Diversity, stratification and *in situ* metabolism of anaerobic methanotrophic archaea in Nyegga cold seeps

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Contents

Scientific environment

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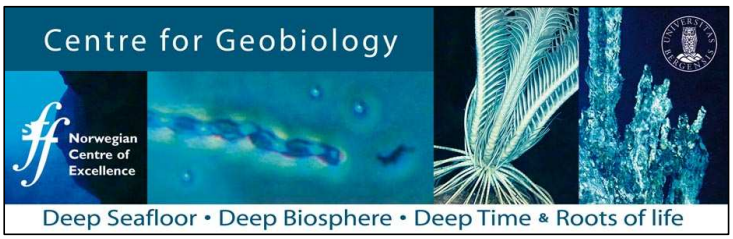

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After 3 years at the Center for Geobiology I realize that being involved in research on exclusive sampling material using the newest technology and interpreting the results from an integrated geological and biological point of view has given me a unique start on my scientific career, and feel privileged to have had this opportunity.

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Abstract

Anaerobic methanotrophic Archaea (ANME) have considerable importance in a global environmental context as they are considered the major biological sink for methane due to their ubiquitous distribution in anaerobic methane-enriched sediments world-wide. Together with a sulfate-reducing partner, ANME perform anaerobic oxidation of methane (AOM) coupled to sulfate reduction. Knowledge about the syntrophic lifestyle, adaptations to varying geochemical conditions and the biochemistry of these uncultivated archaea is limited. In this study, the microbial communities were sampled from sediment cores that were retrieved from two locations at the Nyegga cold seep area. The cores were sampled inside the G11 pockmark and at the CN03 area, which are characterized by differences in the methane fluid flux. The parallel pyrosequencing of 16S rRNA gene amplicons provided taxonomic information with high resolution for analyses of the microbial communities. New information about the vertical transition of ANME clades in sediments, as well as information about their co-occurring microbial assemblages was obtained. While the dominating ANME-1 population was observed as free-living cells in chains, high abundances of either *Deltaproteobacteria* and Candidate division JS-1, or Deep Sea Archaeal Group (DSAG) and Marine Group 1 (MG-1) were found in ANME-2 dominated horizons in the cores. The ANME-2a/b population seemed most sensitive to elevated sulfide concentrations, and the presence of different dominating ecotypes of ANME-2a/b at the two locations could indicate adaptations to the site-specific geochemical conditions. The free-living ANME-1 clade, tolerating elevated sulfide concentration, accounted for up to 89.2% of the retrieved 16S rRNA gene sequences in the sediment core sampled inside the G11 pockmark. A coupled metagenomic and metaproteomic approach was developed to study the *in situ* metabolism of this microbial community. Pyrosequencing of the total DNA resulted in 16.6 Mbp of sequence information that was assembled into contigs and subsequently used as a custom database, in addition to the publically available ANME sequence

information, for protein identification. The results showed that the enzymes in the reverse methanogenesis pathway (except methylene tetrahydromethanopterin reductase), associated electron-accepting complexes, and proteins possibly involved in cold adaptations and production of gas vesicles were expressed by ANME-1. In addition, a novel adenylyl-suflate (APS) reductase, affiliated with a Gram-positive sulfate reducing bacteria (SRB) was identified. Overall, the results challenge the hypothesis of an obligate syntrophy with SRB for ANME-1. Future studies are needed to reveal if the free-living ANME-1 is both a methanotrophic and a sulfate-reducing archaeon that completes the process of AOM itself. However, the coupled metagenomic and metaproteomic approach was effective in assessing the major *in situ* metabolic processes in the cold seep sediments.

List of publications

Paper I:

Roalkvam, I., Jørgensen, S. L., Chen, Y., Stokke, R., Dahle, H., Hocking, W. P., Lanzén, A., Haflidason, H. & Steen, I. (2011) New insights into stratification of anaerobic methanotrophs in cold seep sediments. *FEMS Microbiology Ecology* 78(2):233-43

Paper II:

Stokke, R., Roalkvam, I., Lanzén, A., Haflidason, H. & Steen, I. (2012) Integrated metagenomic and metaproteomic analyses of an ANME-1 dominated community in marine cold-seep sediments. *Environmental Microbiology* 14(2):1333-46

Paper III:

Roalkvam, I., Dahle, H., Chen, Y., Jørgensen, S. L., Haflidason, H. & Steen, I. (2012) Fine-scale community structure analysis of ANME in Nyegga sediments with high and low methane flux. *Frontiers in Extreme Microbiology* (In revision)

List of abbreviations

ANME	anaerobic methanotrophic archaea
AOM	anerobic oxidation of methane
BSR	bottom simulating reflector
cmbsf	centimeters below the seafloor
DNA	deoxyribonucleic acid
DSAG	Deep-Sea Archaeal Group
FISH	fluorescence in situ hybridization
GHSZ	gas hydrate stability zone
H_4MPT	tetrahydromethanopterin
IPL	intact polar lipids
LGT	lateral gene transfer
mbsf	meters below the seafloor
mcrA	gene encoding methyl coenzyme M reductase, α subunit
MG-1	Marine Group 1
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SMTZ	sulfate methane transition zone
SRB	sulfate-reducing bacteria

Introduction

1. Background

More than 70% of the earth's surface is covered with seawater, but this enormous habitat and the seafloor beneath it are not well explored. In 1948 Frances P. Shepard stated: "*Until recent years much more was known about the surface of the moon than about the vast areas that lie beneath three-fourths of the surface of our own planet*" (Shepard, 1948). Since then, the records of marine species (both prokaryotes and eukaryotes) from the water column has increased to ~7 million individual records from ~172000 locations, according to the Ocean Biogeographic Information System (OBIS) (Webb *et al.*, 2010). In addition, the water column has been estimated to contain $7.3x10^{29}$ prokaryotes (Whitman *et al.*, 1998). The sediments that constitute the sea floor are an important part of the marine system, and are located at 4000 meters depth on average where the sediment depth ranges from 0 m at mid-ocean ridges to >10 km at the continental shelves (Orcutt *et al.*, 2011). Sediments constrain a wide range of physical parameters, where factors such as pressure, temperature, sediment grain size and distribution of minerals can create regional variations in a larger area. Other features, such as the concentration of organic carbon, and local input of nutrients and reduced compounds could directly influence the microbial community composition at different sites, and could thereby support different kinds of ecosystems. Taking these potential habitat variations into account, marine sediments can support a wide range of niche-specific organisms, whereby sediments in the world's oceans have been estimated to contain $3.8x10^{30}$ prokaryotes (Whitman *et al.*, 1998). Microbial life has been detected in sediment horizons as deep as 500-800 meters below the seafloor (mbsf) (Parkes *et al.*, 1994; Taylor *et al.*, 1999), however, the number of cells in the deepest sediment horizons are 2-3 orders of magnitude lower than in horizons closer to the sediment surface (D'Hondt *et al.*, 2002), and the cells seems to be inactive or adapted to having low

metabolic activity (D'Hondt *et al.*, 2004). Analyses of intact polar membrane lipids (IPL) from sediments world-wide indicate that the biomass in deeper sediment horizons is highly abundant in *Archaea* (Lipp *et al.*, 2008), which supports the hypothesis of archaeal lineages being adapted to chronic energetic stress, meaning that they require a lower minimum energy in general to maintain cellular processes and the energy conservation potential compared to *Bacteria* (Valentine, 2007). However, we still have much to learn about the microbial diversity in marine deep sea and subsurface habitats. The knowledge we obtain today might be valuable for the future by giving us insight to potential resources that could be derived from marine systems for requirements such as food, gas and minerals, or for bioprospecting to develop novel pharmaceuticals or enzymes for industrial use.

In marine methane-enriched sediments, molecular and isotopic approaches have provided evidence that anaerobic methanotrophic archaea (ANME) play a vital role in the global carbon cycle, where they act as methane sinks and thereby reduce the amount of methane emitted to the atmosphere (Knittel & Boetius, 2009; Reeburgh, 2007; Taupp *et al.*, 2010). Together with sulfate-reducing bacteria they perform anaerobic oxidation of methane (AOM) with sulfate reduction, a process that was recognized already in the mid 1970's based on profiles of dissolved methane and sulfate in marine sediments (Martens & Berner, 1974; Reeburgh, 1976). Despite ANME being directly involved in global processes that influence the environment on Earth, little is known about their physiology, environmental adaptations and how their distribution and abundance in methane-enriched environments could change over time. The new high-throughput DNA sequencing technologies that have been developed in recent years have provided a unique opportunity to obtain deeper insights into the diversity, stratification and potential functions of microbial communities. In parallel, the increasing number of whole-community DNA sequencing projects being undertaken globally has encouraged the development of environmental proteomic and metaproteomic tools, which are increasingly used to explore the *in situ* functions of microbial communities.

In the work presented in this thesis, pyrosequencing tools integrated with geochemical profiles was applied to marine sediments from Nyegga in order to achieve a detailed understanding of the factors influencing the stratification of ANME dominated microbial communities and the abundance of co-occurring microbial taxa in horizons dominated by specific ANME subgroups. Furthermore, a coupled metagenomic and metaproteomic approach was applied to increase our knowledge of the *in situ* metabolism and physical adaptations in the ANME-1 clade.

2. Cold Seeps

Cold seeps can be defined as areas with vertical transport pathways for dissolved components that migrate through the sediments towards the seafloor, and usually occur at lower temperatures and lower flow rates than average hydrothermal vent systems (Suess, 2010). Cold seeps are found world-wide, in both active areas with plate convergence (especially between oceanic and continental plates) and passive areas, such as continental margins, slopes and shelves (Judd & Hovland, 2007; Suess, 2010). Geological surveys have revealed cold seeps in areas such as Gullfaks (North Sea plateau), Eckernförde Bay (Baltic Sea), the Mediterranean Sea and the Black Sea, Hydrate Ridge (Cascadia Margin), the Eel River Basin (coast of California), the Peru Margin, the Gulf of Mexico, the South China Sea, the Timor Sea (Australia) and the Nankai Trough (Japan) (Judd & Hovland, 2007). They include features such as shallow gas accumulations, seeping fluids dominant in methane, pockmarks on the surface of the seafloor, and sometimes associated macrofauna (e.g. bivalves and tubeworms) (Judd & Hovland, 2007). The fluids originate from groundwater aquifers, from deep subsurface regions below subduction zones of tectonic plates, or from reservoirs rich in hydrocarbons or brines located under the continental shelves (Suess, 2010). The fluids that migrate through the sediments contain high concentrations of methane and other hydrocarbons in addition to

remineralized nutrients that can be utilized by microorganisms, including silica, phosphate, ammonia, hydrogen sulfide and the intermediates from degradation of sedimentary organic matter (Suess, 2010). In marine sediments, sulfate is the most dominating terminal electron acceptor, and can fuel the sub-surface microbial communities by vertical diffusion from seawater where the sulfate concentration is 28 mM on average, or it could reach the communities through upward advection from ancient brines in deeper sediment horizons (D'Hondt *et al.*, 2004; Knittel & Boetius, 2009; Orcutt *et al.*, 2011). Consequently, cold seeps are capable of sustaining unique ecosystems of microorganisms, which in turn can support the seep-associated macrofauna in areas below the photic zone. The methane in cold seeps can be found as free gas in porous and permeable sediment layers or as clathrates, also called gas hydrates (Bohrmann & Torres, 2006; Hovland *et al.*, 2005; Hustoft *et al.*, 2007). In gas hydrates, the methane (or other gas molecules) is enclosed between water molecules in a cage-structure, where the gas molecules constitute ~15% of the ice block (Bohrmann & Torres, 2006). The stability of gas hydrates is dependent on several factors, including the temperature, pressure, gas composition and salinity of the surrounding water (Bohrmann & Torres, 2006; Sloan, 1998). Environmental conditions that fulfill these requirements are considered to be within the gas hydrate stability zone (GHSZ). As the sediment temperature increases with depth, the solid gas hydrates will cross their phase boundary, freeing gas and pore-water at certain depths (Bohrmann & Torres, 2006; Hyndman & Davis, 1992). Seismic observations of this transition zone has revealed specific strata of high-velocity sediments containing the gas hydrates overlaying lower–velocity sediments containing free gas, hence the name Bottom Simulating Reflector (BSR) for this zone (Bohrmann & Torres, 2006; Hyndman & Davis, 1992). Studies of the seafloor topography using high-resolution multibeam, side-scan sonar and sub-bottom profilers have revealed high density of pockmarks and seeping structures on the seafloor world-wide (Hovland, 1981; Hovland *et al.*, 1997; King & MacLean, 1970; Max *et al.*, 1992; Roberts & Carney, 1997; Werner,

1978; Xie *et al.*, 2003; Yun *et al.*, 1999). The formation of pockmarks is not fully understood, however several theories have been suggested: (**I**) a pressure build-up in a local gas pocket causes the seafloor to elevate into a dome-shape (Cathles *et al.*, 2010; Judd & Hovland, 2007). At a certain partial pressure seeping fluids break the surface through small fractures, which are gradually enlarged due to erosion by the passing fluids (Judd & Hovland, 2007). Eventually, a violent out-burst of escaping fluids causes an ejection of gas, sediment and pore-water from the site, leaving the newly formed pockmark behind. (**II**) ground water movements through glacial outwash sands causes aquifers that breaches the seafloor where the sediment layers are thinnest, and fluvial erosion over time forms pockmarks (Judd & Hovland, 2007; Whiticar & Werner, 1981). (**III**) a decrease in gas-saturated fluid flow to the hydrates or altered temperature or pressure causes dissolution of the gas hydrates in subsurface sediments (Sultan *et al.*, 2010). This process increases the pressure in the pore-water surrounding the hydrate-bearing sediments, causing the pore-water to migrate towards the seafloor thereby forming a pockmark as the sediments overlaying the hydrates collapse into a depression (Sultan *et al.*, 2010).

The original conduits under a cold seep pockmark can be blocked due to mineral precipitation over time (Foucher *et al.*, 2009; Hovland *et al.*, 2005; Mazzini *et al.*, 2006). Authigenic carbonate deposits can be found in shallower horizons in established seeping structures, derived through chemical reactions or activity of microorganisms in deeper horizons (Mazzini *et al.*, 2006). These methane-derived authigenic carbonate rocks may cause pressure increases inside the sealed-off chimney, generating new pathways for fluids along the rims of the pockmark, forming smaller unit pockmarks alongside the main pockmark, or alternating causing violent eruptions of gas and sediments that break the seal (Hovland *et al.*, 2005; Hovland *et al.*, 2010). Pockmarks can periodically have short outbursts followed by long periods of inactivity or micro-seepage until the pressure has reached the threshold for out-burst again (Hovland *et al.*, 2005; Reiche *et al.*, 2011). The permeability of the fluids can

also be reduced due to the accumulation of gas hydrates within the chimney structures (Nimblett & Ruppel, 2003). During the early phases of seepage, the fluids are guided through conduits in the GHSZ that are lined with hydrates (Foucher *et al.*, 2009). However, further development of the pockmark chimneys could increase the size of hydrate formations until new outward seepage pathways are formed due to fluid flow blockages (Foucher *et al.*, 2009), or the local accumulation of hydrates causes the seafloor to elevate into a pingo structure (Hovland & Svensen, 2006). A pingo structure forms in areas with high methane fluid flow over time, where the temperature and pressure is adequate for hydrate formation (Hovland & Svensen, 2006). It is fuelled by seawater entering through adjacent sediments, causing the seafloor to raise due to hydrate development (Hovland & Svensen, 2006).

3. The Nyegga area

The Nyegga area is located on the upper Mid-Norwegian continental slope, at ~730 meters water depth. The area is close to the northeastern flank of the Storegga Slide, and adjacent to the Vøring Basin to the north and the Møre Basin to the south (Bünz *et al.*, 2003; Hovland *et al.*, 2005; Ivanov *et al.*, 2010) (Fig. 1a). The area developed during several rifting phases in the late Jurassic and the late Cretaceous, which was followed by the continental brake-up between Scandinavia and Greenland during late Paleocene and Early Eocene and subsequent sedimentation of the Mid-Norwegian margin (Bjørnseth *et al.*, 1997; Faleide *et al.*, 2008; Gay & Berndt, 2007). The Cenozoic sediment package is disordered due to polygonal faulting (Berndt *et al.*, 2003), and has been subdivided into the Naust, Kai and Brygge formations (Dalland *et al.*, 1988). The Naust formation (2.8 million years old) includes 600-700 m of hemipelagic and glaciomarine sediments (Hustoft *et al.*, 2007; Rise *et al.*, 2006; Rise *et al.*, 2010).

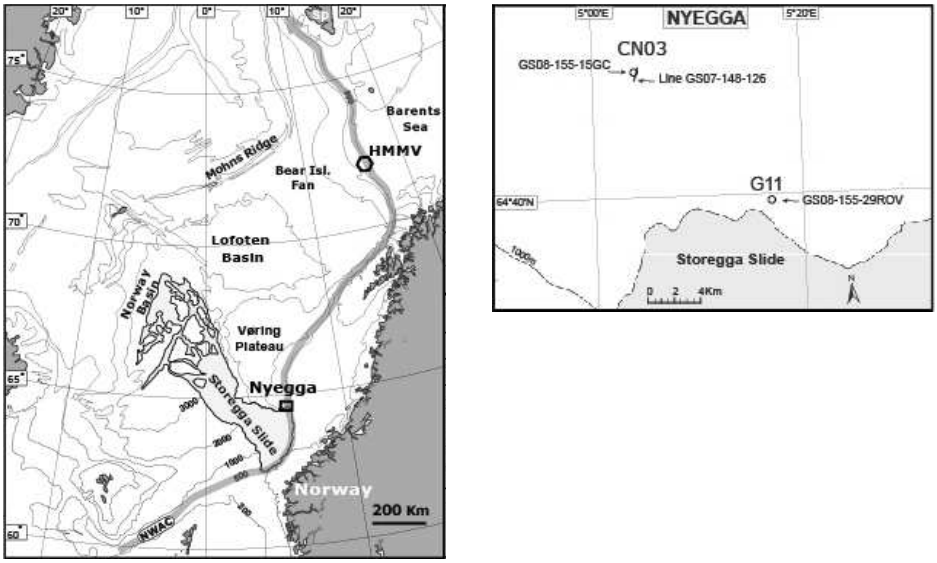

Figure 1: Overview map of the Norwegian Sea where the main seafloor topography and characteristic features, such as the Norwegian Water Atlantic Current (NWAC), are outlined. The Nyegga area is located on the continental slope, at the north-east flank of the Storegga Slide, at ~730m depth (**A**). The sampling site at CN03 area is located approximately 14 km northeast of the G11 pockmark (**B**). Adapted from **Paper III**.

Recent 2D/3D seismic and multibeam mapping of Nyegga has revealed an area with a high density of pockmark structures (>250), many with underlying gas blanking areas, which are also called fluid conduits or chimney structures (Hjelstuen *et al.*, 2010; Hustoft *et al.*, 2007; Hustoft *et al.*, 2009; Hustoft *et al.*, 2010; Reiche *et al.*, 2011). The chimney structures extend down to a pronounced BSR at 250-300 mbsf (Bünz *et al.*, 2003; Hustoft *et al.*, 2007; Hustoft *et al.*, 2010; Plaza-Faverola *et al.*, 2010). Pockmarks are described as circular and elongated depressions in the seafloor that measure 30-600 m in width and 4-15 m in depth, and have seemingly no preferred spatial orientation (Cathles *et al.*, 2010; Hjelstuen *et al.*, 2010; Hovland *et al.*, 2005). In addition, numerous unit pockmarks (<5 m wide) have been observed, which are mainly found as isolated small depressions outside larger, more complex pockmarks (Hovland & Judd, 1988; Hovland *et al.*, 2010). The pockmark structures in

Nyegga are the result of vertical seeping of methane enriched fluids over time, and the methane source in this cold seep area is probably a combination of deeper thermogenic methane from gas hydrates that is mixed with shallower biogenic methane during the advection towards the sediment surface (Mazzini *et al.*, 2005; Vaular *et al.*, 2010). The cold seeps at Nyegga seem to be fueled by over-pressured reservoirs of hydrocarbons, mostly methane, but also small amounts of ethane, propane and isobutane have been detected (Hovland *et al.*, 2005; Ivanov *et al.*, 2010; Suess, 2010; Vaular *et al.*, 2010).

4. The sampling sites at Nyegga

The sediment cores used in this work were sampled at two locations at Nyegga: the G11 pockmark (64°39.788' N, 05°17.317' E) and the CN03 area (64°45.274' N, 05°04.088' E). The CN03 area is located approximately 14 km northeast of the G11 pockmark (Fig. 1b). The G11 pockmark is nearly circular in size (260x310 m) and is 15 m deep (Hovland *et al.*, 2005). It is characterized by two carbonate ridges protruding up to 10 m from the seafloor, which are separated by a central basin with smaller carbonate blocks (up to ~24m^3) (Hovland *et al.*, 2005). The methane flux in the sediments underlying the G11 pockmark is not substantial enough to cause a constant stream of visible gas bubbles, however, other distinctive marks of an active seeping area have been observed (H. Haflidason, pers. comm.). Gas hydrates are present in the sediment horizons below ~75-100 cmbsf (Chen *et al.*, 2010) and several pingo structures are observed within the pockmark, appearing as irregular dome-shaped structures (up to 1m high) (Hovland & Svensen, 2006). Several phyla of pockmark-associated macrofauna have been observed within the G11 pockmark, such as tube-worms (*Polychaeta* and *Pogonophora*), stalked sea lilies (*Crinoidea*), sponges (*Porifera*), mollusks (*Bivalvia* and *Gastropoda*), crustaceans (*Amphipoda*, *Isopoda* and *Tanaidacea*), peanut worms (*Spuncula*), sea spiders (*Pycnogonida*), basket stars (*Ophiuroidea*) and sea

stars (*Pteraster*) (Decker *et al.*, 2011; Hovland *et al.*, 2005; Hovland & Svensen, 2006; Krylova *et al.*, 2011). Furthermore, patchy areas with whitish microbial mats situated on the seafloor have been observed in the G11 pockmark, indicating rich microflora communities supported by the diffusing fluids in combination with the oxic seawater (Hovland *et al.*, 2005). A push core covered in white microbial mats was retrieved from a pingo structure inside the G11 pockmark (Fig. 2). The core was collected using the Argus Bathysaurus ROV system on the research vessel G.O. Sars., and the 22 cm sediment core was designated 29ROV (GS-08-155-29ROV) (**Paper I**).

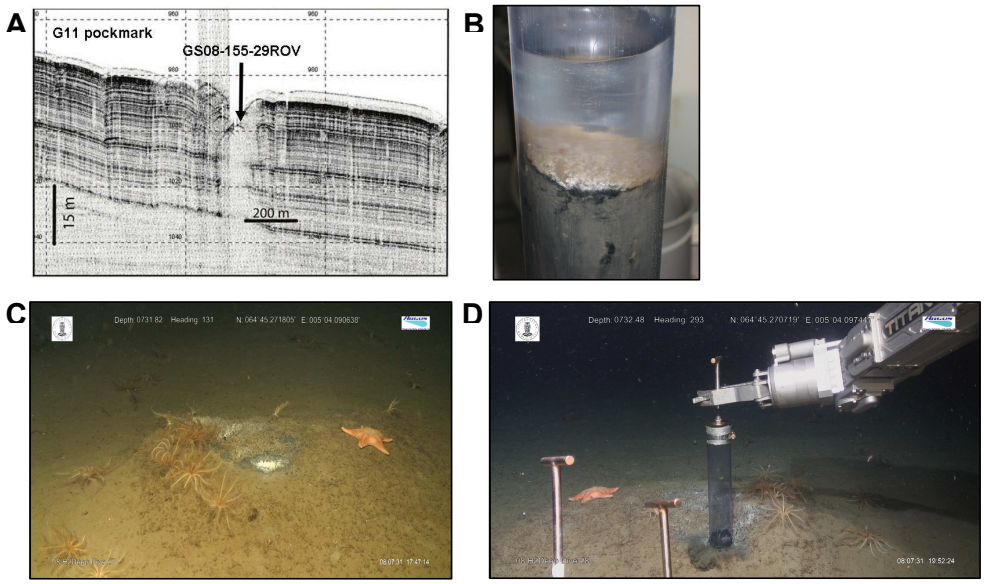

Figure 2: The sediment core 29ROV was sampled inside the G11 pockmark at Nyegga (**A**). The core was retrieved from a pingo-structure covered with microbial mats and diverse macrofauna (**C**) using a push-core sampler guided by a remote operating vehicle (ROV) (**D**). The 22 cm core comprised gray sediments with fluid escape structures, and the microbial mat remained intact in the sediment-water interface after sampling (**B**). Adapted from **Paper I** (B), and photo courtesy of Haflidi Haflidason (A, C and D).

The 300 m wide CN03 area is located northeast of the G11 pockmark and appears as a moderately mounded structure with a central crater where no

pockmark-like depressions were observed (Ivanov *et al.*, 2010; Plaza-Faverola *et al.*, 2010; Westbrook *et al.*, 2008). A clear chimney structure is observed under the central part of the CN03 area, where hydrates occupy ~35% of the pore-space on average (Ivanov *et al.*, 2007; Westbrook *et al.*, 2008). The area is less active than the G11 pockmark, with fewer seeping structures and a thicker layer of recent sediments, although possible gas hydrates are present at ~130-140 cmbsf and below (Ivanov *et al.*, 2010). In some areas of CN03, the upper 10 cm of sediments contain high numbers of tubeworms (*Pogonophora*), however, the macrofauna in general is less diverse than the community inside the G11 pockmark (Ivanov *et al.*, 2010). The core designated 15GC (GS-08-155-15GC) (**Paper III**) was sampled ~50 m from the center of the seeping structure in the CN03 area, using a 300 cm long gravity corer (Fig. 3).

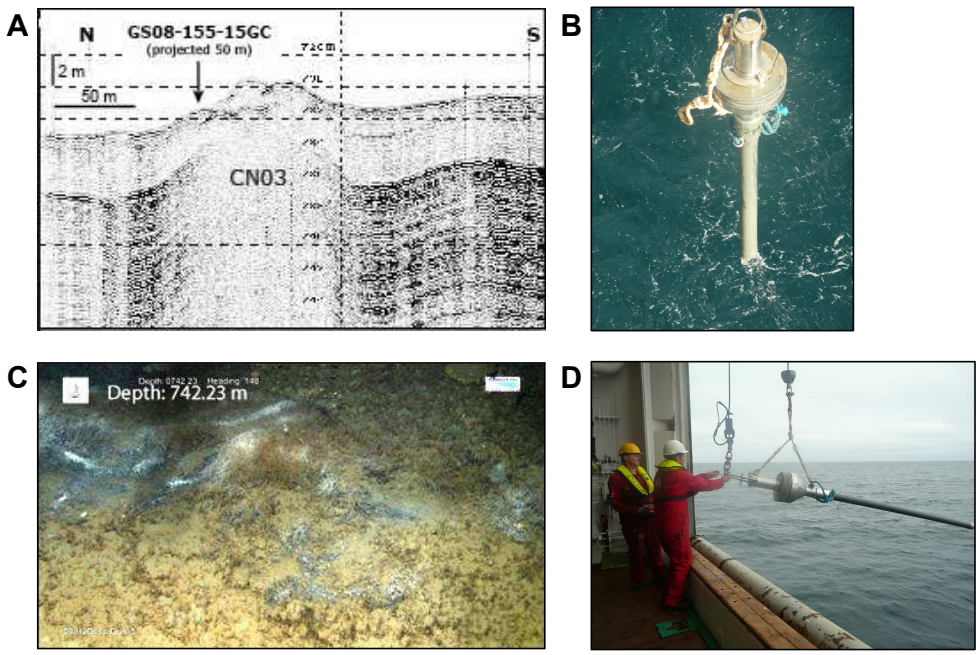

Figure 3: The sediment core 15GC was sampled at the CN03 area (**A**) by using a 3 meter long gravity corer (**B**, **D**). The sampling site is located 50 m north of the central crater (**A**), where the lower methane flux sustains fewer microbial mats and lower diversity of macrofauna compared to G11 pockmark (**C**). Photo courtesy of Haflidi Haflidason (A-D).

5. Anaerobic methanotrophic archaea (ANME)

5.1 Phylogeny

Three clades of ANME; classified as ANME-1, ANME-2 and ANME-3, are described, of which ANME-1 is distantly affiliated with *Methanosarcinales* and *Methanomicrobiales*, while ANME-2 and ANME-3 belong to *Methanosarcinales* (Knittel *et al.*, 2005; Orphan *et al.*, 2001) (Fig. 4a).

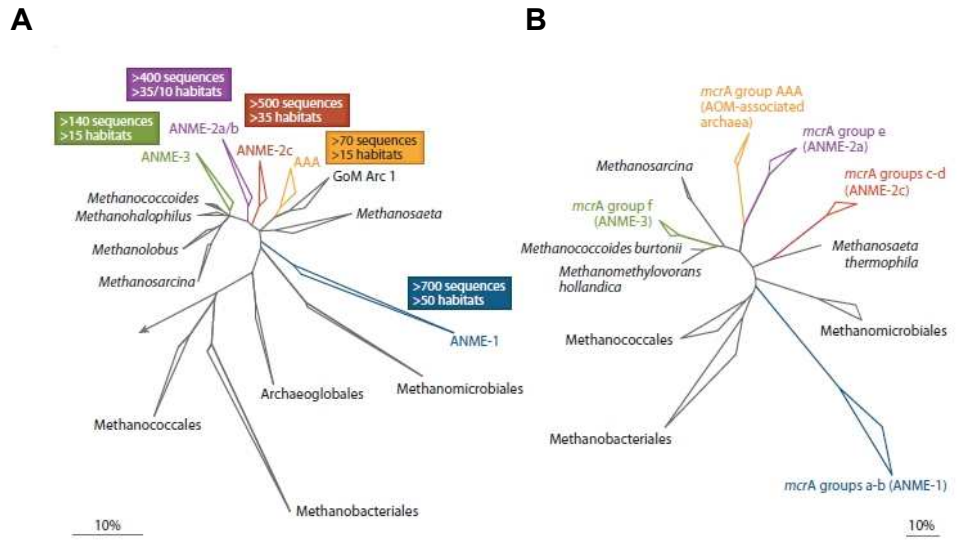

Figure 4: A phylogenetic tree of selected taxa within *Euryarchaeota* shows that ANME-2 and ANME-3 have higher sequence similarity than the ANME-1 clade, based on 16S rRNA gene information (**A**). The clades named AOM-associated archaea (AAA) and Gulf of Mexico Archaea I (GoM Arch I) are closely related to the ANME-2 clade, however their physiology remains unknown. In the phylogenetic tree based on the gene encoding the α subunit of methyl-coenzyme M reductase (*mcrA*) (**B**), ANME-1 and different ANME-2 subgroups are clustered in distinct groups, which are distantly related to the methanogens. Adapted from “Anaerobic oxidation of methane: Progress with an unknown process”, by K. Knittel and A. Boetius (2009).

Phylogenetic studies based on the 16S rRNA gene have shown that the ANME is not a monophyletic group, with a sequence similarity of 75-92%

between the different clades (Knittel & Boetius, 2009). Sequences affiliated with the ANME-1 and ANME-2 have been clustered into subgroups designated 1a-1b and 2a-2b-2c, respectively (Knittel & Boetius, 2009). A fourth subgroup within ANME-2, ANME-2d, has also been suggested, including sequences previously defined as GoM Arch I. However, the ability to perform AOM or to form consortia with a sulfate-reducing partner has not yet been demonstrated for this subgroup (Knittel & Boetius, 2009).

In addition to the 16S rRNA gene, the gene encoding the α subunit of methyl coenzyme M reductase (*mcrA*) can be used as a taxonomical marker gene in phylogenetic studies (Hales *et al.*, 1996; Luton *et al.*, 2002). The Mcr is the key enzyme in methanogenesis (Reeve *et al.*, 1997), and the *mcr* gene is expressed by all known methanogens and methanotrophic archaea (Ferry, 1999; Hallam *et al.*, 2003; Reeve *et al.*, 1997). The recovery of *mcr* operons associated with ANME metagenomes has expanded the phylogenetic range of the marker gene to include methanotrophic archaea (Taupp *et al.*, 2010). Reconstruction of the evolutionary relationships between ANME clades and methanogens has revealed congruence between phylogenetic trees based on the *mcrA* gene and the 16S rRNA gene (Fig. 4a-b), where ANME-1 and the different ANME-2 subgroups are clustered in distinct groups (Hallam *et al.*, 2003; Knittel & Boetius, 2009).

5.2 Anaerobic oxidation of methane (AOM)

Based on incubation experiments using sediments characterized by high fluid flux, it has been suggested that AOM coupled to sulfate reduction is performed by archaea affiliated with methanogens and a sulfate-reducing partner in a proposed syntrophy (Reaction 1) (Hoehler *et al.*, 1994). In a syntrophic lifestyle two microorganisms exchange molecules or metabolites in order to degrade a substance that neither cell can degrade alone (Bryant *et al.*, 1967; McInerney *et al.*, 2008; McInerney *et al.*, 2009). Furthermore, synthrophy in an

anaerobic environment can also involve metabolisms where degradation of a substance by one microorganism is only thermodynamically favorable if the end-product of the pathway (e.g. H_2, formate or acetate) is maintained at low concentrations by other microorganisms (McInerney *et al.*, 2009). The AOM process has since been linked to consortia comprising anaerobic methanotrophic archaea (ANME) and deltaproteobacterial lineages using results from *in vitro* rate measurements of AOM and sulfate reduction in combination with fluorescence *in situ* hybridization (FISH) (Boetius *et al.*, 2000; Michaelis *et al.*, 2002; Nauhaus *et al.*, 2002).

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad (1)$$

($\Delta G° = -21$ kJ/mol)

The amount of free energy available to support life can vary according to the amount of available methane in each ecosystem, and can be up to -35 kJ/mol in sedimentary methane seeps where the methane concentration is high, or as low as -18 kJ/mol in non-seep sediments where the concentration of methane is low (Alperin & Hoehler, 2009; Caldwell *et al.*, 2008; Wang *et al.*, 2010a). The generation time of ANME-1 and ANME-2 in aggregates is relatively long due to the low levels of free energy available through AOM with sulfate reduction, and has been estimated to be between 22-40 days or 2-3.4 months, based on enrichment studies of cold seep sediments in a continuous-flow bioreactor or in isotopic labeling experiments, respectively (Girguis *et al.*, 2005; Krüger *et al.*, 2008b). For the thermophilic ANME-1 phylotype the generation time is estimated to be up to 68 days (Holler *et al.*, 2011). Despite the low energy yield, up to $9x10^7$ ANME aggregates pr. cm^3 sediment have been quantified in cold seeps world-wide (Boetius *et al.*, 2000; Knittel & Boetius, 2009; Lösekann *et al.*, 2007; Niemann *et al.*, 2006; Treude *et al.*,

2005; Wegener *et al.*, 2008b) while >10^{10} ANME cells have been detected pr. cm^3 of microbial mat in the methane-enriched environment of the Black Sea (Knittel *et al.*, 2005; Michaelis *et al.*, 2002).

Calculations of the rates of AOM and sulfate reduction in methane-enriched environments are based on the amounts of incorporated isotopes during *in vitro* incubations of sample material, where the AOM rate overall is higher in dense biomass mats from the Black Sea than it is generally in cold seep sediments (Knittel & Boetius, 2009). The highest AOM rate in Black Sea mats are determined to up to 9.3 µmol/cm^3·day (Michaelis *et al.*, 2002; Treude *et al.*, 2007), while the highest rate in sediments measured so far is estimated to be up to 3 µmol/cm^3·day in Hydrate Ridge sediments (Knittel *et al.*, 2005; Treude *et al.*, 2003). Furthermore, the rate of sulfate reduction at the two sites is determined to be up to 8.4 µmol/cm^3·day in Black Sea mats and 3.6 µmol/cm^3·day in Hydrate Ridge sediments (Michaelis *et al.*, 2002; Treude *et al.*, 2003). The methane production in marine systems by methanogens is estimated to be 85-300x10^{12} gram/year, of which >90% is anaerobically oxidized by ANME (Knittel & Boetius, 2009). These values demonstrate that ANME reduce the emission of methane to the atmosphere, and thus have a major environmental impact on Earth as methane is one of the greenhouse gases involved in climate change.

Although, the first described mechanism of AOM involved methane oxidation coupled to sulfate reduction, this process can also be linked to the global nitrogen cycle. Incorporation of isotopically labeled nitrogen-compounds by ANME-2/DSS consortia (see section 5.4 for details) showed that dinitrogen (N_2) was converted to ammonia (NH_3) for assimilation, which indicates that these consortia could be involved in nitrogen-fixation in cold seeps (Dekas *et al.*, 2009; Orphan *et al.*, 2009). Furthermore, studies of sedimentary enrichment cultures revealed that AOM coupled to nitrogen-reduction is performed by consortia of archaea closely affiliated with ANME and a novel bacterium, where the estimated free energy yield is -928 kJ/mol and -765

kJ/mol with nitrite and nitrate acting as electron acceptors, respectively (Raghoebarsing *et al.*, 2006). This process can also be performed by free-living *Bacteria* without a partner, as *in vitro* inhibition experiments excluded the reverse methanogenese in the archaeal phylotype as the biochemical mechanism for AOM (Ettwig *et al.*, 2008). By using metagenomic tools, it was recently shown that "*Candidatus* Methylomirabilis oxyfera" couples AOM to the reduction of nitrite to dinitrogen (Ettwig *et al.*, 2010). In the proposed pathway, two NO molecules derived from nitrate are converted into O_2 and N_2, where only the oxygen is involved in oxidation of methane (Ettwig *et al.*, 2010). The reaction is thermodynamically favorable, with an estimated energy yield of -173.1 kJ/mol O_2, however, the novel NO dismutase enzyme required for this metabolism has not yet been identified (Ettwig *et al.*, 2010). Furthermore, AOM can also be coupled to other forms of anaerobic respiration, such as the reduction of Fe^{3+} and Mn^{4+} (Beal *et al.*, 2009), where the potential free energy of -270.3 kJ/mol and -556 kJ/mol for the two compounds is substantially higher than when coupled to sulfate. In recent *in vitro* studies using Eckernförde Bay sediments, ANME-1 cells were enriched in cultures provided with substrates such as acetate, pyruvate or butyrate with thiosulfate as the electron acceptor (Jagersma *et al.*, 2011). In summary, the metabolic range of the uncultured ANME clades has not been well described. However, there are indications of consortia being adapted to perform AOM coupled to other metabolisms besides sulfate reduction, which could increase the free energy yield for these taxa. If these possible couplings are verified for ANME, these metabolisms could possibly decrease the generation time and increase the habitat range for these clades.

AOM coupled with sulfate reduction is considered the main energy generating process in methane enriched habitats, especially within the sulfate methane transition zone (SMTZ), which are the horizons where sulfate-rich seawater migrating down the sediments through diffusion, meets methane-rich fluids migrating towards the seafloor through advection (Berelson *et al.*, 2005; Biddle *et al.*, 2006; Knittel & Boetius, 2009). However, areas with deeper SMTZ, such

as the Peru margin where the SMTZ is located at 10-40 mbsf (Biddle *et al.*, 2006), metabolic activity associated with general non-seep sediments, such as ammonification, manganese reduction and iron reduction, is observed in the shallower horizons (D'Hondt *et al.*, 2004; Orcutt *et al.*, 2011). In cold seeps the sulfide produced by AOM with sulfate migrates towards the seafloor where it can sustain microbial mats. Large orange- or white-pigmented mats of aerobic sulfide oxidizing *Beggiatoa* (*Gammaproteobacteria*) are observed in cold seeps at Hydrate Ridge, the Gulf of Mexico, Eckernförde Bay and the Haakon Mosby mud volcano (Knittel *et al.*, 2005; Larkin *et al.*, 1994; Lösekann *et al.*, 2007; Mills *et al.*, 2004; Preisler *et al.*, 2007). Sulfide oxidizing bacteria can also be found as endosymbionts in the gills of animals, such as polychaetes and bivalves, which are commonly found in seeping areas (Cordes *et al.*, 2010; Dubilier *et al.*, 2008). The microorganisms provide the animals with organic carbon through chemoautotrophy with the oxidation of sulfide, and in return the animals position themselves in shallow horizons to provide the microorganisms with an efficient supply of reduced sulfur compounds from the sediments below and oxygen from the seawater above.

5.3 Biogeography

The high abundance and wide distribution of ANME in methane-enriched environments world-wide has been verified by using 16S rRNA gene sequence information, selective probes for FISH and IPL's. Cold seep sediments at the Eel River Basin (Hinrichs *et al.*, 1999; Orphan *et al.*, 2001), the Gulf of Mexico (Mills *et al.*, 2004), Hydrate Ridge (Boetius *et al.*, 2000; Knittel *et al.*, 2005), the Eckernförde Bay (Treude *et al.*, 2005), the Kazan mud volcano (Mediterranean Sea) (Heijs *et al.*, 2007), the Hakon Mosby mud volcano (North Sea) (Lösekann *et al.*, 2007), the Nias Basin (Indian Ocean) (Siegert *et al.*, 2011) and the Gullfaks and Tommeliten methane seeps (Wegener *et al.*, 2008b) are some of the best studied ANME-dominated sediments. However, ANME are also abundant in other habitats, such as

microbial mats and the anoxic water column in the Black Sea (Blumenberg *et al.*, 2004; Michaelis *et al.*, 2002; Schubert *et al.*, 2006; Treude *et al.*, 2007), terrestrial systems and mud volcanoes (Chang *et al.*, 2011; Niederberger *et al.*, 2010; Takeuchi *et al.*, 2011) and on less active chimneys at the Lost City hydrothermal vent field (Brazelton *et al.*, 2006). Whereas these systems are characterized by a low temperature (<22℃), ANME clades are also located on chimneys from hydrothermal vent fields (Reed *et al.*, 2009), and observed in warm (30-35℃) and hot (60-95℃) sediments in the Guaymas Basin (Biddle *et al.*, 2011a; Holler *et al.*, 2011) and in warm (50-55℃) sediments at the Newfoundland Margin (Roussel *et al.*, 2008).

In some ANME-dominated habitats, uncultivated microbial lineages such as the Deep-Sea Archaeal group (DSAG), the Marine Benthic Group D, the Miscellaneous Crenarchaeotic Group (MCG) and the Marine Group 1 (MG-1) within *Archaea* are also present. Furthermore, 16S rRNA gene sequences from *Proteobacteria*, *Planctomycetes*, *Bacteriodetes*, *Chloroflexi*, *Firmicutes* and "*Candidate division* JS-1" within *Bacteria* are frequently retrieved (Harrison *et al.*, 2009; Inagaki *et al.*, 2006; Lloyd *et al.*, 2006; Orcutt *et al.*, 2011; Pachiadaki *et al.*, 2011; Roussel *et al.*, 2009; Wang *et al.*, 2010b; Webster *et al.*, 2011; Yanagawa *et al.*, 2011). A lifestyle involving organotrophic sulfate reduction, which possibly involves oxidation of methane without carbon assimilation, has been suggested for DSAG, (Biddle *et al.*, 2006; Inagaki *et al.*, 2006). The MCG, *Chloroflexi* and JS-1 may be heterotrophs that utilize the organic matter buried in the sediments (Teske & Sørensen, 2008; Webster *et al.*, 2011; Yamada & Sekiguchi, 2009). *Planctomycetes* and MG-1 may perform anaerobic and aerobic ammonium oxidation, respectively (Agogue *et al.*, 2008; Kuypers *et al.*, 2003; Nicol & Schleper, 2006; Spang *et al.*, 2010), while certain sulfate-reducing phyla within *Deltaproteobacteria* are described as syntrophic partners of ANME during AOM (Boetius *et al.*, 2000; Orphan *et al.*, 2001; Schreiber *et al.*, 2010).

One of the criteria for the ANME abundance is the available methane for AOM, which can be compared between ANME habitats through methane fluid flux estimations. The flux is rather high in ANME-dominated areas including Hydrate Ridge (11-33x10^3 mmol/m^2·year) (Torres *et al.*, 2002), the Gulf of Mexico (500-2300 mmol/m^2·year) (Lloyd *et al.*, 2010) and Eckernförde Bay (240-690 mmol/m^2·year) (Treude *et al.*, 2005). In areas with lower flux, ANME are not among the dominant taxa, e.g. the Santa Barbara Basin (182 mmol/m^2·year) which is dominated by DSAG despite the high abundance of ANME-1 (Harrison *et al.*, 2009), or the Peru Margin (1.6-9 mmol/m^2·year) where ANME affiliated sequences were not detected (Biddle *et al.*, 2006). Hence, a certain threshold of methane-seeping is apparently necessary to sustain an ANME dominated community.

The distribution of ANME in cold seeps can vary: (**I**) the microbial community in some areas is dominated by one ANME clade, while other ANME clades or subgroups are present in low amounts. This is the case in ANME-1 dominated habitats, such as the Gulf of Mexico (Lloyd *et al.*, 2006), the Tommeliten cold seep (Wegener *et al.*, 2008b) and ANME-2 dominated habitats such as the Gullfaks cold seep (Wegener *et al.*, 2008b), Garden Banks (Gulf of Mexico) (Martinez *et al.*, 2006) and Eckernförde Bay (Treude *et al.*, 2005). (**II**) other seeping areas provide niches highly abundant in different ANME clades due to varying geochemical conditions within the lateral or vertical space. Examples include the ANME-1 and ANME-2 dominance at different depths in the Eel River Basin (Orphan *et al.*, 2001), specific areas with high abundance of ANME-2a or ANME-2c at Hydrate Ridge (Knittel *et al.*, 2005) and ANME-2 and ANME-3 communities at different regions surrounding the center at the Hakon Mosby mud volcano (Lösekann *et al.*, 2007). Other environmental factors that can influence the presence of the ANME population are temperature, oxygen and concentration of sulfate and sulfide (Rossel *et al.*, 2011). In general, ANME-1 dominated habitats are characterized by low oxygen concentrations or anoxic conditions and higher temperatures than ANME-2 and ANME-3 dominated habitats (Rossel *et al.*, 2011). These observations also agree with

previous studies where ANME-1b is suggested to be adapted to a higher optimum temperature for AOM than ANME-2 (Nauhaus *et al.*, 2005). Enrichment cultures with ANME-2 dominated sediments from Eckernförde Bay showed that increased concentration of sulfide caused inhibition of both AOM and sulfate reduction rates (Meulepas *et al.*, 2009). This is supported by results from a community study at the Guaymas Basin where ANME-2a/b is negatively correlated with increasing concentrations of sulfide and methane (Biddle *et al.*, 2011a). In the same study, the abundance of ANME-1a seemed to be affected by temperature, whereas the ANME-1b correlated positively with high methane concentrations and negatively with high concentrations of sulfate (Biddle *et al.*, 2011a).

5.4 Morphology and syntrophy

The ANME clades have been observed as single cells, chains, mono-aggregates or in consortia, where there is an association of more than one species into aggregates. The sulfate-reducing bacteria (SRB) that constitute the partners of ANME are affiliated with *Deltaproteobacteria*, where *Desulfosarcina* and *Desulfococcus* species (DSS) are mainly associated with ANME-1 and ANME-2 (Knittel *et al.*, 2005; Schreiber *et al.*, 2010), while *Desulfobulbus* species (DBB) are associated with ANME-3 (Lösekann *et al.*, 2007; Niemann *et al.*, 2006). In addition, a novel deltaproteobacterial cluster (HotSeep-1) has been described as a sulfate-reducing partner for the thermophilic ANME-1 in the Guaymas Basin (Holler *et al.*, 2011). A study by Pernthaler *et al*. (2008) suggests that the bacterial partner of ANME may extend beyond *Gammaproteobacteria*, as magneto-FISH captures of ANME-2c consortia revealed high abundance of α- and betaproteobacterial sequences. However, the details regarding the syntrophic lifestyle and how the consortia are formed are not yet described, though it is assumed that the energy yield from the metabolic activity has to be divided between the

contributing cells (Caldwell *et al.*, 2008; Knittel & Boetius, 2009; Nauhaus *et al.*, 2002).

ANME-1 appear as rod shaped cells in FISH images, measuring 0.6-1 µm wide and 1-3 µm long, and are covered in external sheets consisting of a resistant biopolymer (Knittel & Boetius, 2009; Orphan *et al.*, 2002; Reitner *et al.*, 2005). ANME-1 occur as single cells or chains of 2-4 cells (Knittel & Boetius, 2009; Orcutt *et al.*, 2005), although multicellular mono-aggregates and chains up to 100 µm long have been observed in Black Sea mats and Eel River Basin sediments (Orphan *et al.*, 2002; Reitner *et al.*, 2005; Treude *et al.*, 2007). This clade can also form consortia or loose associations with a sulfate-reducing bacterial partner in order to perform AOM coupled to sulfate reduction (Knittel & Boetius, 2009; Michaelis *et al.*, 2002; Orphan *et al.*, 2002).

The cocci shaped ANME-2 cells are approximately 0.5 µm in diameter (Orphan *et al.*, 2002), while the ANME-3 cocci are slightly larger, 0.7 µm in diameter (Lösekann *et al.*, 2007). For ANME-2, single cells and mono-aggregates (4-10 µm in diameter) with pseudo-sarcina morphology have been described (Knittel & Boetius, 2009; Orphan *et al.*, 2002; Treude *et al.*, 2007), however, ANME-2 most often occur in consortia with SRB. In general, consortia involving ANME-2a are usually composed of the mixed-type aggregates without a fixed organization of cells or specific size or shape, while ANME-2c represent the shell-type aggregates where the archaeal inner core is partially or fully surrounded by SRB (Knittel & Boetius, 2009; Orcutt & Meile, 2008). Enrichment experiments with sediments from Hydrate Ridge showed that the ANME-2 consortia can increase in size from <6 µm to >25 µm in diameter, and can involve up to 100,000 cells (Knittel & Boetius, 2009; Nauhaus *et al.*, 2007). When the consortia reach a specific size they tend to fissure, releasing cells and smaller aggregates to the environment (Knittel & Boetius, 2009). Single cells of the ANME-3 clade have been observed in marine sediments, (Omoregie *et al.*, 2008), however, the shell-type consortia is far more common where relatively few bacterial cells are affiliated with the

ANME-3 core (Knittel & Boetius, 2009; Lösekann *et al.*, 2007; Niemann *et al.*, 2006).

During the syntrophic lifestyle, methane (CH_4) is oxidized to bicarbonate (HCO_3^-) by ANME. This is followed by an electron transfer to the sulfate-reducing partner, which uses sulfate as a terminal electron acceptor for the metabolism in total (Nauhaus *et al.*, 2002). The form whereby the electrons are released to the sulfate-reducing partner is unknown. In one model, methyl sulfide is suggested as the electron shuttle (Moran *et al.*, 2008), but production of methyl sulfide by ANME has not been detected, and the compound did not support growth of the syntrophic deltaproteobacterial lineges (Taupp *et al.*, 2010). Inter-species transfer of compounds, such as formate, acetate, glucose, lactate, hydrogen (H_2), carbon monoxide (CO), methylamines, methanol, phenazines and humic acids has been suggested, however, addition of these metabolites to environmental samples enriched in ANME-1 and ANME-2 populations has not increased the sulfate reduction rates compared with the control, thereby experimentally ruling out such a suggestion (Meulepas *et al.*, 2010; Nauhaus *et al.*, 2005). Other hypotheses suggest a direct electron transfer between cells through nanowires, c-type cytochromes or other redo-ox shuttles or excretion of extracellular polymeric substances as a conductive matrix (Heller *et al.*, 2008; Krüger *et al.*, 2008a; Meyerdierks *et al.*, 2010; Thauer & Shima, 2008; Wegener *et al.*, 2008a), however, this remains to be shown.

5.5 AOM via reversed methanogenesis

The details of the AOM mechanism is unknown, but several results based on methangenomic, transcriptomic and proteomic analyses of AOM habitats have suggested that AOM is performed by reversing the steps of methanogensis (Hallam *et al.*, 2003; Hallam *et al.*, 2006; Krüger *et al.*, 2003; Meyerdierks *et al.*, 2010). Genes encoding a nearly complete reverse methanogenesis

pathway have been found for ANME-1 and ANME-2 in metagenomes and a composite genome of ANME-1 (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2010). The gene encoding the key enzyme methylene-tetrahydromethanopterin (H_4MPT) reductase (*mer*) is however lacking (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2005; Meyerdierks *et al.*, 2010). Instead, a bypassing mechanism has been suggested for ANME, based on the bypass-hypothesis for the *Methanosarcina barkeri mer* deletion mutant where methanol is oxidized to formaldehyde and subsequently to methylene H_4MPT (Meyerdierks *et al.*, 2010; Welander & Metcalf, 2008). However, this hypothesis was not supported by the ANME-1 composite genome, as genes encoding enzymes involved in methylene H_4MPT formation (formaldehyde-activating enzyme and hexulose-6-phosphate synthase), formaldehyde formation (putative methanol dehydrogenase or alcohol dehydrogenase) or methanol formation (all subunits in the coenzyme B12-dependent enzymes) were not identified (Meyerdierks *et al.*, 2010). Other hypotheses for the lacking *mer* gene in ANME include: (**I**) the bypass-mechanism involves a novel enzyme yet to be identified. (**II**) the step involving methylene H_4MPT reductase may be avoided if methane is converted to acetyl-coA in the upper oxidative part of the pathway. (**III**) the reverse methanogenesis could be coupled to other metabolisms in ANME-1, such as the gluconeogenesis pathway, for the formation of methylene- H_4MPT (Meyerdierks *et al.*, 2010).

Recently, the key enzyme in the methanogenese pathway, Mcr, was purified from *Methanothermobacter marburgensis* and found to catalyze the oxidation of methane with a sufficiently high specific activity to support AOM (Scheller *et al.*, 2010). All the enzymes in the methanogenesis pathway have been confirmed to catalyze the reversible reactions, except a coenzyme M-S-S-coenzyme B heterodisulfide reductase coupled to a [NiFe]-hydrogenase (MvhADG/HdrABC) (Thauer, 2011). Purification and characterization of MCR from microbial mats enriched in ANME-1 from the Black Sea, revealed 2 isoenzymes, each with a different variant of the F_{430} cofactor (Krüger *et al.*, 2003), which further supported the argument of an AOM mechanism via the

reverse methanogenesis. The enzyme identified as Ni-protein I was associated with the heavy variant of F_{430} and Ni-protein II with the lighter form of F_{430} (Krüger *et al.*, 2003). Both proteins were purified in high yields, comprising 7% and 3% of the total extractable proteome, respectively (Krüger *et al.*, 2003; Mayr *et al.*, 2008). The structure of the two variants of F_{430} has been resolved by NMR, where the light form is similar to the cofactor found in methanogens, and the heavy form had modifications involving a methylthio-group substitute at position $C17^2$ of the F_{430} pentamyl ester (Mayr *et al.*, 2008). Resolution of the 3D structure of Mcr with the modified F_{430} revealed a highly similar structure to the methanogenic Mcr, with small subtle differences including a modification of F_{430}, a cysteine-rich patch, and an altered post-translational amino acid modification (Shima *et al.*, 2012). These modifications were unique for this ANME-1 Mcr, whereas the Mcr from ANME-2 and ANME-3 is apparently more similar to the methanogenic Mcr (Shima *et al.*, 2012). The structural differences have been suggested to reflect the more distant phylogenetic relationship of ANME-1 to the methanogens, or they could play an ecological role in fine-tuning the enzymes for their function in different biological contexts (Shima *et al.*, 2012). These results illustrate how detailed knowledge on the 3D-structure of enzymes may provide clues about ecological niche selection. The redox potential (E°') for the Ni(II)/Ni(I) couple in the F_{430} prosthetic group of Mcr has been determined to be below -0.6V, which means that the reverse methanogenesis coupled to sulfate reduction is thermodynamically feasible due to the negative redox potential for the adenylylsulfate/sulfite couple (-0.06V) and the sulfite/hydrogen sulfide couple (-0.12V) (Thauer & Shima, 2008). AOM coupled to reduction of nitrite, nitrate, iron or manganese has previously been verified (Beal *et al.*, 2009; Ettwig *et al.*, 2010; Raghoebarsing *et al.*, 2006), however, the redox potentials for these electron-acceptor couples are positive (E°= >0), e .g. nitrate/nitrite (+0.43V), N_2O/N_2 (+1.36V), Fe(III)/Fe(II) (+0.2V), Mn(IV)/Mn(II) (+0.41V) (Thauer & Shima, 2008). Hence, AOM coupled to reduction of nitrite, nitrate, iron or

manganese is biologically possible, but the pathway is unlikely to involve Mcr as this mechanism is thermodynamically problematic.

The composite genome of ANME-1, estimated to represent 82-90% of the complete genome, provide more detailed information about the C1-metabolism, electron-transfer and membrane-associated complexes, amino acid metabolism, central carbon metabolism and stress response (Meyerdierks *et al.*, 2010). Identified genes encoding homologues of electron accepting complexes, such as a putative [FeFe]-hydrogenase, subunits MvhD and FrhB of electron shuttling [NiFe]-hydrogenases, a putative Mo-containing oxidoreductase, membrane-associated multiheme c-type cytochromes and $F_{420}H_2$:quinone oxidoreductase, and two heterodisulfide reductase complexes (HdrABC-type), indicate that the electron flow coupled to the reverse methanogenesis pathway in ANME-1 is different from the electron-accepting complexes expressed by methanogens (Meyerdierks *et al.*, 2010; Thauer *et al.*, 2010). Furthermore, the yet incomplete reductive acetyl-CoA pathway has been suggested to be involved in anabolic reactions and in carbon-assimilation from methane and carbon dioxide, while nearly complete versions of the gluconeogenesis pathway, a putative glycolysis pathway and amino acid biosynthesis pathways are identified in the genome (Meyerdierks *et al.*, 2010). Genes encoding glutamate dehydrogenase and glutamine synthetase indicate that ammonium is used by ANME-1 as an inorganic nitrogen source. Finally, ANME-1 seem to be adapted to anoxic environments, as genes encoding putative enzymes involved in oxygen detoxification, such as superoxide reductase, catalase, flavodoxin, peroxiredoxin and rubredoxin, were identified (Meyerdierks *et al.*, 2010).

It has also been suggested that ANME-1 could use the reverse methanogenesis pathway for AOM within the SMTZ and the methanogenesis pathway for methane production in the sediments below (Lloyd *et al.*, 2011), however, utilization of H_2 and CO_2 through methanogenesis has not been shown for ANME-1.

6. Recent developments in molecular microbial ecology

Use of the 16S rRNA gene as a taxonomic and phylogenetic marker has increased our knowledge of microbial evolution and ecology. Today the gene is used world-wide to identify the members of microbial communities in various habitats. Much of our current understanding of the microbial community-structure in methane-enriched environments stems from the analyses of clone libraries constructed with bacteria- or archaea-specific primers (Hinrichs *et al.*, 1999; Knittel *et al.*, 2005; Lloyd *et al.*, 2006; Orphan *et al.*, 2001; Wegener *et al.*, 2008b). However, the application of the clone library approach, typically involving the sequencing of up to a few hundred sequences, will not provide information about low abundant taxa in a community. The high-throughput pyrosequencing technology has been commercially available since 2005 (Kircher & Kelso, 2010; Margulies *et al.*, 2005), and due to the development of new and rapid sequencing technologies, it is now possible to perform *in-depth* sequencing of PCR products from environmental samples in order to study the extremely rare microbes (Sogin *et al.*, 2006). This makes it feasible to study organismal stratification at a much higher resolution than has been done previously with conventional clone library construction. Approaches involving barcodes or unique DNA sequence identifiers have been developed for multiplex sequencing (Hamady *et al.*, 2008; Huber *et al.*, 2007; Parameswaran *et al.*, 2007) allowing the parallel analysis of multiple samples.

Linking the information about microbial diversity with the functional details of microbial ecosystems is a huge challenge. In order to obtain information about the metabolic capacity and physiological adaptations of microbial communities in unexplored habitats or in environments abundant in uncultivated taxa, a metagenomic approach can be used, whereby the genomic content (DNA) from all microorganisms in an environment is studied (Streit & Schmitz, 2004; Torsvik & Ovreas, 2002). Metagenomics is increasingly being viewed as a baseline technology for understanding the ecology and evolution of microbial ecosystems, providing a platform upon which hypothesis and experimental strategies can be based (Kunin *et al.*, 2008; Warnecke & Hugenholtz, 2007).

One of the first large-scale metagenomic studies in marine systems was the whole-genome sequencing of surface water samples from the Sargasso Sea (Venter *et al.*, 2004). In total, the almost 2 million reads generated from the 7 sampling sites were assigned to 1800 genomic species, including 148 novel bacterial phylotypes. The study also provided new information about physical adaptations in this environment, such as genes for ammonium monooxygenase, utilization of polyphosphates and pyrophosphates, several rhodopsin-like photoreceptors and phosphorus transport systems (Venter *et al.*, 2004). In 2006, the first implementation of pyrosequencing in a metagenomic study was published (Poinar *et al.*). Shortly afterwards, the overall diversity and community composition in several sediment depths at Peru Margin were studied using the same sequencing technology (Biddle *et al.*, 2008). In recent studies, metagenomics has been used to gain understanding of how geochemical factors in marine sediments can influence the microbial community structures at different locations (Biddle *et al.*, 2011b), and to characterize specific members of the communities, such as sulfur oxidizing clades (Jung *et al.*, 2010), uncultivated Euryarchaeota (Iverson *et al.*, 2012) and methanotrophic microorganisms (Håvelsrud *et al.*, 2011). As described in section 5.5 metagenomics has been successfully used to reveal that methane oxidation in ANME is performed through the reversed methanogenesis pathway, and has also provided deeper insights into their physiological properties (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2005).

Furthermore, new knowledge about the genes expressed in an environment, as well as information about the *in situ* activities of the whole microbial community is often considered an ultimate goal in microbial ecology studies. A metaproteomics approach could provide the basis for a deeper understanding of the interplay between the microbial taxa and how the community interacts with its physical environment. However, the use of metaprotoemics as a technique for studies of environmental samples is lagging far behind the accumulation of metagenomic data from various habitats. This fact may be explained by: (**I**) the use of protein-based techniques in functional studies of

microbial communities relies on the effective recovery of proteins from environmental samples (Maron *et al.*, 2007). (**II**) the bioinformatic assignment of mass spectrometrically determined peptide masses of environmental samples, and thereby identification of expressed proteins in the habitat, relies on the presence of known peptide sequences in the reference database (Schweder *et al.*, 2008). Metaproteomic approaches have, however, been applied to several ecosystems, such as human gut microbiota (Verberkmoes *et al.*, 2009), soil samples (Benndorf *et al.*, 2007; Wang *et al.*, 2011), leaf litter (Schneider *et al.*, 2012), biofilm communities (Banfield *et al.*, 2005; Jiao *et al.*, 2011), marine systems world-wide (Morris *et al.*, 2010; Sowell *et al.*, 2011) and aquatic systems (Habicht *et al.*, 2011; Lauro *et al.*, 2011).

Aims of the study

The cold seep area called Nyegga has previously been a site for several multidisciplinary geology studies. However, the microbial community structures in the G11 pockmark and the CN03 area remained unexplored.

The overall goal of this study was to provide new information and knowledge about the diversity and function of the microbial communities in sediments from Nyegga by applying new and powerful molecular techniques to sediment cores taken in the active G11 pockmark and the less active CN03 area.

Subgoal I was to study how high and low methane fluxes and chemical profiles could influence the composition and stratification of the microbial communities by using 454-pyrosequencing of 16S rRNA gene tag-encoded amplicon libraries integrated with quantitative PCR and FISH on several subsamples from each sampling site.

Based on the results obtained, including the identification of a sediment horizon dominated by free-living ANME-1, a coupled metagenomic and metaproteomic approach was developed. **Subgoal II** was thus to make hypotheses about the metabolic properties and *in situ* metabolisms involved in sustaining a sedimentary community dominated by free-living ANME-1, based on successful functional assignment of genes in the metagenome and identification of as many expressed proteins as possible from ANME-1 in this horizon.

Results and Discussion

1 Taxonomy and OTU classification

The stratification and structure of the microbial communities in the Nyegga cold seeps were studied using the 454-pyrosequencing technology (Roche[1]) on barcoded 16S rRNA gene amplicons (**Paper I** and **III**). Extracted DNA from the 7 subsamples from each sediment core was amplified using universal primers that target both archaeal and bacterial 16S rRNA genes, thus making it possible to estimate their relative abundance in the microbial communities. It is well-known that the PCR amplification may introduce bias in the distribution of amplified sequence types (Acinas *et al.*, 2005; Bru *et al.*, 2008; Suzuki & Giovannoni, 1996; Teske & Sørensen, 2008), which may largely influence microbial diversity estimates (Hong *et al.*, 2009; Kunin *et al.*, 2010). However, different sequencing error rates can be linked to specific variable regions on the 16S rRNA gene that is targeted by primers, where the error rates for the V5/V6 and V7/V8 region amplified in Nyegga subsamples (**Paper I** and **III**) are lower than e.g. the V3/V4 region of the gene (Claesson *et al.*, 2010). Differences in diversity and community structure due to bias introduced by the primers used in **Paper I** and **III** were assessed in a study by Lanzén *et al.* (2011), where the 16S rRNA content was analyzed by pyrosequencing of PCR-derived amplicon libraries and by a PCR-independent approach involving the synthesis of complementary DNA from total RNA extracted from the same sample. The study revealed that certain taxa were consistently under-represented in the amplicon dataset, and multidimentional linear regression showed that approximately 50% of the variation between datasets could be explained by PCR bias caused by primer mismatch and preferential binding to the template (Lanzén *et al.*, 2011). In addition, diversity estimations based on Simpson's diversity index showed that the diversity in general was lower in

[1] www.454.com

samples analyzed by the PCR-independent approach (Lanzén *et al.*, 2011). However, the relative abundance of the dominating epsilonproteobacterial taxa corresponded well in the datasets obtained by the different approaches. To further assess the relative abundance of *Archaea* and *Bacteria* in the Nyegga sediments, quantitative PCR was used (**Paper I** and **III**), and in one sediment horizon (14-16 cmbsf in the G11 pockmark) the relative abundances were assessed using FISH (**Paper I**). Congruent data were found for this horizon using all three approaches, indicating limited bias in the 16S rRNA gene amplicon dataset. In addition, the cell counts of 3 DAPI-stained subsamples from one of the cores agreed with quantified *Archaea* and *Bacteria* from corresponding horizons, obtained by real-time PCR (data not shown).

Clearly, the new sequencing technologies may offer deeper insights into the community structure by providing comprehensive datasets, however, such datasets require the development of new biocomputing methods. In this work quality filtering of the dataset was needed prior to both the taxonomical classification and the calculation of diversity indices in order to correct for errors introduced during PCR and sequencing (Quince *et al.*, 2011). In total, the quality filtering of amplicon reads excluded up to 22.6% (**Paper I**) and 29% (**Paper II**) of the sequences in the raw dataset. Barcode and primer sequences were removed prior to taxonomic classification, yielding an average read length of ~230bp for the reads (**Paper I**). Despite the reduced sequence length of the amplicon reads compared to near full-length 16S rRNA gene sequences obtained through clone libraries, the sequencing depth is considerably improved with 922-21400 reads (after filtering) for each of the subsamples (**Paper I** and **III**). While there are other technologies available for high-throughput sequencing of amplicons, such as Genome Analyzer II/IIx (Illumina[2]), SOLiD (Applied Biosystems[3]) and Ion Torrent (Life Technologies[4]),

[2] www.illumina.com

[3] www.appliedbiosystems.com

[4] www.iontorrent.com/

the 454-pyrosequencing instrument provides longer reads (<450 bp) compared to the other instruments (<150 bp). The length of the amplified section of the 16S rRNA gene is highly significant for the resolution of the taxonomic classification of reads, and a comparison between 454 and Illumina sequencing technology revealed that most of the Illumina reads could not be classified down to genus level (Claesson *et al.*, 2010). These observations were explained by the higher error rate in reads including more than 60 nucleotides and the shorter reads achieved with the Illumina technology (Claesson *et al.*, 2010). Furthermore, a study of community structures using 16S rRNA gene amplicon libraries with different read lengths (100, 400 and 1000 bp) revealed that the library comprising the shortest reads contained a higher number of unique sequences, higher diversity estimates and a different community structure compared to the other, more similar amplicon libraries (Huber *et al.*, 2009). The continuous development of high-throughput sequencing technology, especially with respect to increased read length, accuracy and lower cost, will provide datasets with improved sequencing depth for future studies of microbial communities.

In this study, taxonomic assignments of amplicon reads were done in Megan (Huson *et al.*, 2007) using the Lowest Common Ancestor (LCA) algorithm and the SilvaMod database (Lanzén *et al.*, 2011). There was a high abundance of uncultured taxa in the communities analyzed, where <22.8% (**Paper I**) and <88.6% (**Paper III**) of the reads were unclassified at order level in subsamples from the G11 pockmark and the CN03 area, respectively. The share of unassigned reads in datasets from both cores increased even further at lower taxonomical levels. These results illustrate the limitation of the amplicon library approach, as many of the uncultivated taxa in 15GC (**Paper III**) could not be assigned to lower taxonomical levels due to limited sequence information in the database. The communities in 29ROV were mainly dominated by taxa closely related to cultivated representatives, or ANME clades that were manually added to the Silva database (**Paper I**), thereby increasing the amount of classified reads at order level.

2 Stratification of the microbial communities

By using frequent vertical subsampling of the cores sampled at Nyegga in combination with pyrosequencing of 16S rRNA gene amplicons, it was possible to study the transitions in microbial community composition with increasing sediment depth (**Paper I** and **III**), and also to compare the communities from the active seeping area at the G11 pockmark with the communities in the CN03 area with less seeping activity (**Paper III**). Overall, the core from the G11 pockmark was dominated by aerobic and anaerobic methanotrophs (**Paper I**), suggesting that the community is adapted to an environment rich in methane, as indicated by previous geochemical analyses (Chen *et al.*, 2010; Ivanov *et al.*, 2010). In addition, the shallowest horizons had a high abundance of sulfur oxidizing *Epsilonproteobacteria,* which are associated with the microbial mat located on the seafloor. The ε-proteobacterial reads were assigned to *Sulfurovum* and *Sulfurimonas*, which have previously been described as chemolithoautotrophs genera that are associated with marine hydrothermal vents and adjacent sediments (Campbell *et al.*, 2006). It is possible that these microbes to have an opportunistic life strategy by overlying fluid chimneys in Nyegga cold seeps, as the optimum growth temperature for cultivated representatives are within the mesophilic range (Inagaki *et al.*, 2003; Inagaki *et al.*, 2004; Takai *et al.*, 2006; Yamamoto *et al.*, 2010). However, *Sulfurovum* and *Sulfurimonas* are not commonly observed in microbial mats at cold seeps. Instead, mats composed of *Beggiatoa* (*Gammaproteobacteria*) have been described at cold seeps world wide (Boetius *et al.*, 2000; Hovland, 2007; Larkin *et al.*, 1994; Lösekann *et al.*, 2007). Furthermore, microbial mats including cells similar to *Arcobacter sulfidicus* (*Epsilonproteobacteria*), *Beggiatoa* and *Thiomargarita* (*Gammaproteobacteria*) have been observed in the area at the Nile Deep Sea Fan (Grünke *et al.*, 2011; Omoregie *et al.*, 2008), and Grünke *et al.* (2011) suggested that different mat-forming sulfur-oxidizing bacteria could be adapted to different oxygen-sulfide gradients in the transition between the oxic and anoxic zone. A different theory was presented by Macalady et al. (2008),

stating that the presence of different mat-forming taxa is limited by their physical properties. The lack of holdfasts in *Beggiatoa* species may cause the filaments to drift in areas with high fluid flow or currents, while *Thiothrix* and ε-proteobacterial taxa might require water-movements to avoid burial by accumulating sediments in sheltered areas. Similar factors could have influenced the mat-formation at the Nyegga cold seeps, creating optimum growth conditions for epsilonproteobacterial mats rather than gammaproteobacterial mats due to the high fluid flow, however, this deduction should be addressed in future studies. The aerobe methanotrophs, which dominated the sediment horizons between seafloor and 3 cmbsf, were affiliated with *Methyloccocales* within *Gammaproteobacteria*, however, the taxomomical classification was unsuccessful below order level (**Paper I**). Below this zone, a stratification of ANME-clades was observed with a transition between an ANME-2a/b dominated community (4-7 cmbsf) to an ANME-1 dominated community (9-16 cmbsf), followed by an ANME-2c dominated community (20-22 cmbsf) (**Paper I**). **Paper I** was published almost simultaneously with a second study of the microbial communities in the G11 pockmark by Lazar *et al.* (2011), although the coordinates of the sampling site shows that the core used in the study by Lazar *et al.* was retrieved approximately 1 km north of the center of the G11 pockmark. The 10 cm long core is half the length of the core in **Paper I**, and subsamples were taken every 2 cm (Lazar *et al.*, 2011). However, the total dataset of 9 sequences originating from dominant DGGE bands (n=5) and 46 archaeal RNA-derived 16S rRNA sequences (n=2) provided poor resolution for the community study compared to the results present in **Paper I**. The results presented by Lazar *et al.* showed the presence of ANME-2, MBG-D and MG-1 in the sallower horizons and ANME-1 in the two deepest horizons, which is consistent with the order of ANME-clades and presence of MG-1 in the shallower horizons in the core from the G11 pockmark (**Paper I**). The approach of frequent sampling through a sediment core was also used at the Amsterdam mud volcano and the Kazan mud volcano (Mediterranean Sea) (Pachiadaki *et al.*, 2010;

Pachiadaki *et al.*, 2011), and Garden Banks (Gulf of Mexico) (Martinez *et al.*, 2006) with 3-7 subsamples in each study, revealing high abundance of different ANME clades at different depths. However, these studies have low resolution as the community composition analysis is based on less than 60 clones of archaeal 16S rRNA gene content from each subsample. This fact has a large impact on the relative abundance of each ANME-clade, and therefore the trend of specific zones involving one dominant ANME-clade, equivalent to the situation at Nyegga, was not observed in these cases.

Although the estimated methane fluid flux in the CN03 area is ~4-5 times lower than the flux inside the G11 pockmark (Chen *et al.*, 2010; Chen *et al.*, 2011), the same transition between an ANME-2a/b and ANME-1 dominated community was observed (**Paper III**). The abundance of ANME-2c was lower in the core from the CN03 area, however the trend of increasing abundance with increasing depth indicate that an ANME-2c dominated community could be present in deeper horizons (**Paper III**). The relative abundance of ANME-2a/b and ANME-1 in each core were within the same range, though the quantified of archaeal 16S rRNA gene copies were 2 orders of magnitude lower in the ANME-dominated horizons at the CN03 area compared to the G11 pockmark (**Paper III**). Furthermore, the ANME-specific stratification zone was found at a deeper sediment depth in the core from the CN03 area, where the zone of each ANME-clade extended over a wider depth interval. This could indicate that the different fluid flow regimes at the two sampling sites did not influence the stratification of ANME in Nyegga, but may rather affect the total biomass of ANME, as has been previously suggested (Constan, 2009). The observed stratification could be correlated to the geochemical profiles of the cores, showing increasing concentration of hydrogen sulfide (H_2S) with increasing depth (**Paper I** and **III**). This corresponds well with the study by Meulepas *et al.* (2009) indicating that AOM by the ANME-2 clade could be limited by increased H_2S concentration. In this study, the AOM rates and sulfate reduction rates in enrichment cultures with ANME-2 dominated sediments were inhibited at sulfide concentrations above 2.4 mM. The sulfide

concentrations measured in ANME-2a/b dominated horizons of the Nyegga cores were higher, <2.5 mM in the G11 pockmark (**Paper I**) and <8.1 mM in the CN03 area (**Paper III**), but the concentrations increased even further in the ANME-1 dominated horizons below, thereby supporting the *in situ* observations by Meulepas *et al.* (2009).

The major differences between the sediment cores were related to the dominating taxa in horizons above the ANME stratification zone, and also co-occurring taxa within this zone (**Paper III**). The microbial community structures in the horizons between 10 and 120 cmbsf in the CN03 core had a high abundance of DSAG and MG-1 that are associated with a heterotrophic lifestyle, possibly with sulfate reduction, (Biddle *et al.*, 2006; Inagaki *et al.*, 2006) and anaerobic ammonia-oxidation (Nicol & Schleper, 2006; Spang *et al.*, 2010), respectively. This could indicate that most of the methane has been utilized by the ANME clades below before it reaches the shallower horizons. Similar observations are recorded for other cold seeps characterized with lower fluid flow, such as the Peru Margin, the South China Sea, the Santa Barbara Basin and the New Caledonia Basin where the communities are dominated by DSAG and MG-1 (Harrison *et al.*, 2009; Inagaki *et al.*, 2006; Roussel *et al.*, 2009; Sørensen & Teske, 2006; Wang *et al.*, 2010b). The highest abundances of ANME-1 in cores from the G11 pockmark and the CN03 area were 89.2% and 82.2% (**Paper I** and **III**), respectively. FISH images of the ANME-1 dominated horizon in the G11 pockmark revealed free-living ANME-1 cells affiliated in chains (**Paper I**) (Fig. 5). This suggests that this clade might perform AOM independently of a sulfate-reducing partner.

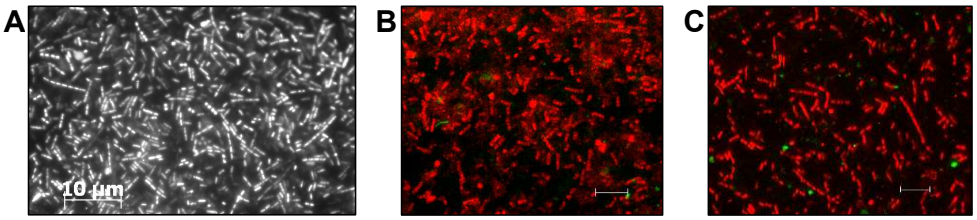

Figure 5: The microbial community at 14-16 cmbsf in the G11 pockmark, which is characterized by high methane fluid flux, was visualized by microscopy. The DAPI stained community (**A**) revealed high numbers of microorganisms with mostly rod-shaped morphology. The selective FISH probes showed that the community was dominated by Archaea (red) (**B**), of which the majority of cells were identified as ANME-1 (red) (**C**). The number of bacterial cells (green) was minimal in the FISH stained community (**B, C**). Scale bar is 5 µm if not specified in the image. Adapted from **Paper I**.

In contrast, horizons dominated by ANME-2a/b and ANME-2c had a higher overall diversity and lower relative abundance of the methanotrophic clades (**Paper I** and **III**), suggesting that they might perform AOM with a syntrophic partner. In the core from the G11 pockmark, these horizons had an intermediate abundance (5-10%) of the co-occurring *Deltaproteobacteria* (**Paper I**), which have been previously found to include the bacterial partners for ANME-2 (Knittel & Boetius, 2009; Schreiber *et al.*, 2010). In addition, there was a correlation between the abundance of JS-1 and ANME-2, which indicates that JS-1 might be involved in AOM through syntrophy or that JS-1 benefits indirectly from ANME-associated metabolisms (**Paper I**). The relationship between ANME-2 and the potential partners were not examined in detail, however, the possibility of taxa other than *Deltaproteobacteria* being involved in the syntrophic metabolism has been suggested (Pernthaler *et al.*, 2008), and this option should therefore not be excluded for the Nyegga cold seeps. In the core from the CN03 area, the abundance of *Deltaproteobacteria* in ANME-2 dominated horizons was low (<1%), while taxa such as DSAG and MG-1 were abundant, indicating that other metabolisms could co-occur with AOM (**Paper III**). Furthermore, OTU clustering of all ANME affiliated 16S rRNA

gene sequences revealed that both the G11 core and the CN03 core were dominated by a common ecotype of ANME-1 and different ecotypes of ANME-2a/b (**Paper III**). This could indicate that ANME-2a/b is a diverse subgroup, including various ecotypes that could adapt to different geochemical conditions, where regional differences in methane concentrations could influence the distribution of the unique ANME-2a/b ecotypes in Nyegga. The system at the G11 pockmark is rich in methane, even in the *Methylococcales*-dominated horizons near the seafloor, while the methane concentration in the CN03 core was lower, ranging from <3.6mmol/L in ANME-1 dominated horizons to 0.05-0.3mmol/L in the ANME-2a/b dominated horizons above (Vaular, 2011). Similarly, a study of the vertical variations in the community structures at the Amsterdam mud volcano, which had a gradual increase in the concentration of methane with increasing depth, revealed that different phylotypes within each of the observed ANME clades were dominating at different depths throughout the core (Pachiadaki *et al.*, 2011).

Overall, the observation of unique ANME-2a/b ecotypes and different co-occurring taxa in the cores from the Nyegga cold seep might challenge the theory that ANME-2 is obligate methanotrophic. Future studies using a metagenomic approach or *in vitro* cultivation of ANME-2 might be required to determine if this taxon is solely capable of AOM or if they can perform other metabolisms dependent on the geochemical conditions. Given that the ANME-2a/b community at the CN03 area could be sustained at lower methane concentrations, they might play an important role as potential seed populations in newly developed methane-enriched systems, as suggested by Knittel & Boetius (2009).

3 The coupled metagenomic and metaproteomic approach

In order to obtain a deeper understanding of the physiological aspect in the free-living ANME-1 community dominating the sediment horizon at 14-16

cmbsf in the core from the G11 pockmark (**Paper I**), a coupled metagenomic and metaproteomic approach was developed (**Paper II**). Successful extraction of proteins from sediment samples can be difficult as the proteins tend to adhere irreversibly to the clay particles (Ding & Henrichs, 2002), hence the manufacturers protocol for Tri-reagent solution was modified prior to the application on the Nyegga sediment samples for efficient protein extraction (150-700 µg/g sediment) (**Paper II**). Proteins were separated by 1D-gelelectrophoresis followed by tryptic digestion, and the peptides were subsequently analyzed using a nanoLC system connected to the linear quadrupole ion trap orbitrap mass spectrometer (see **Paper II** for details).

In order to optimize the protein identification for the Nyegga sediment sample, a metagenome from the same sample material was constructed to compensate for the limited sequence information of functional genes in the publically available databases when the project was initiated (**Paper II**). Although, the metagenomic work was started before the composite genome of ANME-1 was published (Meyerdierks *et al.*, 2010), the number of identified proteins in the Nyegga cold seeps improved when sequences from the ANME-1 composite genome were included in the database (**Paper II**), due to the apparently close phylogenetic relationship between the ANME-1 population in the Nyegga and the Black Sea ecosystems. Pyrosequencing of the extracted DNA from the sediments resulted in 830 000 reads, with an average length of 450 bp, of which ~43% were assembled into contigs using the Newbler software (Roche) (**Paper II).** This is comparable to other environmental studies, such as the whole genome shotgun sequencing of a sludge community (Martin *et al.*, 2006) and Illumina sequencing of paired-end libraries from human gut microbes (Qin *et al.*, 2010) where 68-74% and 42.7% of the reads could be assembled, respectively. For future studies, the amount of assembled sequence information could be increased by using additional sequencing technologies, such as the paired-end sequencing, which improves assembly due to a internal marker sequence (Kircher & Kelso, 2010), or the

Single Molecule Real Time (SMR) DNA sequencing (Pacific Biosciences), which provides sequences up to 10 000 bp long (Flusberg *et al.*, 2010; McCarthy, 2010). Due to the conserved nature of the 16S rRNA gene and the presence of similar gene sequences affiliated with the various taxa in environmental samples, the 16S rRNA gene sequences were collapsed on to highly conserved contigs by the Newbler software, which excluded all 16S rRNA genes from the contigs with functional genes (**Paper II**). This problem is avoided when using fosmid libraries where the 16S rRNA gene is often included in longer sequences containing several kbp of genetic information. By using this approach it might be possible to link function with taxonomy, e.g. linking genes associated with the methanogenesis pathway to ANME (Hallam *et al.*, 2004) or linking the key enzyme in nitrification (ammonia monooxygenase) to the 1.1b group of *Crenarchaeota* (now *Thaumarchaeota*) (Treusch *et al.*, 2005).

Furthermore, the assembled sequences were aligned to the NCBI Refseq protein database using BlastX, followed by taxonomical classification in MEGAN. Only 66.3% of the sequences in the metagenome could be assigned to ANME-1 (**Paper II**), which is significantly lower than the share of 89.2% ANME-1 reads deduced from the amplicon library from the same horizon (**Paper I**). This discrepancy could be due to selective primers or to the introduction of bias during amplicon library construction, lower sensitivity in taxonomical assignment of metagenomic sequences than for 16S rRNA genes tag-encoded amplicons due to limited genomic information for ANME-1 in the database, or that ANME-1 might have a higher copy number of the 16S rRNA gene compared to the remaining genes in the genome. In total, 3 custom databases were constructed for protein identification, based open reading frame (ORF) prediction (all 6-frames) of assembled contigs from the metagenome, selected sequences from the NCBI Refseq protein database and published sequences assigned to ANME-1 and ANME-2 (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2005; Meyerdierks *et al.*, 2010) (see **Paper II** for details). The search engine used, Mascot, identifies proteins by comparing

experimental MS/MS ion scores with theoretical ion scores based on *in silico* tryptic digestion of sequences in a defined database (Aebersold & Mann, 2003). For each query submitted, Mascot will report significant scores to peptides in the database, where the score is based on the probability that the observed match is a random event (Pappin *et al.*, 1993). However, all peptide matches are not necessarily above the threshold, meaning that the hit is significant only if the event is expected to randomly occur with a frequency of less than 5%. In addition, the search is repeated by Mascot using a decoy database (Elias *et al.*, 2005), which includes reversed and randomised sequences from the original database. Here, positive hits represent the number of false positives in the search result from the true database, which makes it possible to estimate the false discovery rate. Mascot also provides a non-probabilistic protein score, which is based on the combined peptide scores that can be assigned to each protein. Due to the algorithm implemented in Mascot, whereby spectra obtained from the MS/MS instrument are linked to proteins in the database, the selected database can impact the threshold level and false discovery rate, and thereby influence the number of identified proteins. Hence, a non-redundant database including sequences of closely related taxa is preferred. For these reasons, the translated ORF's from the metagenome gave the highest number of peptide hits over threshold (4459) and the lowest false discovery rate (3.16%) (**Paper II**). The low number of peptide hits over threshold (1360) and high false discovery rate (6.10%) achieved when using the NCBI non-redundant protein database emphasize the advantage of having closely related sequences from the uncultivated phyla in the environment in the database, in addition to the sequenced representatives in the public available databases (**Paper II**).

In total, 356 proteins were identified from the Nyegga sediment sample, which is within the range of the number of proteins identified in previous studies, e.g. a total of 1790 proteins from 3 samples in the study of human gut microbes using custom databases (Kolmeder *et al.*, 2012), 122 proteins in soil using the NCBI non redundant protein database (Wang *et al.*, 2011), 481 unique protein

families in marine systems using metagenomes from the global ocean survey (GOS) in costal environments as the database (Rusch *et al.*, 2007; Sowell *et al.*, 2011), and 428 (±158 per sample) proteins in surface seawater using GOS combined assembly protein database (Morris *et al.*, 2010).

4 Metabolism and physical adaptations in ANME-1

The 356 proteins identified gave an overview of the *in situ* activity of the ANME-1 dominated community, including proteins for energy metabolism and for mechanisms involved in an optimized lifestyle in cold seep environments, which reflected the strength of the coupled proteomic and metagenomic approach. The reverse methanogenesis pathway was found to be the main C1 metabolism in ANME-1, as all enzymes involved in this pathway (except methylene H_4MPT reductase) were expressed by ANME-1 (**Paper II**), which agrees with previous observations (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2005; Meyerdierks *et al.*, 2010). A *mer* gene with 82% sequence identity to *Methanococcoides burtonii* was identified in the metagenome and metaproteome (**Paper II**). However, this gene is possibly expressed by ANME-2, which constitutes 3.6% of the community based on data from the amplicon library of this horizon (**Paper I**). Alternative bypassing mechanisms to complete the reverse methanogenesis pathway have been suggested, including formation of methylene H_4MPT from methanol via formaldehyde (Welander & Metcalf, 2008), however, this was mechanism was not supported by the metaproteome in **Paper II**. Instead, *mer* could be replaced by the putative methylene tetrahydrofolate (H_4F) reductase gene that was found expressed by ANME-1, and also identified in the ANME-1 composite genome (Meyerdierks *et al.*, 2010). The H_4MPT and the H_4F are structural analogues at the binding site for methylene, however, they are functionally distinct as the methylene carried by H_4MPT is more readily oxidized than the one on H_4F (Maden, 2000). Thus, biochemical characterization of the methylene

tetrahydrofolate (H_4F) reductase is needed to confirm this hypothesis. Furthermore, several electron-accepting complexes were expressed by ANME-1 (**Paper II**), such as homologues of the $F_{420}H_2$:quinone oxidoreductase (Fqo) complex and two heterodisulfide reductase complexes (Hdr1 and Hdr2), which are also previously observed in ANME-1 (Hallam *et al.*, 2004; Meyerdierks *et al.*, 2010) and ANME-2 (Meyerdierks *et al.*, 2005). However, the membrane-associated Hdr found in methanogens (Thauer *et al.*, 2010) was not identified in either the Nyegga metagenome (**Paper II**) or in the ANME-1 composite genome (Meyerdierks *et al.*, 2010).

In the metagenome from Nyegga sediments, all genes for dissimilatory sulfate reduction were identified, where the genes for adenylyltransferase (Sat), adenosine 5'-phosphosulfate (Apr) and dissimilatory sulfite reductase (Dsr) were found expressed by sulfate-reducing *Deltaproteobacteria* (**Paper II**), which is not unexpected in a cold seep environment where sulfate is available as the terminal electron acceptor. In addition, an expressed Apr subunit with high similarity to *Syntrophobacter fumaroxidans* was present in the metaproteome, and this protein was found to be affiliated with Gram positive sulfate-reducing bacteria rather than *Deltaproteobacteria* in the phylogenetic tree that was generated (**Paper II**). AprBA-based phylogenetic trees comprising various sulfate-reducing prokaryotes indicates that the *apr* gene has been transferred between sulfate-reducing lineages through lateral gene transfer (LGT) (Meyer & Kuever, 2007). Furthermore, the sulfate-reducing euryarchaeon, *Archaeoglobus sp*., may have acquired the genes for dissimilatory sulfate reduction from Gram positive *Firmicutes* through LGT (Basen *et al.*, 2011; Meyer & Kuever, 2008). Hence, the Apr identified in the Nyegga proteome may originate from a novel Gram positive SRB, or it might be affiliated with ANME-1 as the FISH images in **Paper I** indicated high abundance of be free-living ANME-1 in this horizon.

The coupled metaproteogenomic approach also revealed proteins important for the cold seep lifestyle besides enzymes involved in metabolism, such as

proteins for stress response and gas vesicle development (**Paper II**). Proteins involved in the oxidative stress response, such as putative peroxiredoxin, putative flavodoxin, putative NADH oxidase and a conserved hypothetical protein with significant similarity to rubrerythrin from *Methanohalobium evestigatum* were expressed by ANME-1 (**Paper II**). Genes encoding peroxiredoxin and flavodoxin homologues were also identified in the ANME-1 composite genome (Meyerdierks *et al.*, 2010). Furthermore, several chaperones were identified from the Nyegga metaproteome, including thermosome (subunit alpha and beta) and dnaK expressed by ANME-1, as well as GroEL expressed by *Deltaproteobacteria* (**Paper II**). Chaperones are identified in all archaeal genomes so far where they assist in protein folding by providing an enclosed cavity where the proteins can transform into a folded state without interaction from the intracellular environment or other partners that could result in insoluble aggregates (Lund, 2011). Chaperones are constantly expressed, as correct protein folding is vital for successful maintenance and reproduction of the cell, but the transcribed genes are highly up-regulated during increased temperatures as a stress response to heat-shock (Lund, 2011). A novel characteristic of ANME-1 was revealed by the metaproteogenomic approach, where the expression of the gas vesicle synthesis family protein and gas vesicle protein GvpN indicates that ANME-1 can develop intracellular gas vesicles (**Paper II**). Gas vesicles are gas-permeable rigid structures that can provide buoyancy in aquatic prokaryotes, especially in phototrophic lineages, or be involved in stress-response in halophilic archaea (Walsby, 1994). Gas vesicles has also been observed in methanogenic taxa, such as *Methanosarcina* and *Methanothrix*, which are closely related to the ANME clades, however their function in these taxa remains unknown (Archer & King, 1984). Interestingly, transmission electron microscopy (TEM) images of ANME-1 cells revealed stacks of intracellular membranes with unknown function (Reitner *et al.*, 2005), though the morphology of the structures resemble gas vesicles in aquatic prokaryotes

(Walsby, 1994) rather than the intracytoplasmic membranes in aerobe methanotrophs suggested by Reitner *et al*. (2005).

In summary, based on the results from the metaproteogenomic approach it is possible to make predictions about the *in situ* metabolic pathways and adaptations for taxa in environmental samples. The ANME-1 clade in the Nyegga cold seeps thus performs AOM through the reversed methanogenesis with associated electron-accepting complexes, as previously suggested. Furthermore, ANME-1 seems to be adapted to permanently cold environments and anoxic conditions, but could possibly position itself in a sulfide gradient for optimal growth conditions due to intracellular gas vesicles. The metaproteogenomic approach did unfortunately not reveal the mechanism involving a terminal electron acceptor in ANME-1, although the expression of APS reductase by ANME-1 cannot be ruled out.

Concluding remarks

The integrated approach used to study the microbial communities in the Nyegga cold seeps, involving 16S rRNA tag-encoded amplicon libraries, quantitative PCR and FISH, revealed an overall dominance of free-living aggregates of ANME-1. The observation of ANME-1 without a close association to a sulfate-reducing partner challenges the hypothesis of ANME having an obligate syntrophic lifestyle, and also raises questions about the possibilities of novel metabolic pathways for this clade.

ANME-1 is the main consumer of methane in the Nyegga cold seeps, as the coupled metagenomic and metaproteomic approach revealed that most of the genes involved in the reverse methanogenesis pathway were expressed by this clade. However, this approach could not shed light on possible biochemical mechanisms involved in a terminal electron-transport chain. Previously supported hypotheses, including nano-wires, conductive matrix and various compounds as redo-ox shuttles, were not supported by the dataset. An APS-reductase gene associated with dissimilatory sulfate reduction was found expressed in the environment, but the gene could not be directly linked to ANME-1 in the metagenome. Additional sequencing, using e.g. a paired-end sequencing approach or SMRT Time DNA Sequencing that could provide longer contigs, might be able to verify if the genes encoding enzymes for sulfate reduction are present in the ANME-1 genome. However, future research is needed to reveal if ANME-1 is both a methanotrophic and a sulfate-reducing clade that completes the process of AOM itself. OTU clustering of ANME affiliated reads revealed that a common ecotype of free-living ANME-1 was abundant in both the sampling sites. For ANME-2a/b, different ecotypes were associated the high or low methane fluid flow at each site. These observations could indicate that the ANME-2a/b community is sustained by lower methane concentrations at the CN03 area, or that this clade is not an obligate methanothrophic archaeon, which is supported by the

clear differences in co-occurring taxa at the two sites. Despite the differences in methane fluid flow at the sites, a similar vertical stratification of ANME clades were observed in both cores. Based on the geochemical profiles, it is likely that the positioning of the ANME clades in specific horizons at Nyegga cold seeps is influenced by increasing concentrations of sulfide with depth rather than the differences in the concentration of methane.

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