to new PCR tube pre-loaded with 37.5 µL of neutralization buffer.
6. Wash remaining sample with 50 µL of 50˚C water.
7. Centrifuge in mini-fuge for 30 sec at 25˚C.
8. Transfer supernatant to the PCR tube from step (5).
9. Add 50 µL of lysis solution to remaining sediment in PCR tube.
10. Heat for 20 min at 70˚C.
11. Centrifuge in mini-fuge for 30 sec at 25˚C.
12. Transfer supernatant to new PCR tube pre-loaded with 37.5 µL of neutralization buffer.
13. Wash remaining sample with 50 µL of 70˚C water.
14. Centrifuge in mini-fuge for 30 sec at 25˚C.
15. Transfer supernatant to the PCR tube from step (12).
16. Combine supernatant from (8) and (15) into one tube.

Primers used for MISA assay.
The MISA assay involves two PCR steps, both targeting the pmoC-pmoA intergenic spacer region. Since the original MISA publication in 2010 (Tavormina et al. 2010), the primers have been further modified. The primers employed in this study were:

PCR#1, Forward primer, spacer_pmoC_599f:	`AAY GAR TGG GGH CAY RCB TTC`
PCR#1, Reverse primer, spacer_pmoA_192r:	`TCD GMC CAR AAR TCC CAR TC`
PCR#2, Forward primer, spacer_pmoC626_mod_f:	`RCB TTC TGG HTB ATG GAA GA`
PCR#2, Reverse primer, spacer_pmoA_189r:	`CCA RAA RTC CCA RTC NCC`

A1.9 SUPPLEMENTAL MATERIAL: TABLES

Table S1. Relative abundance of 16S rRNA gene iTag data for all samples in the study.

Supplementary Table 1 can be found in .xlsx format in the Caltech repository along with this thesis.

A1.10 REFERENCES

Birrien, J.-L., X. Zeng, M. Jebbar, M. A. Cambon Bonavita, J. Quérellou, P. Oger, N. Bienvenu, X. Xiao, and D. Prieur. 2011. Pyrococcus yayanosii sp. nov., an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol **61**: 2827–2881.

Campbell, B. J., A. S. Engel, M. L. Porter, and K. Takai. 2006. The versatile ε-proteobacteria: key players in sulphidic habitats. Nat Rev Micro **4**: 458–468.

Carini, S., N. Bano, and G. LeCleir. 2005. Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). Environmental Microbiology **7**: 1127-1138.

Case, D. H., A. L. Pasulka, J. J. Marlow, B. M. Grupe, L. A. Levin, and V. J. Orphan. 2015. Methane Seep Carbonates Host Distinct, Diverse, and Dynamic Microbial Assemblages. mBio **6**: e01348–15.

De Angelis, M., and M. Lilley. 1993. Methane oxidation in deep-sea hydrothermal plumes of the Endeavour Segment of the Juan de Fuca Ridge. Deep Sea Research I **40**: 1169-1186.

Devol, A. H. 1983. Methane oxidation rates in the anaerobic sediments of Saanich Inlet1. Limnology and Oceanography **28**: 738–742.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl. Acids Res. **32**: 1792–1797.

Gamo, T., and Y. Horibe. 1983. Abyssal Circulation in the Japan Sea. Journal of the Oceanographical Society of Japan **39**: 220–230.

Girguis, P., V. Orphan, S. Hallam, and E. F. DeLong. 2003. Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. Applied and Environmental Microbiology **69**: 5472–5482.

Harrits, S. M., and R. S. Hanson. 1980. Stratification of aerobic methane-oxidizing organisms in Lake Mendota, Madison, Wisconsin1. Limnology and Oceanography **25**: 412–421.

Hoehler, T. M., M. J. Alperin, D. B. Albert, and C. S. Martens. 1994. Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium. Global Biogeochemical Cycles **8**: 451.

Ijiri, A., N. Harada, A. Hirota, U. Tsunogai, N. O. Ogawa, T. Itaki, B.-K. Khim, and M. Uchida. 2012. Biogeochemical processes involving acetate in sub-seafloor sediments from the Bering Sea shelf break. Organic geochemistry **48**: 47–55.

Iversen, N., R. S. Oremland, and M. J. Klug. 1987. Big Soda Lake (Nevada). 3. Pelagic methanogenesis and anaerobic methane oxidation. Limnology and Oceanography **32**: 804-814.

Joye, S. B., T. L. Connell, L. G. Miller, R. S. Oremland, and R. S. Jellison. 1999. Oxidation of ammonia and methane in an alkaline, saline lake. Limnology and Oceanography **44**: 178–188.

Kato, C., Y. Nogi, and S. Arakawa. 2008. Isolation, Cultivation, and Diversity of Deep-Sea Piezophiles, p. 203–217. *In* C. Michiels, D.H. Bartlett, and A. Aertsen [eds.], High-Pressure Microbiology. ASM Press.

Kato, D. C. 2011. Cultivation Methods for Piezophiles, p. 719–726. *In* Extremophiles Handbook. Springer Japan.

Kim, S. J., and C. Kato. 2010. Sampling, Isolation, Cultivation, and Characterization of Piezophilic Microbes, p. 3869–3881. *In* K.N. Timmis [ed.], Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg.

Kubo, Y., Y. Mizuguchi, F. Inagaki, and K. Yamamoto. 2014. A new hybrid pressure-coring system for the drilling vessel *Chikyu*. Sci. Dril. **2**: 1–7.

Kyo, M., Y. Tuji, H. Usui, and T. Itoh. 1991. Collection, Isolation And Cultivation System For Deep-sea Microbes Study. Proceedings of the Ocean Technologies and Opportunities in the Pacific. IEEE. 419–423.

Mason, O. U., D. H. Case, T. H. Naehr, R. W. Lee, R. B. Thomas, J. V. Bailey, and V. J. Orphan. 2015. Comparison of Archaeal and Bacterial Diversity in Methane Seep Carbonate Nodules and Host Sediments, Eel River Basin and Hydrate Ridge, USA. Microbial Ecology **70**: 776–784.

Matsumoto, R., Y. Okuda, C. Aoyama, A. Hiruta, Y. Ishida, M. Sunamura, H. Numanami, H. Tomaru, G. T. Snyder, J. Komatsubara, R. Takeuchi, and M. Hiromatsu. 2005. Methane plumes over a marine gas hydrate system in the eastern margin of Japan Sea: A possible mechanism for the transportation of sub-surface methane to shallow waters. 749–754.

Morono, Y., T. Terada, T. Hoshino, and F. Inagaki. 2014. Hot-Alkaline DNA Extraction Method for Deep-Subseafloor Archaeal Communities. Applied and Environmental Microbiology **80**: 1985–1994.

Nagata, T., C. Tamburini, J. Arístegui, F. Baltar, A. B. Bochdansky, S. Fonda-Umani, H. Fukuda, A. Gogou, D. A. Hansell, R. L. Hansman, G. J. Herndl, C. Panagiotopoulos, T. Reinthaler, R. Sohrin, P. Verdugo, N. Yamada, Y. Yamashita, T. Yokokawa, and D. H. Bartlett. 2010. Emerging concepts on microbial processes in the bathypelagic ocean – ecology, biogeochemistry, and genomics. Deep Sea Research Part II: Topical Studies in Oceanography **57**: 1519–1536.

Nauhaus, K., A. Boetius, and M. Kruger. 2002. In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. Environmental Microbiology **4**: 296-305.

Ohtomo, Y., A. Ijiri, Y. Morono, Y. Ikegawa, H. Suenaga, D. Case, H. Machiyama, F. Yamamoto, S. Goto, T. Fukuba, M. Nagasawa, M. Matsumoto, and F. Inagaki. 2015. CO_2 emission and shallow-type methane hydrate decomposition experiment on deep-sea floor. JAMSTEC-R **20**: 61–71.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. Minchin, R. B. OHara, G. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. vegan: Community Ecology Package.

Orphan, V., W. Ussler, T. H. Naehr, C. H. House, Hinrichs, and C. K. Paull. 2004. Geological, geochemical, and microbiological heterogeneity of the seafloor around methane vents in the Eel River Basin, offshore California. Chemical Geology **205**: 265–289.

Picard, A., and T. G. Ferdelman. 2012. Microbial activity in deep marine sediments: does pressure make the difference? J. Phys.: Conf. Ser. **377**: 1–5.

R Core Team. 2014. R: A language and environment for statistical computing.

Reeburgh, W., B. Ward, and S. Whalen. 1991. Black Sea methane geochemistry. Deep Sea Research **38**: 1189-1210.

Rudd, J., and R. Hamilton. 1974. Measurement of microbial oxidation of methane in lake water. Limnology and Oceanography **19**: 519-524.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 1-2.

Tavormina, P., W. Ussler, S. Joye, B. Harrison, and V. Orphan. 2010. Distributions of putative aerobic methanotrophs in diverse pelagic marine environments. The ISME Journal **4**: 700–710.

Valentine, D., D. Blanton, and W. Reeburgh. 2001. Water column methane oxidation adjacent to an area of active hydrate dissociation, Eel River Basin. Geochimica et Cosmochimica Acta **65**: 2633-2640.

Ward, B., and K. Kilpatrick. 1993. Methane oxidation associated with mid-depth methane maxima in the Southern California Bight. Continental Shelf Research **13**: 1111–1122.

Yanagawa, K., M. Sunamura, M. A. Lever, Y. Morono, A. Hiruta, O. Ishizaki, R. Matsumoto, T. Urabe, and F. Inagaki. 2011. Niche Separation of Methanotrophic Archaea (ANME-1

and -2) in Methane-Seep Sediments of the Eastern Japan Sea Offshore Joetsu. Geomicrobiology Journal **28**: 118–129.

Yanagibayashi, M., Y. Nogi, L. Li, and C. Kato. 1999. Changes in the microbial community in Japan Trench sediment from a depth of 6292 m during cultivation without decompression. FEMS Microbiology Letters **170**: 271–279.

Yayanos, A. A., A. S. Dietz, and R. Van Boxtel. 1981. Obligately barophilic bacterium from the Mariana trench. Proceedings of the National Academy of Sciences **78**: 5212–5215.

Zeng, X., J.-L. Birrien, Y. Fouquet, G. Cherkashov, M. Jebbar, J. Quérellou, P. Oger, M. A. Cambon Bonavita, X. Xiao, and D. Prieur. 2009. Pyrococcus CH1, an obligate piezophilic hyperthermophile: extending the upper pressure-temperature limits for life. The ISME Journal **3**: 873–876.

Zhang, Y., X. Li, D. H. Bartlett, and X. Xiao. 2015. Current developments in marine microbiology: high-pressure biotechnology and the genetic engineering of piezophiles. Current Opinion in Biotechnology **33**: 157–164.

Zobell, C. E., and R. Y. Morita. 1957. Barophilic Bacteria in Some Deep Sea Sediments. Journal of Bacteriology **73**: 563.