Methane Oxidation and Carbon Assimilation in Marine Sediments

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften

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There is a theory which states that if anybody ever discovers exactly the nature of AOM, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

Freely adapted from
Douglas Noel Adams (1952 – 2001)

Acknowledgments

I started my PhD training as a Geologist and had to become a combination of Biologist, Biogeochemist, Organic Geochemist and Seaman. This metamorphosis required lots of support – here is probably an incomplete list of the people I would like to thank for this exciting transformation.

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Zusammenfassung

Methan, ein starkes Treibhausgas, entsteht bei der mikrobiellen und thermogenen Mineralisation von Biomasse unter anoxischen Bedingungen. Über diese Prozesse werden im Meeresboden große Mengen des Methans akkumuliert. Dort liegt Methan entweder als Gashydrat vor, ist im Porenwasser gelöst, oder nimmt den Porenraum als Gasphase ein.

Marine Methanemissionen sind jedoch nur für ca. 3% der atmosphärischen Methanzufuhr verantwortlich, da der Kohlenwasserstoff im Sediment von einer hocheffektiven Gemeinschaft aerober und anaerober Mikroorganismen oxidiert wird. Diese Arbeit befasst sich mit der anaeroben Oxidation von Methan (AOM), die im Meeresboden für ca. 80% des Methanabbaus verantwortlich ist. AOM wird von Konsortien methanotropher Archaeen und sulfatereduzierenden Bakterien katalysiert, welche Methan und Sulfat in syntrophen Reaktionen zu Karbonat und Sulfid umsetzen.

Im ersten Kapitel wird die Rolle von Methan im Kohlenstoffkreislauf sowie die Quellen und Senken von Methan mit besonderer Berücksichtigung von AOM beschrieben. Wichtige Erkenntnisse zur AOM und Methoden zur Erforschung dieses Phänomens, insbesondere isotopengeochemische Ansätze, werden vermittelt.

Im zweiten Kapitel wird die Aktivität verschiedener Gasaustrittsstellen in der Nordsee beschrieben. Aufgrund der relativ geringen Wassertiefe von 70 bis 150 Metern ist die Methanlöslichkeit im sedimentären Porenwasser relativ gering. Der Methantransport findet somit überwiegend in der Gasphase (in Form von Gasblasen) statt. Dabei wird ein großer Teil des Methans nicht im Sediment oxidiert, sondern in die Wassersäule abgegeben. Der kurze Transportweg zur Wasseroberfläche macht einen direkten Beitrag zum atmosphärischen Methanhaushalt wahrscheinlich.

Zwei der aktivsten Gasaustritte, Gullfaks in der nördlichen Nordsee und Tommeliten in der zentralen Nordsee, wurden zur geochemischen und mikrobiologischen Charakterisierung ausgewählt. Die Methanaustritte von Gullfaks liegen in einer mächtigen

glazialen Sandschicht. Die Gasaustritte sind in einem Bereich von ca. 0.5 km^2 konzentriert, in dessen Zentrum von 0.1 km^2 ein teils dichter Bewuchs durch schwefeloxidierende Bakterien (*Beggiatoen*) hohe Sulfidflüsse aus dem Sediment anzeigt. Die großen Sulfidmengen entstehen bei der anaeroben Methanoxidation.

Methan steigt durch Risse in einem glazialen Mergel an die Oberfläche auf. Die Methanaustritte beschränken sich so auf wenige kleinere Bereiche (<0.3 m^2), die meist mit dichten Matten sulfidoxidierender Bakterien bedeckt sind. Mit Hilfe eines Tauchroboters wurden diese Bakterienmatten und die darunter liegenden Sedimente erstmals beprobt.

An den untersuchten Methanaustritten der Nordsee wurde im Unterschied zu vielen Tiefsee-Methanaustritten keine chemosynthetische Fauna gefunden. Die hohen, gezeitenbedingten Transportenergien verhindern offenbar die Besiedlung durch benthische Organsimen. Jahreszeitlich schwankende und durchschnittlich höhere Temperaturen in der Nordsee sind weitere Unterschiede zu Tiefsee-Methanaustritten. Die mikrobielle Gemeinschaft der Methanquellen von Gullfaks und Tommeliten wurde mit Hilfe von Lipidbiomarkeranalysen und molekularbiologischen Methoden beschrieben. Die gefundenen Bakterien- und Archaeenbiomarker deuten auf eine Dominanz von anaeroben Methanoxidierern, deren sulfatreduzierenden Partnerbakterien sowie sulfidoxidierenden Bakterien in den Sedimenten hin. Im Durchschnitt sind die Kohlenstoff-Isotopenwerte der Lipidbiomarker in den Methanaustrittssedimenten sehr niedrig, wobei insbesondere die Archaeenbiomarker mit $\delta^{13}C$-Werten bis -120‰ die deutlichsten Abreicherungen in der Kohlenstoffisotopie zeigen. Die ^{13}C-abgereicherten Biomarker zeigen die Aufnahme von Kohlenstoff aus dem schon isotopisch leichten Methan und eine weitere starke Fraktionierung während der Kohlenstofffixierung an. Die auf 16S-rRNA-Gensequenzierung basierende Analyse der mikrobiellen Gemeinschaft zeigt, dass sich die Organismen der Nordseemethanaustritte von denen der Tiefsee kaum unterscheiden. In den aktiven

Sedimenten dominieren Anaerobe Methanoxidierer (ANME-2), die in Gemeinschaft mit Sulfatreduzierern leben.

Im dritten Kapitel wird ein Durchflusssystem zur Untersuchung der anaeroben Methanoxidation in Sedimenten vorgestellt. In der Durchflusszelle wird Sediment permanent mit methangesättigtem anaeroben Medium durchströmt. Durch die Messung von Methanzehrung und Sulfidbildung wurde die jeweilige Aktivität des Sediments ermittelt. Bei gleichbleibenden Methankonzentrationen im Zufluss von ca. 2 mM wurden über Monate konstante Methanoxidation gemessen. Ein merkliches Wachstum der Bakterienpopulation erfolgte nicht, obwohl weder Methan noch Sulfat limitierend waren. Auch in anderen Publikationen wurde oft von sehr geringen Wachstumsraten der anaeroben Methanoxidierer berichtet. Ein Grund hierfür wird der besonders geringe Energiegewinn bei der anaeroben Methanoxidation (ΔG_R ~ -15 bis -40 kJ mol^{-1} CH_4 Umsatz) sein, der zudem von zwei Organismen geteilt werden muss. Aus diesem Grund werden für ein merkliches Wachstum der mikrobiellen Population vermutlich Methankonzentrationen im Bereich mehrerer Millimolar benötigt.

Methanotrophe Sedimente wurden über sechs Wochen mit methanfreiem Medium durchströmt. Nach der Wiederaufnahme der Methanzufuhr wurden innerhalb weniger Tage die vorherigen Methanoxidationsraten gemessen. Substratmangel über lange Zeit zu tolerieren scheint eine wichtige Eigenschaft der methanoxidierenden Organismen zu sein, um die in der Natur oftmals schwankenden Methankonzentrationen zu überleben. Für die Umwelt zeigt es, dass die Mikroorganismen auch bei wechselnden Methanflüssen eine Barriere für das aufsteigende Methan darstellen.

Im vierten Kapitel werden die Methoden der Lipidbiomarkeranalyse mit der Isotopenmarkierung von Substraten kombiniert, um die Rolle anorganischen Kohlenstoffs und Methans als Kohlenstoffquelle der methanoxidierenden Mikroorganismen zu untersuchen. In den Archaeenlipiden wurden der Einbau von ^{13}C-markierten Methans und

anorganischen Kohlenstoffs in ungefähr gleichen Mengen beobachtet. Die sulfatreduzierenden Bakterien nahmen hingegen ausschließlich ^{13}C-markierten anorganischen Kohlenstoff in ihre Lipide auf.

Die Ergebnisse der Isotopenmarkierungsversuche können die an Umweltproben gemessenen sehr niedrigen Isotopien der Bakterienbiomarker ($\delta^{13}C$ bis ca. -100‰) und und Archaeenlipide ($\delta^{13}C$ bis ca. -130‰) erklären. Archaeen bauen hauptsächlich stark ^{13}C-abgereichertes Methan und/ oder direkt aus Methan stammendes CO_2 ein. Daneben sorgt eine hohe Fraktionierung während der Kohlenstofffixierung für die extrem ^{13}C-abgereicherte Lipide. Die Sulfatreduzierer bauen ihre Biomasse aus CO_2 auf, welches aus dem leichter abgereicherten inorganischen Kohlenstoffpools des Porenwasser entstammt.

Die Ergebnisse zeigen, dass der Energietransfer zwischen den methanoxidierenden Archaeen und den Sulfatreduzierern nicht auf Monomeren wie Acetat oder Format beruhen. Wären diese Stoffe ein Intermediat, so müsste sich isotopisch markiertes Methan auch in der Isotopensignatur der Biomarker der partizipierenden sulfatreduzierenden Bakterien wiederfinden.

Aus den Labelingversuchen konnte eine Wachstumseffizienz ermittelt werden. Unter unseren Versuchsbedingungen ist der Biomasseaufbau mit Kohlenstoffaufnahmeeffizienz von nur 0,3% bis 1,3% äußerst gering. Diese einzigartig niedrige Wachstumseffizienz lässt sich durch die extrem niedrigen Energiegewinn bei der anaeroben Oxidation des Methans erklären.

Im letzten Kapitel werden die Ergebnisse der wissenschaftlichen Arbeit zusammengefasst und im Kontext der bisherigen Forschungsarbeiten beschrieben. Zuletzt wird ein Ausblick auf weitere Schritte der Erforschung des Kohlenstoffkreislaufs in der anaeroben Methanoxidation gegeben. Einen Schwerpunkt bilden dabei Methoden der Isotopenmarkierung.

Summary

Methane, a strong greenhouse gas, is produced by microbial fermentation of organic matter under anoxic conditions and by thermal driven decay of organic matter. Both biogenic and thermogenic methanogenesis lead to the accumulation of large quantities of methane in the seafloor in the form of gas hydrate, dissolved in the pore water, and as gas phase.

Despite the large methane content of the sediment, its contribution to water column and atmospheric budgets is rather small (about 3%). This is because aerobic and anaerobic microbes consume much of the seafloor methane. In this thesis I focus on the anaerobic oxidation of methane (AOM), which is, in the marine context, responsible for about 80% of methane consumption. AOM describes the microbially mediated consumption of methane and sulfate with products carbonate and sulfide. The responsible microorganisms are believed to consist of syntrophic consortia of methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB).

In **Chapter I**, I discuss methane as part of the marine carbon cycle, including it's sources and sinks, with the main emphasis on AOM. Important experimental methods, including kinetics and growth determination isotope labeling approaches, are discussed in detail in **Chapter IV.**

In **Chapter II**, I describe methane emissions at several shallow seep areas (<160m) of the North Sea. Because of the shallow water (low pressure), methane solubility is low, much lower than necessary for the formation of gas hydrates. This favors the transport of methane in the gas phase, which limits the function of the microbial barrier and makes the export of methane in the form of bubbles to the water column and atmosphere more likely.

The two most active seepage areas of the North Sea: Gullfaks in the Northern North Sea, and Tommeliten in the center of this basin, were chosen for geochemical and microbial characterization. The Gullfaks seep sites were situated on a deep sand layer,

deposited within the last glacial maximum. Gas seepage was found in an area of 0.5 km^2, with the zone of highest seep activity, limited to an area of about 0.1 km^2, marked by mats of sulfide oxidizing bacteria, which are fueled by the high sulfide fluxes due to AOM activity in the sediment layers centimeters below.

Methane seepage at Tommeliten has been studied for roughly 30 years. At Tommeliten the seafloor consists of consolidated marls. Methane emissions to the water column was limited to small, densely covered *Beggiatoa* patches of < 0.3 m^2 , which were spread over an area of about 0.1 km^2. We sampled these patches for the first time using a remotely operated vehicle (ROV).

Chemosynthetic fauna such as (e.g., tube worms and clams), which are often found at deep-sea seeps, were not present at the North Sea sites. Presumably this was because of the high, tidally induced water movement, which prevented settlement of benthic organisms. Water temperatures of the North Sea are higher than in deep water sites and distinct seasonal cycles were present.

Microbial communities at both seep sites were described using lipid biomarker analyses and molecular tools (Fluorescence in situ hybridization (FISH) and 16S rDNA based sequence libraries). The bacterial and archaeal biomarker compositions were typical for seep sites. Biomarkers characteristic of ANMEs and their partner SRBs and sulfide oxidizing bacteria were found. The mean carbon isotope composition of those lipids was highly depleted, whereas lowest ^{13}C-values were measured for the archaeal biomarker archaeol and sn2-hydroxyarchaeol. This is due to the use of already substantially depleted methane as carbon source and further fractionation during carbon assimilation. Notwithstanding the highly different physical factors, the microbial communities at Gullfaks and Tommeliten were very similar to that of the deep sea. The active seep areas were dominated by methanotrophs of the ANME-2 cluster, living in syntrophy with different sulfate reducing bacteria.

In **Chapter III**, a flow-through system for the investigation of AOM in sediments is presented. Sediment columns were constantly percolated with methane saturated anaerobic seawater. By measuring the differences in methane and sulfide concentrations between in- and outflow, metabolic rates were determined. At constant conditions of about 2mM methane in the inflow, no increases in AOM and sulfate reduction rates were measured over more than 80 days, although neither methane nor sulfate were limiting. Extremely low growth rates of methanotrophic communities were also observed in other studies. This might be due to the extremely low energy yield of AOM (ΔG_R ~15 to 40 kJ mol^{-1} CH_4); a yield which presumably has to be shared between the two organisms of the AOM syntrophy. Additionally, carbon fixation (either reduction of CO_2 or oxidation of methane) is highly energy intense, therefore allowing only limited growth.

We found that methanotrophs are highly tolerant to starvation. After 6 weeks of methane free percolation, methanotrophs resumed AOM at similar rates as before, when methane was re-supplied. This behavior might be important in environments where fluctuating methane supply predominates.

In **Chapter IV** the roles of inorganic carbon (DIC) and methane as carbon sources for the microbial community performing AOM were examined. We combined the methods of stable isotope probing (of ^{13}C methane and ^{13}C bicarbonate) with lipid biomarker analyses and found carbon uptake of both labeled carbon sources into archaeal lipids. For archaea it might be energetically favorable to combine the assimilation of both compounds, because the combination of both carbon sources would reduce the input of reducing or oxidizing power. The lipid biomarkers assigned to sulfate reducing bacteria, however, were exclusively labeled by inorganic carbonate. In an additional experiment we found that this bacterial inorganic carbon uptake strictly depends on the oxidation of methane, which shows the tight metabolic relationship between archaea and sulfate reducing bacteria within the consortia.

Our results can explain the natural isotopic compositions of bacterial and archaeal markers. With $\delta^{13}C$ value of down to -130‰, methanotrophic archaea have extremely depleted carbon signatures. This is due to incorporation of already strongly depleted methane and methane derived CO_2 into their biomass and further fractionation in strongly ^{13}C-discriminating fixation pathways. Sulfate reducers assimilate CO_2 from the only slightly depleted DIC of the pore water pool. Our results also help in understanding the syntrophic relationship between the two organisms. As an example, partner-SRBs do not incorporate carbon from methane, from which we can exclude organic monomers (e.g. acetate or formate) as shuttled intermediate in AOM.

From the assimilation into lipids we were able to calculate carbon assimilation efficiencies for the methanotrophic community, which were extraordinarily low with only 0.3% to 1.3%. This low growth yield must be due to the low energy yield of AOM.

In the last chapter, the results of this thesis are summarized and brought into the context of prior work. At the end I include an outlook to the future of AOM research, with a continued emphasis on isotope labeling methods.

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Chapter I

Introduction:

Methane in the Global and Marine Carbon Cycle

Gunter Wegener

Introduction

This chapter starts by introducing the role of methane as part of the global carbon cycle. An overview of stable isotope geochemistry, one of the major tools to track fluxes in the carbon cycle, particularly of methane, follows. The major sources and sinks of methane on earth with emphasis on the biological methane production and consumption are described. Methane-rich habitats, particularly cold seeps, are covered. An overview of important methods used in the examination of methanotrophy. Stable isotope probing, combined with molecular and biomarker tools, is discussed in detail. The chapter concludes with an overview of the manuscripts within this thesis, including a description of my contributions to these works.

1. Methane in the global and marine carbon cycle

Methane is the most abundant hydrocarbon on earth. It is a major greenhouse gas and plays an important role in tropospheric chemistry (Reeburgh, 2007). Figure 1 illustrates important sources of methane as well as their net contributions to the atmospheric methane budget (after Reeburgh et al., 2007). Annual methane production on earth is about 1200×10^9 kg, with more than half of this (ca. 700×10^9 kg) consumed by microbial oxidation before reaching the atmosphere. Microbial degradation of organic matter under anoxic conditions is the largest natural source of methane. Wetlands, tundra and swamps produce 142×10^9 kg yr^{-1} methane, most of which (ca. 115×10^9 kg yr^{-1}) gets emitted to the atmosphere. Methane production by microbial symbionts in animals (e.g., ruminants and termites) contributes 80×10^9 kg yr^{-1} to the atmosphere. Anthropogenic methane emissions account for the dramatic increase of atmospheric methane concentrations from pre-industrial 700 ppb to about 1700 ppb today. Rice farming represents the major human methane source with an annual production of 577×10^9 kg yr, however, only a fraction of this (100×10^9 kg yr^{-1}) is emitted to the atmosphere. Biomass burning (55×10^9 kg yr^{-1}) and

fossil fuel and gas production (75×10^9 kg) also have a large impact on the atmospheric methane budget.

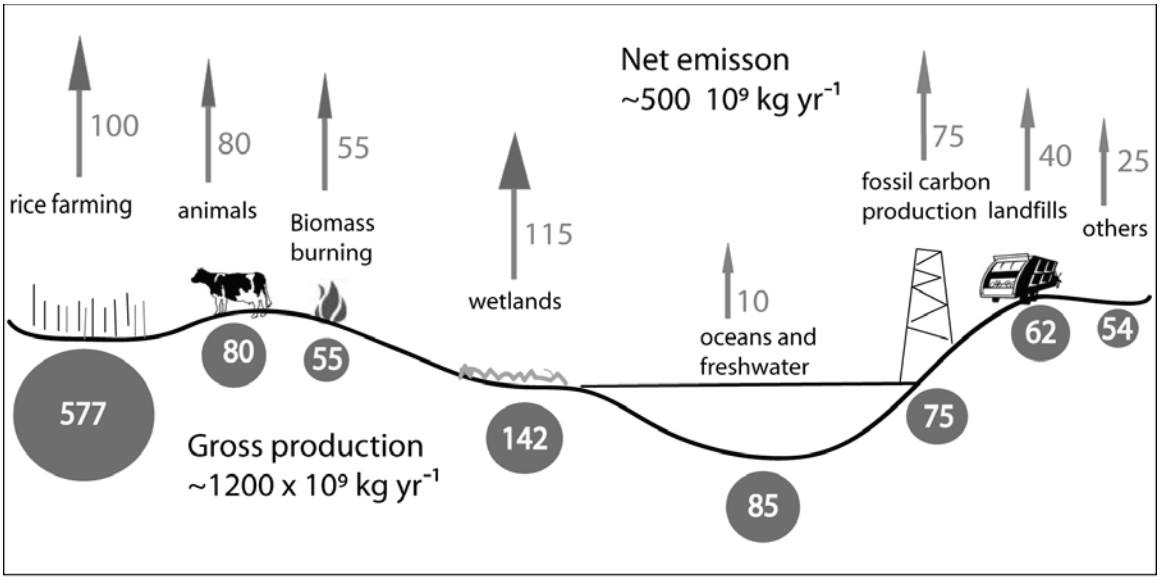

Figure 1. Schematic presentation of the global methane budget. All numbers in 10^9 kg yr^{-1}. Data from Hanson and Hanson, 1996; Nedwell, 1996; Reeburgh, 2007

Compared to marine primary production, which is 60000 $\times10^9$ kg C yr^{-1} (del Giorgio and Duarte, 2002), the role of the oceans in the global methane budget is rather small, with a production of 85 $\times10^9$ kg yr^{-1} and a net flux to the atmosphere of just 10×10^9 kg yr^{-1}. The vast majority of marine organic carbon gets mineralized aerobically in the upper water column and only 1% is exported to the seafloor (Ducklow et al., 2001) where conditions become anaerobic. In marine sediments this residual organic matter is further degraded by microorganisms (fermented to monomers) and mineralized to CO_2 using available electron acceptors in order of decreasing energy yield: oxygen, nitrate, redox active metals such as manganese (MnIV) and iron (FeIII), sulfate and CO_2 (Fenchel and Jørgensen, 1977; Jørgensen, 2006). Seawater concentration of sulfate exceeds other oxidants by orders of magnitude, making sulfate reduction, coupled to the oxidation of a variety of monomeric organic compounds (e.g. acetate and lactate, propionate, butyrate; Finke et al., 2007) of particular relevance. Sulfate reduction is also coupled to anaerobic oxidation of methane

(AOM). In presence of non-limiting sulfate concentrations, sulfate reducers outcompete methanogens on the common substrates hydrogen and acetate because of much higher energy yields when sulfate and not CO_2 is used as terminal electron acceptor (Ward and Winfrey, 1985; Widdel, 1988).

In absence of sulfate, methanogenesis based on microbial reduction of CO_2 or disproportionation of acetate is a major metabolic process in marine sediments (Capone and Kiene, 1988; Nealson, 1997). About 5 to 10% of the marine organic matter reaching the seafloor is mineralized via methanogenesis (Canfield, 1993; Hinrichs and Boetius, 2002; Canfield et al., 2005). Residual organic matter, consisting mostly of highly complex polycyclic hydrocarbons (not previously mineralized by marine microbes), is the major long term carbon sink on earth. Deeper in sediments, temperatures between 50 and 180°C mobilize this refractory organic matter, forming thermogenic methane and higher alkanes. Biogenic and thermogenic methane accumulate in the seafloor, either dissolved in the pore water, as a separate gas phase, or condensed as gas hydrates (Reeburgh, 2007). The amount of hydrate-bound methane is vast, with estimates converging at 1 to 5×10^{15} m^2 (0.7 to 3.6×10^{15} kg; Milkov, 2004 and references therein).

At even higher temperatures (e.g., ocean crust subduction zones) kerogen (organic matter with high molecular weight which is insoluble in usual organic solvents) decays to CO_2 and hydrogen. This CO_2 is then re-supplied to atmospheric carbon cycles via volcanic and hydrothermal activity (Sano and Marty, 1995; de Leeuw et al., 2007).

2. Carbon isotope analysis as a tool in biogeochemistry

Most elements, including the main components of organic biomass C, H, N, S, and O, are mixtures of atoms with different masses as a result of a variable number of neutrons. Isotopes (from Greek *ισο* [iso] – equal, *τόπος* [topos] – location [in the table of elements]) of

each element appear naturally in relatively fixed ratios. Deviations from those values are expressed as δ-notations [‰], which are defined in Eq. 1:

$$\delta X = \left[\left(R_{sample} - R_{reference}\right) - 1\right] * 1000 \quad (1)$$

X is the usually less-abundant isotope of an element (e.g., ^{13}C), and R is the corresponding ratio of this isotope to its more abundant equivalent (e.g., $^{13}C/^{12}C$). The δ notation of the *sample* is measured against a *reference*, which is Vienna PeeDee Belemnite limestone (V-PDB) for carbon. Other reference materials are Canyon Diablo Meteorite (CDM) for sulfur, (Vienna) standard mean ocean water (V-SMOW) for oxygen and hydrogen, and atmospheric N_2 for nitrogen.

Deviations from mean isotope ratios are caused by physical, chemical and biological processes and reactions. A purely physical isotope fractionation effect is the exchange between CO_2 in the air and the dissolved inorganic carbon (DIC = CO_3^{2-} + HCO_3^- + H_2CO_3 + CO_2) in water, which causes a 1‰ $\delta^{13}C$-enrichment of oceanic DIC compared to atmospheric CO_2 (Mook and Tan, 1991). Plant biomass has about 20 to 25‰ lighter $\delta^{13}C$-values than atmospheric CO_2 (Farquhar et al., 1989). This fractionation includes purely physical effects such as CO_2 uptake through stomatal pores and diffusion into the water phase; these effects are small and cause a 1‰ to 2‰ discrimination in ^{13}C (Farquhar et al., 1989). In contrast, enzyme driven, biochemical carbon fixation pathways of the cells catalyze a carbon isotope fractionation of about -20‰ (Rubisco pathway; Farquhar et al., 1989; Guy et al., 1993).

The carbon isotope compositions of microbial biomass can reveal the organisms' carbon sources and even the carbon fixation pathways they use. Heterotrophic bacteria have only slightly lighter $\delta^{13}C$-compositions than that of the carbon substrate they have consumed (e.g., marine algae), having $\delta^{13}C$-values of about -25 to -30‰ (Coffin et al., 1989). Autotrophic microorganisms are typically more strongly $\delta^{13}C$-depleted owing to their use of highly fractionating carbon fixation pathways such as the acetyl-coenzyme A pathway which

has δ^{13}C-discriminations reaching -47‰ in ammonium-oxidizing bacteria (Schouten et al., 2004).

Stable isotope compositions of fossils and limestone, as well as lipid-derived hydrocarbons, can be used to reconstruct carbon cycles and dominant redox processes during earth's history. Dickens (2003) examined development of the δ^{13}C-composition of benthic foraminifera in the Tertiary. The rapid decline of the δ^{13}C-composition at the PETM (Paleocene/Eocene temperature maximum) in foraminifera was explained by massive methane emission during this time. Hayes and Waldbauer (2006) used the δ^{13}C-composition of lipid-derived biomarkers to model the dominant biological redox processes throughout early earth history. Elevated ^{13}C-values in carbonate minerals were interpreted as an indication of the importance of methanogenesis in the late Archaean (2,500 Mya). Understanding the origin of present-day δ^{13}C-values of biomass, lipid biomarkers, methane and DIC may help to reconstruct the evolution of life throughout earth history, the causes of past and present climate changes as well as the functions of organisms.

3. Methane: Sources, sinks and isotope composition

Methane can be produced by biogenic fermentation and thermogenic degradation of organic matter. Figure 2 shows isotope compositions for methane from these various sources, which are explained in further detail below.

Methane sources

Methane formed independent of organic matter and microbial catalysis is defined as abiogenic. Biogenic methane is formed by heat-induced decay of organic matter

(thermogenic methane) or by microbial reduction of CO_2 and organic monomers (biogenic methane).

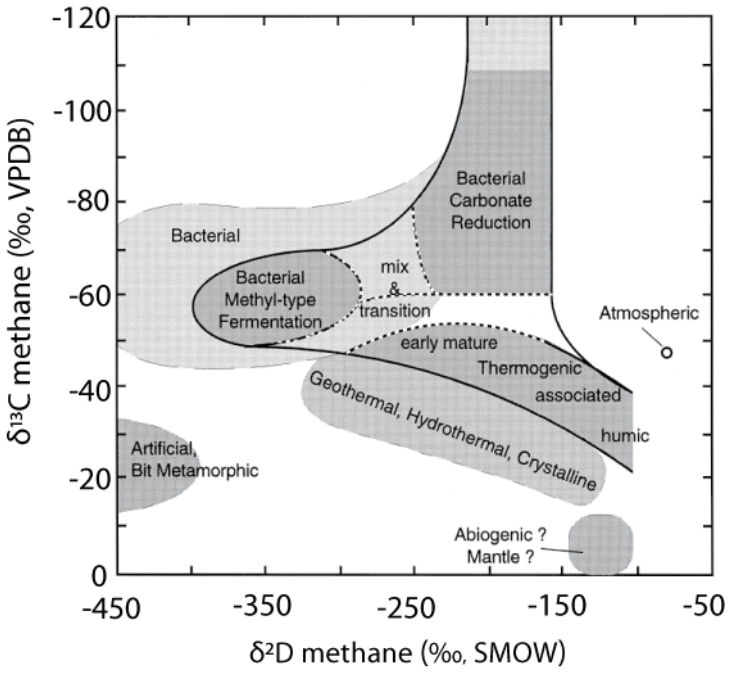

Fig. 2. Carbon and hydrogen isotope composition of methane from different sources (Whiticar, 1999).

Abiogenic methane

Abiogenic methane is predominantly formed by alteration of fresh oceanic rocks, such as is found at the East Pacific Rise (Welhan and Craig, 1977) and the Mid Atlantic Ridge (Rona et al., 1992). It can also be formed from ophiolites (Abrajano et al., 1990) and crystalline rocks such as those in the Canadian Shield (Sherwood Lollar et al., 2002). During diagenesis of these rocks (e.g., conversion of olivine to serpentine) hydrogen is released according to Eq. 2:

$$6\left[\left(Mg_{1.5}Fe_{0.5}\right)SiO_4\right]+7H_2O \rightarrow 3\left[Mg_3Si_2O_5\left(OH\right)_4\right]+Fe_3O_4+H_2 \qquad (2)$$

Serpentinization is a strongly exothermic reaction involving heating of the rock material. At temperatures above 300°C, CO_2 and hydrogen react in a Fischer-Tropsch-like reaction on the surface of catalysts such as iron or chromium according to Eq. 3 (Foustoukos and Seyfried, 2004):

$$CO_{2aq} + [2 + (m/2n)]H_{2aq} \rightarrow \frac{1}{n} C_n H_m + 2H_2O \quad (3)$$

Abiogenic methane is characterized by moderately depleted carbon and strongly depleted hydrogen isotope compositions ($\delta^{13}C$ -34‰, δD < -400‰). Next to methane, substantial amounts of C_{2+}-compounds (ethane, propane, etc.) with inversely depleted isotope compositions are produced (e.g. butane, $\delta^{13}C$ -37‰, δD ~ -250‰; Sherwood Lollar et al., 2002). These isotope compositions have not been found in economically significant hydrocarbon reservoirs, suggesting a relatively minor role for abiogenic methane production in the global methane cycle (Sherwood Lollar et al., 2002). However, in local areas of sea floor spreading such as the Lost City Hydrothermal field (off the Mid Atlantic Ridge), abiogenic hydrogen or methane may be important microbial energy sources (Charlou et al., 2002; Boetius, 2005; Kelley et al., 2005; Proskurowski et al., 2008).

Biogenic methane

The overwhelming majority of earth's methane is formed via decay of organic matter, which in marine environments is mainly plankton such as diatoms, coccolithophorids and foraminifera. When oxygen, nitrate and sulfate are depleted, methanogenic microorganisms utilize a limited number of fermentation products (mainly hydrogen and acetate) according to Eq. 4 and Eq. 5, to form methane.

$$CO_2 + 4H_2 \xrightarrow{-131 kJmol^{-1}} CH_4 + 2H_2O \quad (4)$$

$$CH_3COOH \xrightarrow{-36 kJmol^{-1}} CH_4 + CO_2 \quad (5).$$

In biogenic methanogenesis very few higher hydrocarbons are produced as byproducts, which leads to characteristic C_1/C_{2+} ratios of >>100 among biogenic hydrocarbon gases (Whiticar et al., 1986; Whiticar, 1999).

Methane derived from carbonate reduction (Eq. 4) is highly $\delta^{13}C$-depleted, having values of -60 to -110‰ that arise due to a strong fractionation of -46 to -58‰ during CO_2

reduction (values derived from pore water profiles (Galimov and Kvenvolden, 1983) and Rayleigh experiments (Balabane et al., 1987; Krzycki et al., 1987; Whiticar, 1999)). Since methanogenic archaea preferentially reduce $^{12}CO_2$, the remaining DIC pool enriches in ^{13}C, which causes positive $\delta^{13}C$-DIC-values in highly methanogenic sediments (Blair, 1998). Hydrogen in methane derived from CO_2-reduction has only moderately depleted δD-values.

Methanogenesis that proceeds via fermentation-type reactions (Eq. 5) is characterized by ^{13}C-fractionation between -22‰ (acetogenic methanogenesis) and -74‰ (methane derived from methanol reduction) between carbon source and product (Krzycki et al., 1987). Usually, environmental limitation of fermented reactants prevents high fractionation. Natural samples attributed to organoclastic methanogenesis have $\delta^{13}C$-values of -50‰ to -70‰. Hydrogen in methane produced from fermentation is highly depleted, with δD-values of -200‰ to -400‰ (Whiticar, 1999).

Thermogenic methane

Thermogenic methane is formed by the heat-induced decay of organic matter. Economically valuable oil and gas reservoirs are formed by this process (Tissot and Welte, 1984). Subduction- or sedimentation-driven subsidence of sediments leads to export of refractory organic material (kerogen) into the deep subsurface. Temperatures rise following ambient geothermal gradients, and at depths where temperatures exceed 50°C, kerogen slowly releases gaseous and liquid hydrocarbons (Schoell, 1980; Tissot and Welte, 1984), which in turn increase C:H ratios in the residual organic matter (an indicator of increasing maturity). In the lower temperature range, gas mixtures with high amounts of ethane and propane (i.e., wet gases; C_{2+} >5%) are released. With increasing maturity of the organic matter, higher subsidence and increasing temperatures, dryer hydrocarbon gases with C_{2+} contents as low as 1% are produced (Schoell, 1980). Thermogenic methane typically has only moderately depleted $\delta^{13}C$-values between -55 and -35‰, whereas in general, increasing

maturities are associated with less depleted carbon isotope compositions (Schoell, 1980). Higher hydrocarbons (C_{2+}) formed during thermogenic methanogenesis have less negative $\delta^{13}C$-values. Hydrogen isotopy of thermogenic gases can vary greatly, with $\delta^{2}D$-values of -375 to -100‰.

4. Methane consumption and isotope effects

Abiotic oxidation of methane

Within the troposphere and stratosphere, methane is naturally oxidized by photochemical radical reactions. UV degradation of ozone leads to the formation of hydroxyl radicals that attack methane, forming methyl radicals (Levy, 1971; Le Texier et al., 1988; Lelieveld et al., 1998), which are ultimately oxidized to formaldehyde (Levy, 1971; Grosjean, 1995). Annual photochemical methane oxidation is about 500×10^{9} kg, resulting in an average atmospheric lifetime for methane of 10 years (Crutzen, 1994). As a mixture of all its source carbon isotope compositions, atmospheric methane has an intermediately depleted $\delta^{13}C$-value of -48‰. In contrast, hydrogen in atmospheric methane has a δD value of -30‰, which is significantly less depleted than any of its source methane (Fig. 2). This relatively high value is due to photochemically mediated exchange reactions between hydrogen from methane and other atmospheric hydrogen pools (Whiticar, 1993).

Combustion represents another abiotic pathway for methane oxidation. Natural petroleum gas fires are found in Azerbaijan (*azer* from Persian "fire") where constant methane seepage from deep fossil fuel sources causes the famous "eternal fires". However, the relevance of naturally occurring methane consuming fires is minor compared to human gas extraction and burning of 1.5×10^{12} kg of methane (United Nation Conference on Trade and Development, UNCTAD; 2004; www.unctat.org/infocomm/anglais/gas/market/htm#production).

Biogenic methane consumption

Aerobic methanotrophy. Two-thirds of the biogenic methane produced on earth is consumed as an electron donor by aquatic methylotrophic microbes before escaping to the atmosphere. In terrestrial habitats, such as swamps and rice paddies, aerobic methanotrophy is the main methane sink (Hanson and Hanson, 1996; Reeburgh, 2007), whereas in marine environments, aerobic methanotrophy contributes about 20% of the methane consumption (Reeburgh, 2007). Additionally, methanotrophic bacteria are known to occur as symbionts of fauna living at hydrocarbon seeps such as *Bathymodiolus platifrons* mussels (Barry et al., 2002).

Aerobic methanotrophy can be described by the bulk formula (Eq. 6):

$$CH_4 + 2O_2 \xrightarrow{\Delta G^0 = -842\,kJmol^{-1}} CO_2 + 2H_2O \qquad (6)$$

Methane carbon isotope fractionation factors for different inoculated methanotrophic bacteria range from 1.0130 to 1.0252 (Coleman et al., 1981), resulting in a shift in methane $\delta^{13}C$ from 10 to 20‰ after 50% methane consumption (Barker and Fritz, 1981). Considering hydrogen isotopes, Coleman et al. (1981) measured 8- to 14-fold higher fractionation factors for deuterium. However, since aerobic methanotrophy is usually limited to the top few millimeters of marine sediments (due to the low dissolved oxygen concentrations found there), aerobic oxidation of methane is not recovered in highly depleted carbon compositions in the DIC. Diffusive exchange between the pore water in the methane oxidation zone and the water column blurs any isotope signal.

Anaerobic oxidation of methane (AOM). For decades, methanotrophy was thought to be limited to oxic environments, as described above. However, marine pore water data analyzed by Martens and Berner (1974) revealed simultaneous depletions of methane and sulfate, which indicated the net AOM reaction (Eq. 7):

$$CH_4 + SO_4^{2-} \xrightarrow{\Delta G_0^R = -16 kJmol^{-1}} HCO_3^- + HS^- + H_2O \quad (7)$$

This stoichiometry was confirmed in laboratory studies (Iversen and Jørgensen, 1985; Nauhaus et al., 2002) and field measurements of methane oxidation (Treude et al., 2003). In marine sediments, AOM is the dominant sink of methane, accounting for 80 to 85% of marine methane oxidation (Hinrichs and Boetius, 2002). According to Eq. 7, AOM increases alkalinity which causes precipitation of carbonates. The other product of AOM, sulfide, provides an energy source for sulfide-oxidizing bacteria (e.g., *Beggiatoa* spp.).

Anaerobic methanotrophy is found in zones of sediment where methane and sulfate are both present. Methanotrophic habitats may be broadly classified according to the dominant fluid transport process being either diffusion or advection. Diffusion-driven sulfate methane transition zones (SMTZ) are found in shelf and productive ocean sediments, within sediments depth of less than a meter (Parkes et al., 2007) down to depths of several hundreds of meters (SMTZ of the Eastern Pacific; D'Hondt et al., 2004). The position of the often very narrow AOM horizon is mainly determined by the supply of methane creating normally steep diffusion gradients. A large number of diffusion-driven SMTZs have been studied. Metabolic rates at these sites are in the range of pmol to nmol cm^{-3} d^{-1} with correspondingly small populations of anaerobic methanotrophs.

Higher methane fluxes usually depend on advective pore water transport. Advection is caused by sediment compression, which is forced by high sedimentation or sea floor subduction. Leakage of fossil fuel-derived methane reservoirs or dissolution of gas hydrates leads to bubble-driven advective transport. Both processes are found at continental slope or margin estuaries, permanently anoxic basins and areas with anoxic histories such as the Mediterranean seeps (Judd, 2003).

Laboratory experiments examining the isotope fractionation of methane in AOM metabolism have not been published to date. However, a strong increase of ^{13}C-CH_4 values from below to above AOM zones, obtained in sediment depth profiles, indicates substantial

discrimination of the heavy isotope during anaerobic methane consumption (Fig. 3; Tsunogai et al., 2002; Werne et al., 2004). Corresponding ^{13}C-carbon compositions of DIC are depleted due to the transfer of methane isotope into DIC (Werne et al., 2004).

5. Key microorganisms in methane production and consumption and their carbon composition

Modern 16S rRNA-based techniques (such as FISH (fluorescence in situ hybridization) and full length gene sequencing) have identified many key organisms involved in the majority of carbon mineralization processes. Lipid biomarker patterns have also been shown to have phylogenetic relevance. Isotopic compositions of lipid biomarkers also provide insight into microbial processes (Sinninghe Damste et al., 2002; Pancost and Sinninghe Damste, 2003). Diagnostic lipid biomarker carbon signatures for organisms performing methanogenesis and methanotrophy in methane rich habitats are summarized in Fig. 3 and described in further detail below.

Methanogens. Methanogenesis is limited to archaea, and specifically to the key genera *Methanobacterium, Methanococcus* and *Methanosarcina* within the Euryarchaeota (Balch et al., 1979). Most methanogens are capable of CO_2 reduction, whereas acetoclastic methanogenesis, which is the main methane producing pathway in terrestrial habitats, appears limited to the genus *Methanosarcina Methanothrix* and *Methanosaeta*. Formate, methanol and methylamines are also reduced by methanogens, although those compounds seem to have minor environmental relevance (Conrad et al., 2006). Most methanogens discovered to date prefer moderate temperatures, but there are also hyperthermophilic genera, such as *Methanopyrus,* which grow at temperatures up to 110°C (Kurr et al., 1991).

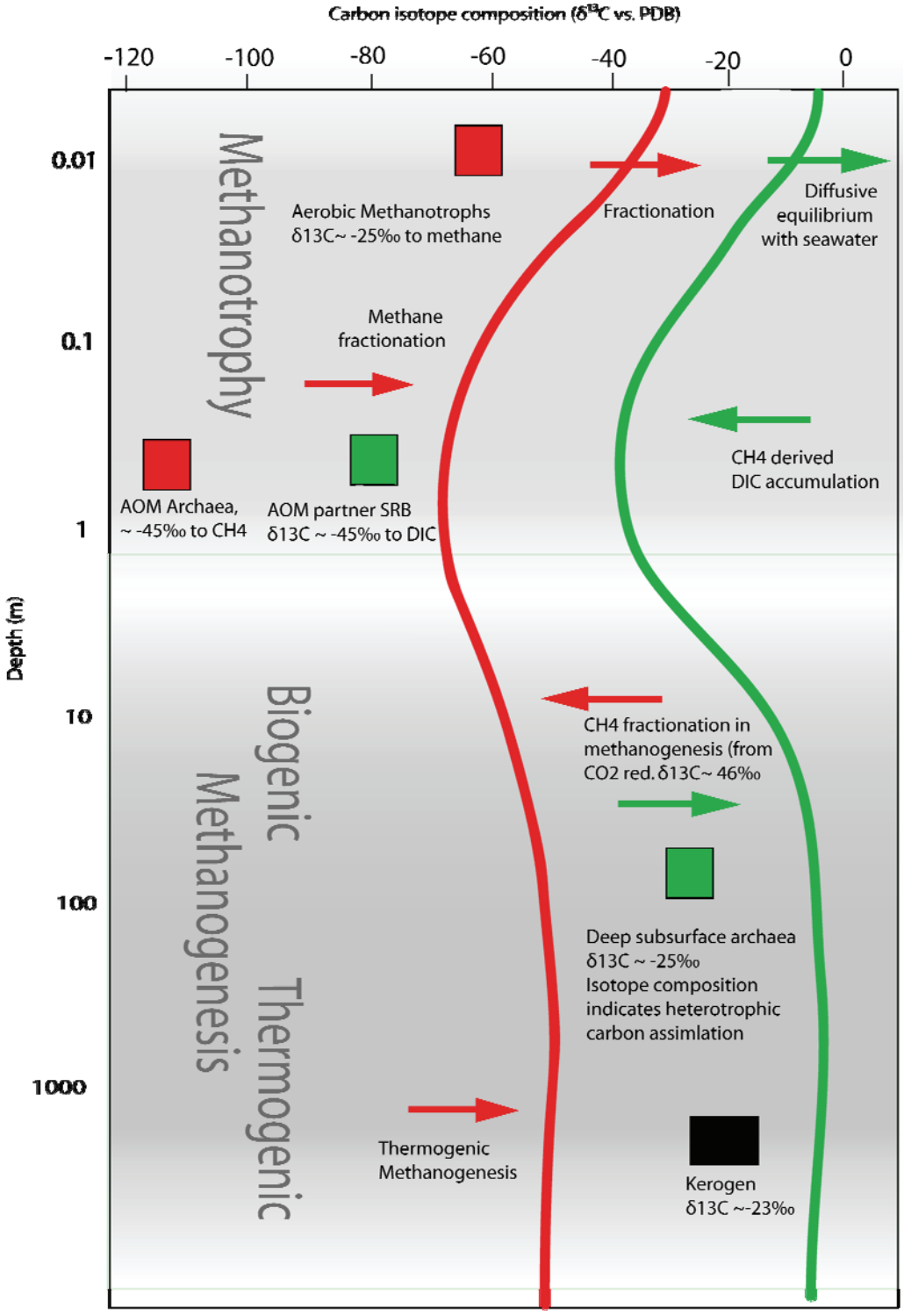

Fig. 3. Schematic plot of isotope compositions of methane, inorganic carbon and lipid biomarker of the dominant microorganism at seep sites. Data from Biddle et al. (2006) (deep biosphere archaea); Galimov and Kvenvolden (1983) (fractionation while methane formation); Hinrichs and Boetius (2002) (methane, biomarker of ANME and partner-SRB lipids); Peters et al. (1978) (mean kerogen isotopy); Kinnaman et al. (2007) (carbon fractionation in aerobic methane consumption); and Werne et al. (2002) (typical ^{13}C-composition of methane and carbonate and bacterial methanotrophs).

The lipid-derived biomarkers of methanogens are the isoprenoidal glycerol diethers isoprenoidal glycerol ethers archaeol (2,3-di-O-phytanyl-sn-glycerol) and *sn2*-hydroxyarchaeol (2-O-(3'-hydroxy-3',7', 11',15'-tetramethyl)hexadecyl-3-O-phytanyl-sn-glycerol) as well as the branched hydrocarbon 2,6,10,15,19-pentamethylicosane (PMI) and crocetane (Ohtsubo et al., 1993; Schouten et al., 1997). The biomass and lipid biomarker of autotrophic methanogens have intermediately depleted carbon compositions (-30 to -60‰). Their carbon source, CO_2, is most often quite heavy, but assimilation of inorganically fixed carbon involves strong isotope fractionation.

Methane oxidizing bacteria are present in all habitats where methane and oxygen overlap (King, 1992; Knief et al., 2003). Methane-oxidizing bacteria (MOB) are either *Gammaproteobacteria* (Type I-MOB) or *Alphaproteobacteria* (Type II-MOB). Type I-MOB (e.g., in *Methylomonas*, *Methylomicrobium* and *Methylobacter*) use the more efficient ribulose monophosphate pathway (RMP) for carbon fixation, whereas Type II-MOB (e.g. *Methlyosinus* and *Methylocystis*) perform carbon fixation via the serine pathway. The latter requires additional ATP as a reducing agent for CO_2. The higher energy demand of Type II manifests in a lower growth yield and a minor ecological relevance of this group (Hanson and Hanson, 1996). A dominance of Type-II-MOB was found in slightly acidic environments and under nitrogen limitation (Hanson and Hanson, 1996; Jahnke et al., 1999). Most MOB have a growth optimum between 25 and 35°C at neutral pHs. Growth of both groups is limited to temperatures of 50°C. Recently, a third group of aerobic methanotrophs, distantly related to the *Verrucomicrobia*, was discovered and have the ability to perform aerobic methane oxidation under highly acidic conditions (down to pH 1), however the ecological relevance of these extremophilic MOB has so far not been evaluated (Dunfield et al., 2007).

Half saturation constants (K_M) for methane in aerobic methanotrophy are in the range of 25-40 ppmv (i.e., 40-70 nM) for soil samples (Bender and Conrad, 1993), and as high as 2 μM for a pure culture of *Methylosinus* (Jørgensen and Degn, 1983). Such low K_M values are due to the high efficiency of the methane monooxygenase enzyme, which catalyzes the first step of methane assimilation in all aerobic methanogens.

Methanotrophic bacteria leave characteristic phospholipid-derived fatty acid patterns (PLFAs) in sediments. Type I MOB predominantly produce monounsaturated C_{16} compounds such as $C_{16:1\omega5t}$, $C_{16:1\omega6c}$ and $C_{16:1\omega8c}$, whereas $C_{18:1\omega8c}$ is diagnostic for Type II MOB (Guckert et al., 1991; Sundh et al., 1995; Hanson and Hanson, 1996). MOB biomass usually has ^{13}C-values of -30 to -80‰. The strong depletion is because MOB usually consume already-depleted methane, and a further fractionation of about -30‰ occurs while assimilating this methane into biomass (as measured for *Methylococcus capsulate, Methylomonas methanica,* both Type I-M. using the RMP cycle (Summons et al., 1994)). Instead of assimilated methane carbon isotopy, the hydrogen isotopy of methane does not substantially impact the biomass δD composition, since in MOB methane-bound hydrogen is not preserved during methane carbon assimilation (Sessions et al., 1999).

Anaerobic methanotrophs (ANMEs) and their partner SRBs. Three major groups of methanotrophic archaea, ANME-1, -2 and -3, have been identified (Hinrichs et al., 1999; Boetius et al., 2000; Niemann et al., 2006). ANME-1 are distantly related to *Methanosarcina* and *Methanomicrobiales* (Hinrichs et al., 1999; Knittel et al., 2005) and appear as single cells (Valentine, 2002; Knittel et al., 2005), filaments (Niemann et al., 2005), or monospecies aggregates (Orphan et al., 2002). ANME-1 was found to dominate diffusion-driven SMTZs (Niemann et al. 2005), and is a main organism in microbial mats of the Black Sea (Michaelis et al., 2002; Knittel et al., 2005). The biomass at most highly active cold seeps is dominated by aggregated methanotrophic consortia of ANME-2 or -3 and SRB. In these

settings archaea and their partner-SRB comprise up to 90% of the microbial biomass (Knittel et al., 2005). ANME-2 belong to the order *Methanosarcinales* and are usually found in consortia with SRB of the order *Desulfosarcinales/Desulfococcuales* (DSS) (Boetius et al., 2000; Knittel et al., 2003, 2005). The recently discovered third group of anaerobic methanotrophs, ANME-3, is most closely related to *Methanococcoides* and *Methanolobus* and is typically found in aggregates with *Desulfobulbus-like SRB* (Niemann et al., 2006; Lösekann et al., 2007).

Methanogenic and methanotrophic archaea are both *Euryarchaeota*. Given this phylogenetic overlap, it is not surprisingly that both groups have similar biomarker patterns. Hence, typical lipid biomarkers of ANMEs are archaeol and *sn2*-hydroxyarchaeol, as well as crocetane and PMIs and their unsaturated homologues (crocetenes and pentamethylicosenes - PMIΔ), and a number of isoprenoidal glycerol dialkylglycerol tetraethers (GDGTs; Niemann and Elvert, in press). In ANME-2 dominated sediments, *sn2*-hydroxyarchaeol: archaeol ratios >3 as well as the presence of substantial amounts of crocetane were reported (Nauhaus et al. 2007, Niemann and Elvert, in press). In contrast, distinct production of PMI:4 and PMI:5 and dominance of archaeol to sn2-hydroxyarchaeol may be indicative of ANME-1 dominated sediments (Blumenberg et al., 2004; Niemann and Elvert, in press).

ANME lipids characteristically have superlight carbon isotope compositions (Hinrichs et al., 1999; Hinrichs et al., 2000; Orphan et al., 2001). Most reported $\delta^{13}C$-values of archaeol and hydroxyarchaeol are between -90‰ and -130‰ (Hinrichs et al., 1999; Elvert et al., 2000; Orphan et al., 2001; Thiel et al., 2001), with a fairly constant methane offset of -40‰ to -50‰ (Hinrichs and Boetius, 2002). This suggests that methane is the direct carbon source comprising the biomass of anaerobic methanotrophs.

The consortial partner-SRBs partners produce a diverse lipid pattern of mainly unsaturated fatty acids and isoprenoids. However, within those, $C_{16:1\omega5}$ and cy-(cyclopropane)$C_{17:0\omega5,6}$, *iso*- and *anteiso*-$C_{15:0}$ as well as some non-isoprenoidal mono- and dialkyl- glycerol ethers (MAGEs, DAGEs) have taxonomic relevance (Niemann and Elvert,

in press). All of these compounds were found with substantially depleted stable carbon isotope compositions (e.g., $\delta^{13}C$ $C_{16:1\omega5c}$ of -75 to -96‰ and $\delta^{13}C$ $C_{17:0\omega5,6}$ of -94 to -103‰ in Hydrate Ridge seep sediments; Elvert et al., 2003), suggesting a tight coupling between consortial SRB and methane oxidation (Orphan et al., 2001; Elvert et al., 2003). A relatively constant offset of -40 to -50‰ between seep specific fatty acids and DIC carbon composition was found, which may indicate autotrophic carbon assimilation by the seep SRB (Hinrichs and Boetius, 2002).

6. Experimental investigation of Methane Oxidation

Apparent kinetics of methane oxidation

Since half saturation constants (K_M) for methane in aerobic methanotrophy and anaerobic methane oxidation are very different, different methods for K_M determination also need to be chosen. For aerobic methanotrophs, methane oxidation kinetics are typically determined in open systems where a constant gas flow provides a defined methane-air mixture (Bender and Conrad, 1993). An inline membrane mass spectrometer is used to measure concentrations of methane and oxygen in the medium. Half saturation constants for methane on the order of 20 to 50 ppmv for non-enriched soil samples (Bender and Conrad, 1993), and up to 54,100 ppmv for cultures of *Methylococcus* (Carlsen et al., 1991) were determined using this approach.

Half saturations of methane in AOM were estimated to be orders of magnitude higher than for aerobic methanotrophy. To determine the response of AOM to methane pressure far above one atmosphere, Nauhaus et al. (2002) incubated samples in hydrostatic pressure chambers. The high methane levels made determination of relatively small methane consumptions impossible, thus, changes in the concentration of produced sulfide were

measured. Within these incubations the authors measured no saturation of methane dependent SR rate up to methane pressures of 1 MPa (~16mM).

Using a semi-continuous flow-through reactor, we tested the development of methane consumption and sulfide oxidation in the low pressure range from 0 up to about 0.15 MPa. The advantage of the flow-through setup was its accommodation of repeated measurements on the same sediments. This is possible since prior experiments have shown that the methane oxidation capacity of the bacterial community, one established, stays constant over longer time scales and can resist longer starving periods (see Chapter III).

Experimental investigation of growth and carbon assimilation in methanotrophy

Growth rates for methanotrophic bacteria are determined by incubating a pure culture in a turbidostatic and oxystatic fermentation system supplying optimized or other defined conditions. By monitoring exponentially increasing methane consumption rates in the fermenter, growth rates up to 0.37 h^{-1} were determined for *Methylococcus* (Jørgensen and Degn, 1983). Up to 1 g biomass was formed while oxidizing 1 g methane (Wilkinson et al., 1974), which is equivalent to a carbon uptake efficiency of almost 50%.

Growth rate and growth yield determinations for anaerobic methanotrophs must tackle very different challenges. Pure cultures of ANMEs do not exist. Hence, experiments rely on natural enrichments of organisms from sediments of highly active seep areas (Hydrate Ridge, Black Sea, etc.). Organisms performing AOM live at the lower limit of energy yields for microbial growth and activity, growth rates are orders of magnitude lower than for aerobic methanotrophs. Furthermore, factors that limit the growth of these organisms are not known. The first results that were determined showed high half saturation constants for methane that were in the range of several mM (Nauhaus et al., 2002), making other experimental setups necessary.

Subsequent growth experiments were performed in pressure incubations supplying about 1MPa CH_4 (Nauhaus et al., 2007). From an almost two-year long incubation growth rates of 0.021 $week^{-1}$ were calculated based on (fluorescence *in situ* hybridization (FISH) counts and biomass weights. Based on sulfide concentration data, carbon assimilation efficiencies of about 1% were measured.

Girguis et al. (2003) examined the dynamics of methanotrophic communities using flow-through reactor incubations. Twenty-four weeks of percolation with methane-saturated water did not result in significant changes to the composition of the microbial community in seep sediments. However, in non-seep sediments, Girguis et al. (2005) were able to quickly stimulate the growth of an AOM community. Using gene copy numbers, they calculated growth rates of 0.17 $week^{-1}$ for ANME-2, 0.22 $week^{-1}$ for ANME-1 and 0.3 $week^{-1}$ for *Desulfosarcina* relatives. The difference of determined growth rates is so far not explainable.

An alternative way to determine growth efficiencies or carbon assimilation efficiencies in anaerobic methanotrophy involves carbon stable isotope probing and lipid biomarker analysis. Using stable isotope probing of methane, Blumenberg et al. (2005) found assimilation of methane derived carbon into both archaeal and sulfate reducing bacterial lipids. Based on the isotopic shift and metabolic activities, a carbon uptake of 1.9% was calculated (Blumenberg et al., 2005). By combining radiotracer incubation, beta microimaging and secondary ion mass spectrometry, Treude and coworkers (2007) demonstrated also substantial uptake of inorganic carbon into methanotrophic biomass. This indicates that carbon assimilation rates, based only on methane uptake, underestimate the growth efficiency of AOM.

Chapter IV describes our calculations of carbon assimilation efficiencies, based on results from parallel ^{13}C-labeling of methane and DIC. To determine methane consumption rates and to reduce the negative effect of rising product concentrations, we used a flow-

through column setup for the incubations. Seep sediments from Gullfaks, Hydrate Ridge and Black Sea were used as inoculates in these experiments.

Radioactive and stable isotope probing (SIP) as tools to study the ecology of methanotrophs

Carbon isotope probing can be used to trace metabolic activity and carbon assimilation in sediments. To link metabolic activity to microbial diversity, labeling is combined with a microbial identification tool, such as DNA or RNA sequencing, FISH, or lipid biomarker analysis.

In **DNA-SIP** and **RNA-SIP** studies, a pure ^{13}C-labeled potential carbon source is added to a sample, usually in relatively high concentrations to support sufficient growth. After incubation, nucleic DNA or RNA is extracted. The newly formed, heavy nucleic acids are separated from the "old" non labeled DNA or RNA using ultracentrifugation (Morris et al., 2002). Separated bands with heavy labeled DNA/RNA are then amplified by polymerase chain reaction (PCR).

A general limit of RNA or DNA-SIP is the generation of sufficient labeled nucleic acids, which limits either approach to fast growing organisms. Examples of successful applications of DNA.SIP include the identification of aerobic methanol oxidizers (Radajewski et al., 2000) and degraders of organic pollutants such as phenol, naphthalene (Jeon et al., 2003; Padmanabhan et al., 2003) and the "ozone killer" methylchloride (Borodina et al., 2005).

The combination of stable isotope probing with labeled nitrogen (^{15}N; Cadisch et al., 2005) and water ($H_2{}^{18}O$; Schwartz, 2007) has extended the field of DNA-SIP. Both methods are independent from the addition of labeled carbon sources.

The combination of SIP and RNA analysis can directly demonstrate gene expression associated with the use of a carbon source (Manefield et al., 2002). For example, RNA-SIP

has been successfully applied in the examination of propionate incorporation into rice field microbiota (Lueders et al., 2004) and in the examination of PCP degradation (Mahmood et al., 2005).

All RNA/DNA SIP studies to date have been performed with terrestrial aerobic inoculates; similar studies for marine environments are lacking. Application of SIP techniques in marine sediments have failed presumably due to the limited microbial growth that occurs in these environments. This is especially true in the case of anaerobic methanotrophy, where cell doubling times are the order of months to years (Nauhaus et al., 2007).

MAR-FISH links microautoradiography (MAR) and FISH. Samples are incubated with radiolabeled carbon sources, similarly as in SIP. Incubated samples are fixed and simultaneously analyzed with FISH and microautoradiography (Lee et al., 1999). Few ^{14}C labeled compounds are commercially available, limiting this method to a few carbon sources. MAR-FISH studies have been performed on aerobic methane-consuming cultures (Stoecker et al., 2006), in addition to other studies where organic monomers such as acetate were incorporated into biomass (Ginige et al., 2005).

Lipid biomarker-SIP, also known as PLFA-(phospholipid derived fatty acids)-SIP combines carbon isotope labeling with lipid biomarker analysis. Lipid biomarker patterns have taxonomic significance (Kaneda, 1991; Moore et al., 1994; Brocks and Pearson, 2005) and the development of chromatography (combustion) isotope ratio mass spectrometry (GC-(c)-IRMS) allows highly accurate isotope composition analysis of lipid compounds (Hayes et al., 1990; Brenna et al., 1997). Using this combination, uptake of minute carbon quantities into lipids can be determined. The limitation of this method is making phylogenetic inferences from lipid derived carbon assimilation data; this is not always possible, especially given the influences of environmental parameters such as temperature, salinity and nutrient supply on microbial lipid patterns (Hazel, 1995; Nicolaus

et al., 2001). Parallel PLFA-SIP and DNA-SIP experiments that combine advantages of both methods (sensitivity and quantification vs. specificity) as shown by Webster et al. (2006) or Singh and Tate (2007) may offer the best way forward.

Successful applications of lipid biomarker-SIP began with pioneering work on coastal North Sea sediments, where Boschker et al. (1998) examined the growth of sulfate-reducing bacteria on certain substrates. Wuchter et al. (2003) showed mainly autotrophic growth of *Crenarchaeota* in North Sea sediments. An in situ ^{13}C-pulse-chase experiment by Middleburg et al. (2000) followed inorganic carbon flow into algae and bacterial biomass. Various studies have examined plant-microbe interactions using lipid biomarker-SIP (Treonis et al., 2004; Prosser et al., 2006).

Lipid biomarker SIP with $^{13}CH_4$ was applied by Deines et al. (2007) to track the carbon flow from methane via methylotrophic bacteria into the larvae of *Stictochironomus pictulus*, an abundant gnat species. Maxfield et al. (2006) and Singh and Tate (2007) described activity of methanotrophic bacteria in fertilized farmland and forests soils, and documented methane carbon flows in these systems.

Besides carbon isotopes, hydrogen isotope compositions of lipid biomarker can also be analyzed by GC-IRMS. Deuterium labeling of styrene (vinyl benzene) was used by Alexandrino et al. (2001) to investigate degradation and assimilation of this anthropogenic compound by *Pseudomonas* spp. The deuterium label from styrene was recovered in strongly deuterium labeled bacterial biomarkers.

The only prior stable carbon isotope labeling study focusing on anaerobic methanotrophy was performed by Blumenberg et al. (2005) who examined the assimilation of methane into biomass under anaerobic conditions. Chapter IV compares the carbon incorporation of labeled methane and inorganic carbon into lipid biomarkers from archaea and their partner SRBs. We incubated sediments from three seepage sites (Black Sea,

Hydrate Ridge and Gullfaks) in a flow-through setup that was developed for anaerobic experiments, and is described in Chapter III.

7. Structure and main objectives of the thesis

A dominant role in the carbon cycle has been attributed to the anaerobic oxidation of methane (AOM). In marine environments AOM contributes between 80% and 85% of the methane consumption. This PhD thesis combines the investigation of methanotrophic habitats with laboratory studies that together focus on the biogeochemistry and physiology of methanotrophic consortia. Chapter I reviews the current knowledge on methane biogeochemistry and the molecular signatures of microorganisms involved in methane production and consumption. Chapter II presents a study of microbial communities at methane seeps at Gullfaks and Tommeliten (Northern and central North Sea) that applies biogeochemical methods, lipid biomarker analysis and 16S rRNA-based molecular methods. Chapter III describes a flow-through reactor and presents first results from incubations using naturally enriched seep sediments from Gullfaks and other sites; it investigates the short term response of anaerobic methane oxidizing communities to different methane and sulfate concentrations. The flow-through setup was also used for experiments presented in Chapter IV, which examines the relevance of methane and inorganic carbon as carbon sources for microbial communities mediating AOM, work that combines stable isotope probing with lipid biomarker analysis.

In summary, three research projects were performed to answer the following questions:

1. What are the geochemical and the biomarker patterns and the microbial characteristics of coastal seep habitats, and how do they compare to deep sea habitats?

2. How do changing availabilities of methane and sulfate influence the activity of anaerobic methanotrophic communities?
3. What are the carbon sources for organisms performing AOM? How can we integrate this knowledge into prior hypotheses on the functioning of AOM?

8. Publication outline

Biogeochemical Processes and Microbial Diversity of Gullfaks and Tommeliten Methane Seeps (Northern North Sea)

G. Wegener, M. Shovitri, K. Knittel, H. Niemann, M. Hovland and A. Boetius

Online at Biogeosciences Discussions (review process for Biogeosciences Journal)

This study was performed in the EU project METROL (Methane flux control in ocean margin sediments), which aims to describe the biological and geochemical controls of methane fluxes. This study describes methane fluxes and the composition and distribution of key microbial communities at various methane seepage systems in the North Sea. Comparison of those shallow water sites to deep-sea seep systems forms part of this manuscript.

Bathymetric measurements and sonar gas flare imaging were carried out as a service by the company Innomar during five research cruises with R/V Heincke (HE 169, HE 180, HE 208) and R/V Alkor (AL 267). Samples for microbiological and geochemical studies were taken during HE 208 and AL 267 by G. Wegener and A. Boetius. Organic and inorganic geochemical analyses were performed by G.Wegener. Molecular methods were performed in by M Shovitri as part of her M.Sc. thesis, supervised by G. Wegener and K. Knittel. The manuscript was written by G. Wegener with input from A. Boetius, K. Knittel and H. Niemann.

Short-Term Responses of Anaerobic Methane-Oxidizing Microbial Communities from Submarine Cold Seeps to Variations in Methane and Sulfate

G. Wegener, T. Ferdelman and A. Boetius.

In preparation for submission to Biogeosciences Journal

Laboratory experiments on short-term responses of methane oxidation and sulfate reduction were performed in a flow-through system ensuring stable conditions and non-invasive sampling of pore waters. The flow-through setup was developed and operated by G. Wegener. Sediment samples were obtained by G. Wegener (Gullfaks, HE 208); additional samples were provided from earlier expeditions. All rate measurements and data analyses were carried out by G. Wegener. Writing of the manuscript was done by G. Wegener with input from T. Ferdelman and A. Boetius.

Assimilation of Methane and Inorganic Carbon by Microbial Communities Mediating the Anaerobic Oxidation of Methane

Submitted to Environmental Microbiology

G. Wegener, H. Niemann, M. Elvert, K.-U. Hinrichs and A. Boetius

This study was undertaken to investigate methane and carbonate assimilation by microorganisms mediating AOM. The project was initiated by G. Wegener, the setup was developed and samples were taken by G. Wegener. Rate measurements and lipid biomarker analyses were performed by G. Wegener with the help of H. Niemann and M. Elvert. Data analysis was carried out by G. Wegener. The manuscript was written by G. Wegener with input from H. Niemann, A. Boetius, K.-U. Hinrichs and M. Elvert.

References

Abrajano, T.A., Sturchio, N.C., Kennedy, B.M., Lyon, G.L., Muehlenbachs, K. and Bohlke, J.K. (1990) Geochemistry of reduced gas related to serpentinization of the Zambales ophiolite, Philippines. *Appl. Geochem. Water-Rock Interactions Special Memorial Issue Ivan Barnes (1931-1989)* **5**: 625-630.

Alexandrino, M., Knief, C. and Lipski, A. (2001) Stable-isotope-based labeling of styrene-degrading microorganisms in biofilters. *Appl. Environ. Microb.* **67**: 4796-4804.

Balabane, M., Galimov, E., Hermann, M. and Letolle, R. (1987) Hydrogen and carbon isotope fractionation during experimental production of bacterial methane. *Org. Geochem.* **11**: 115-119.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. (1979) Methanogens: reevaluation of a unique biological group. *Microbiol. Mol. Biol. Rev.* **43**: 260-296.

Barker, J., and Fritz, P. (1981) Carbon isotope fractionation during microbial methane oxidation. *Nature* **293**: 289-291.

Barry, J.P., Buck, K.R., Kochevar, R.K., Nelson, D.C., Fujiwara, Y., Goffredi, S.K. and Hashimoto, J. (2002) Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami Bay, Japan. *Invertebr. Biol.* **121**: 47-54.

Bender, M., and Conrad, R. (1993) Kinetics of methane oxidation in oxic soils. *Chemosphere* **26**: 687-696.

Biddle, J.F., Lipp, J.S., Lever, M.A., Lloyd, K.G., Sørensen, K.B., Anderson, R. et al. (2006) Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proc. Natl. Acad. Sci.* **103**: 3846-3851.

Blair, N. (1998) The $\delta^{13}C$ of biogenic methane in marine sediments: the influence of C_{org} deposition rate. *Chem. Geol.* **152**: 139-150.

Blumenberg, M., Seifert, R., Reitner, J., Pape, T. and Michaelis, W. (2004) Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. *Proc. Natl. Acad. Sci.* **101**: 11111-11116.

Blumenberg, M., Seifert, R., Nauhaus, K., Pape, T. and Michaelis, W. (2005) In Vitro Study of Lipid Biosynthesis in an Anaerobically Methane-Oxidizing Microbial Mat. *Appl. Environ. Microb.* **71**: 4345-4351.

Boetius, A. (2005) Lost City Life. *Science* **307**: 1420-1422.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623-626.

Borodina, E., Cox, M.J., McDonald, I.R. and Murrell, J.C. (2005) Use of DNA-stable isotope probing and functional gene probes to investigate the diversity of methyl chloride-utilizing bacteria in soil. *Environ. Microbiol.* **7**: 1318-1328.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R. et al. (1998) Direct linking of microbial populations to specific biogeochemical processes by 13C-labelling of biomarkers. *Nature* **392**: 801-805.

Brenna, J.T., Corso, T.N., Tobias, H.J. and Caimi, R.J. (1997) High-precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrom. Rev.* **16**: 227-258.

Brocks, J.J. and Pearson, A. (2005) Building the biomarker tree of life. *Rev.Mineral. Geochem.* **59**: 233-258.

Cadisch, G., Espana, M., Causey, Z., Richter, M., Shaw, E., Morgan, J.A.W. et al. (2005) Technical considerations for the use of 15N-DNA stable-isotope probing for functional microbial activity in soils. *Rapid Commun. Mass Sp.* **19**: 1424-1428.

Canfield, D. (1993) Organic matter oxidation in marine sediments. Interactions of C, N, P and S biogeochemical cycles and global change. *NATO ASI Series* **14**: 33–363.

Canfield, D., Kristensen, E. and Thamdrup, B. (2005) *Aquatic Geomicrobiology*. San Diego, California: Elsevier.

Capone, D.G. and Kiene, R.P. (1988) Comparison of microbial dynamics in marine and freshwater sediments: Contrasts in anaerobic carbon catabolism. *Limnol: Oceanogr.* **33**: 725-749.

Carlsen, H., Jørgensen, L. and Degn, H. (1991) Inhibition by ammonia of methane utilization in Methylococcus capsulatus (Bath). *Appl. Microbiol. Biot.* **35**: 124-127.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P. and Holm, N. (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14'N, MAR). *Chem. Geol.* **191**: 345-359.

Coffin, R., Fry, B., Peterson, B. and Wright, R. (1989) Carbon isotopic compositions of estuarine bacteria. *Limnol. Oceanogr.* **34**: 1305-1310.

Coleman, D., Risatti, J. and Schoell, J. (1981) Fractionation of carbon and hydrogen isotopes by methane-oxidizing bacteria. *Geochim. Cosmochim. Ac.* **45**: 1033-1037.

Conrad, R., Erkel, C. and Liesack, W. (2006) Rice Cluster I methanogens, an important group of archaea producing greenhouse gas in soil. *Curr. Opin. Biotech.* **17**: 262-267.

Crutzen, P.J. (1994) Global budgets for non-CO2 greenhouse gases. *Environ. Monit. Assess.* **31**: 1-15.

de Leeuw, G.A.M., Hilton, D.R., Fischer, T.P. and Walker, J.A. (2007) The He-CO2 isotope and relative abundance characteristics of geothermal fluids in El Salvador and Honduras: New constraints on volatile mass balance of the Central American Volcanic Arc. *Earth Planet. Sc. Lett.* **258**: 132-146.

Deines, P., Bodelier, P.L.E. and Eller, G. (2007) Methane-derived carbon flows through methane-oxidizing bacteria to higher trophic levels in aquatic systems. *Environ. Microbiol.* **9**: 1126-1134.

del Giorgio, P.A. and Duarte, C.M. (2002) Respiration in the open ocean. *Nature* **420**: 379-384.

D'Hondt, S., Jørgensen, B.B., Miller, D.J., Batzke, A., Blake, R., Cragg, B.A. et al. (2004) Distributions of microbial activities in deep subseafloor sediments. *Science* **306**: 2216-2221.

Dickens, G.R. (2003) Rethinking the global carbon cycle with a large, dynamic and microbially mediated gas hydrate capacitor. *Earth Planet. Sc. Lett.* **213**: 169-183.

Ducklow, H.W., Steinberg, D.K. and Buesseler, K.O. (2001) Upper ocean carbon export and the biological pump. *Oceanography* **14**: 50-58.

Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S. et al. (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature* **450**: 879-882.

Elvert, M., Suess, E., Greinert, J. and Whiticar, M.J. (2000) Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone. *Org. Geochem.* **31**: 1175-1187.

Elvert, M., Boetius, A., Knittel, K. and Jørgensen, B.B. (2003) Characterization of specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. *Geomicrobiol. J.* **20**: 403-419.

Farquhar, G.D., Ehleringer, J.R. and Hubick, K.T. (1989) Carbon isotope discrimination and photosynthesis. *Annu.Rev. Plant Phys.* **40**: 503-537.

Fenchel, T. and Jørgensen, B.B. (1977) Detritus food chains of aquatic ecosystems: The role of bacteria. In *Advances in Microbial Ecology*. Alexander, M. (ed). New York: Plenum Press, pp. 1-58.

Finke, N., Vandieken, V. and Jørgensen, B.B. (2007) Acetate, lactate, propionate, and isobutyrate as electron donors for iron and sulfate reduction in Arctic marine sediments, Svalbard. *FEMS Microb. Ecol.* **59**: 10-22.

Foustoukos, D.I. and Seyfried, W.E., Jr. (2004) Hydrocarbons in hydrothermal vent fluids: The role of chromium-bearing catalysts. *Science* **304**: 1002-1005.

Galimov, E.M. and Kvenvolden, K.A. (1983) Concentrations and carbon isotopic compositions of CH4 and CO: in gas from sediments of the Blake Outer Ridge, Deep Sea Drilling Project Leg 76. In *Initial Reports DSDP*. Washington, pp. 402-403.

Ginige, M.P., Keller, J. and Blackall, L.L. (2005) Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent in situ hybridization-microautoradiography. *Appl. Environ. Microb.* **71**: 8683-8691.

Girguis, P.R., Cozen, A.E. and DeLong, E.F. (2005) Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Appl. Environ. Microb.* **71**: 3725-3733.

Girguis, P.R., Orphan, V., Hallam, S. and DeLong, E.F. (2003) Growth and Methane Oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. *Appl. Environ. Microb.* **69**: 5472-5482.

Grosjean, D. (1995) Atmospheric chemistry of biogenic hydrocarbons: Relevance to the Amazon. *Quím. Nova, l8 (2)*: 184-2011.

Guckert, J.B., Ringelberg, D.B., White, D.C., Hanson, R.S. and Bratina, B.J. (1991) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J. Gen. Microbiol.* **137**: 2631-2641.

Guy, R.D., Fogel, M.L. and Berry, J.A. (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiol.* **101**: 37-47.

Hanson, R.S. and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol. Rev.* **60**: 439-471.

Hayes, J.M. and Waldbauer, J.R. (2006) The carbon cycle and associated redox processes through time. *Philos. T. Roy. Soc. B.* **361**: 931-950.

Hayes, J.M., Freemann, K.H., Popp, B.N. and Hoham, C.H. (1990) Compound-specific isotopic analyses: A novel tool for reconstruction of ancient biogeochemical processes. *Org. Geochem.* **16**: 1115-1128.

Hazel, J.R. (1995) Thermal adaption in biological membranes. Is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**: 19-42.

Hinrichs, K.-U., Hayes, J.M., Silva, S.P., Brewer, P.G. and DeLong, E.F. (1999) Methane-consuming archaebacteria in marine sediments. *Nature* **398**: 802-805.

Hinrichs, K.-U., Summons, R.E., Orphan, V., Sylva, S.P. and Hayes, J.M. (2000) Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. *Org. Geochem.* **31**: 1685-1701.

Hinrichs, K.-U. and Boetius, A. (2002) The anaerobic oxidation of methane: New insights in microbial ecology and biogeochemistry. In *Ocean Margin Systems*. Wefer, G., Billett, D., Hebbeln, D., Jørgensen, B.B., Schlüter, M. and van Weering, T. (eds). Berlin Heidelberg: Springer-Verlag, pp. 457-477.

Iversen, N. and Jørgensen, B.B. (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnol. Oceanogr.* **30**: 944-955.

Jahnke, L.L., Summons, R.E., Hope, J.M. and Des Marais, D.J. (1999) Carbon isotopic fractionation in lipids from methanotrophic bacteria II: the effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. *Geochim. Cosmochim. Ac.* **63**: 79-93.

Jeon, C.O., Park, W., Padmanabhan, P., DeRito, C., Snape, J.R. and Madsen, E.L. (2003) Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. *Proc. Natl. Acad. Sci.* **100**: 13591-13596.

Jørgensen, L. and Degn, H. (1983) Mass spectrometric measurements of methane and oxygen utilization by methanotrophic bacteria. *FEMS Microbiol. Lett.* **20**: 331-335.

Jørgensen, B.B. (2006) Bacteria and marine biogeochemistry. In *Marine Geochemistry*. Schulz, H.D. and Zabel, M. (eds). Berlin, Heidelberg: Springer, pp. 169-206.

Judd, A.G. (2003) A global importance and context of methane escape from the seabed. *Geo-Mar. Lett.* **23**: 147-154.

Kaneda, T. (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Mol. Biol. Rev.* **55**: 288-302.

Kelley, D.S., Karson, J.A., Fruh-Green, G.L., Yoerger, D.R., Shank, T.M., Butterfield, D.A. et al. (2005) A serpentinite-hosted ecosystem: The Lost City hydrothermal field. *Science* **307**: 1428-1434.

King, G.M. (1992) Ecological aspects of methane oxidation, a key determinant of global methane dynamics. *Adv. Microb. Ecol.* **12**: 431-468.

Kinnaman, F.S., Valentine, D.L. and Tyler, S.C. (2007) Carbon and hydrogen isotope fractionation associated with the aerobic microbial oxidation of methane, ethane, propane and butane. *Geochim. Cosmochim. Ac.* **71**: 271-283.

Knief, C., Lipski, A. and Dunfield, P.F. (2003) Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microb.* **69**: 6703-6714.

Knittel, K., Lösekann, T., Boetius, A., Kort, R. and Amann, R. (2005) Diversity and distribution of methanotrophic archaea at cold seeps. *Appl. Environ. Microb.* **71**: 467-479.

Knittel, K., Boetius, A., Lemke, A., Eilers, H., Lochte, K., Pfannkuche, O. et al. (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia margin, Oregon). *Geomicrobiol. J.* **20**: 269-294.

Krzycki, J.A., Kenealy, W.R., DeNiro, M.J. and Zeikus, J.G. (1987) Stable carbon isotope fractionation by *Methanosarcina barkeri* during methanogenesis from acetate, methanol, or carbon dioxide-hydrogen. *Appl. Environ. Microb.* **53**: 2597-2599.

Kurr, M., Huber, R., König, H., Jannasch, H.W., Fricke, H., Trincone, A. et al. (1991) *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Arch. Microbiol.* **156**: 239-247.

Le Texier, H., Solomon, S. and Garcia, R.R. (1988) The role of molecular hydrogen and methane oxidation in the water vapour budget of the stratosphere. *Quarterly Journal of the Royal Meteorological Society* **114**: 281-295.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H. and Wagner, M. (1999) Combination of fluorescent in situ hybridization and microautoradiography---a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microb.* **65**: 1289-1297.

Lelieveld, J., Crutzen, P.J. and Dentener, F.J. (1998) Changing concentration, lifetime and climate forcing of atmospheric methane. *Tellus B* **50**: 128-150.

Levy, H. (1971) Normal Atmosphere: Large radical and formaldehyde concentrations predicted. *Science* **173**: 141-143.

Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B., Niemann, H., Boetius, A. and Amann, R. (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. *Appl. Environ. Microb.* **73**: 3348-3362.

Lueders, T., Pommerenke, B. and Friedrich, M.W. (2004) Stable-isotope probing of microorganisms thriving at thermodynamic limits: Syntrophic propionate oxidation in flooded soil. *Appl. Environ. Microb.* **70**: 5778–5786.

Mahmood, S., Paton, G.I. and Prosser, J.I. (2005) Cultivation-independent in situ molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environ. Microbiol.* **7**: 1349-1360.

Manefield, M., Whiteley, A.S., Griffiths, R.I. and Bailey, M.J. (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microb.* **68**: 5367-5373.

Maxfield, P.J., Hornibrook, E.R.C. and Evershed, R.P. (2006) Estimating high-affinity methanotrophic bacterial biomass, growth, and turnover in soil by phospholipid fatty acid ^{13}C-labeling. *Appl. Environ. Microb.* **72**: 3901-3907.

Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M. et al. (2002) Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science* **297**: 1013-1015.

Middleburg, J.J., Barranguet, C., Boschker, H.T.S., Herman, P.M.J., Moens, T. and Heip, C.H.R. (2000) The fate of intertidal microphytobenthos carbon: An in situ ^{13}C-labeling study. *Limnol. Oceanogr.* **45**: 1224-1234.

Milkov, A.V. (2004) Global estimates of hydrate-bound gas in marine sediments: how much is really out there? *Earth-Sci. Rev.* **66**: 183-197.

Mook, W. and Tan, F. (1991) Stable carbon isotopes in rivers and estuaries. In *Biogeochemistry of Major World Rivers*. Degens, E.T., Kempe, S. and Richey, J.E. (eds): John Wiley & Sons Hoboken, NJ.382pp.

Moore, L., Bourne, D. and Moore, W. (1994) Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic gram-negative bacilli. *Int. J. Syst. Bacteriol.* **44**: 338-347.

Morris, S.A., Radajewski, S., Willison, T.W. and Murrell, J.C. (2002) Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Appl. Environ. Microb.* **68**: 1446-1453.

Nauhaus, K., Boetius, A., Krüger, M. and Widdel, F. (2002) In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediments from a marine gas hydrate area. *Environ. Microbiol.* **4**: 296-305.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A. and Widdel, F. (2007) In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environ. Microbiol.* **9**: 187-196.

Nealson, K. (1997) Sediment bacteria: Who's there, what are they doing, and what's new? *Annu. Rev. Earth Planet. Sc.* **25**: 403-434.

Nedwell, D. (1996) Methane production and oxidation in soils and sediments. In Murrell, J.C. and Kelly, D P (eds): *Microbiology of Atmospheric Trace Gases*. Berlin, Germany. Springer

Nicolaus, B., Manca, M.C., Lama, L., Esposito, E. and Gambacorta, A. (2001) Lipid modulation by environmental stresses in two models of extremophiles isolated from Antarctica. *Polar Biology* **24**: 1-8.

Niemann, H. and Elvert, M. (in press) Diagnostic lipid biomarker and stable carbon isotope signatures of microbial communities mediating the anaerobic oxidation of methane with sulphate. *Org. Geochem.* doi:10.1016/j.orggeochem.2007.11.003.

Niemann, H., Elvert, M., Hovland, M., Orcutt, B., Judd, A., Suck, I. et al. (2005) Methane emission and consumption at a North Sea gas seep (Tommeliten area). *Biogeosciences* **2**: 335-351.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K. et al. (2006) Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* **443**: 854-858.

Ohtsubo, S., Kanno, M., Miyahara, H., Kohno, S., Koga, Y. and Miura, I. (1993) A sensitive method for quantification of aceticlastic methanogens and estimation of total methanogenic cells in natural environments based on an analysis of ether-linked glycerolipids. *FEMS Microbiol. Ecol.* **12**: 39-50.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F. (2001) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**: 484-487.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F. (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc. Natl. Acad. Sci.*: 072210299.

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R. et al. (2003) Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. *Appl. Environ. Microb.* **69**: 1614-1622.

Pancost, R.D. and Sinninghe Damste, J. (2003) Carbon isotopic compositions of prokaryotic lipids as tracers of carbon cycling in diverse settings. *Chem. Geol.* **195**: 29-58.

Parkes, R.J., Cragg, B.A., Banning, N., Brock, F., Webster, G., Fry, J.C. et al. (2007) Biogeochemistry and biodiversity of methane cycling in subsurface marine sediments (Skagerrak, Denmark. *Environ. Microbiol.* **9**: 1146-1161.

Peters, K.E., Sweeney, R.E. and Kaplan, I.R. (1978) Correlation of carbon and nitrogen stable isotope ratios in sedimentary organic matter. *Limnol. Oceanogr.* **23**: 598-604.

Proskurowski, G., Lilley, M.D., Seewald, J.S., Fruh-Green, G.L., Olson, E.J., Lupton, J.E. et al. (2008) Abiogenic hydrocarbon production at Lost City Hydrothermal Field. *Science* **319**: 604-607.

Prosser, J.I., Rangel-Castro, J.I. and Killham, K. (2006) Studying plant-microbe interactions using stable isotope technologies. *Curr. Opin.Biotech.* **17**: 98-102.

Radajewski, S., Ineson, P., Parekh, N.R. and Murrell, J.C. (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* **403:** 646-649.

Reeburgh, W. (2007) Oceanic methane biogeochemistry. *Chem Rev.* **107**: 486-513.

Rona, P.A., Bougault, H., Charlou, J.L., Appriou, P., Nelsen, T.A., Trefry, J.H. et al. (1992) Hydrothermal circulation, serpentinization, and degassing at a rift valley-fracture zone intersection; Mid-Atlantic Ridge near 15 degrees N, 45 degrees W. *Geology* **20**: 783-786.

Sano, Y., and Marty, B. (1995) Origin of carbon in fumarolic gas from island arcs. *Chem. Geol.* **119**: 265-274.

Schoell, M. (1980) The hydrogen and carbon isotopic composition of methane from natural gases of various origins. *Geochim. Cosmochim. Ac.* **44**: 649-661.

Schouten, S., van der Maarel, M.J.E.C., Huber, R., and Damste, J.S.S. (1997) 2,6,10,15,19-Pentamethylicosenes in *Methanolobus bombayensis*, a marine methanogenic archaeon, and in *Methanosarcina mazei*. *Org. Geochem.* **26**: 409-414.

Schouten, S., Strous, M., Kuypers, M.M.M., Rijpstra, W.I.C., Baas, M., Schubert, C.J. et al. (2004) Stable carbon isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria. *Appl. Environ. Microb.* **70**: 3785-3788.

Schwartz, E. (2007) Characterization of growing microorganisms in soil by stable Isotope probing with $H_2{}^{18}O$. *Appl. Environ. Microb.* **73**: 2541-2546.

Sessions, A.L., Burgoyne, T.W., Schimmelmann, A. and Hayes, J.M. (1999) Fractionation of hydrogen isotopes in lipid biosynthesis. *Org. Geochem.* **30**: 1193-1200.

Sherwood Lollar, B., Westgate, T.D., Ward, J.A., Slater, G.F. and Lacrampe-Couloume, G. (2002) Abiogenic formation of alkanes in the Earth's crust as a minor source for global hydrocarbon reservoirs. *Nature* **416**: 522-524.

Singh, B.K. and Tate, K. (2007) Biochemical and molecular characterization of methanotrophs in soil from a pristine New Zealand beech forest. *FEMS Microbiol. Lett.* **275**: 89-97.

Sinninghe Damste, J., Schouten, S., Hopmans, E.C., van Duin, A.C.T. and Geenevasen, J.A.J. (2002) Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. *J. Lipid Res.* **43**: 1641-1651.

Stoecker, K., Bendinger, B., Schöning, B., Nielsen, P.H., Nielsen, J.L., Baranyi, C. et al. (2006) From the cover: Cohn's Crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc Natl. Acad. Sci.* **103**: 2363-2367.

Summons, R.E., Jahnke, L.L. and Roksandic, Z. (1994) Carbon isotopic fractionation in lipids from methanotrophic bacteria: Relevance for interpretation of the geochemical record of biomarkers. *Geochim. Cosmochim. Ac.* **58**: 2853-2863.

Sundh, I., Borga, P., Nilsson, M., and Svensson, B.H. (1995) Estimation of cell numbers of methanotrophic bacteria in boreal peatlands based on analysis of specific phospholipid fatty acids. *FEMS Microbiol. Ecol.* **18**: 103-112.

Thiel, V., Peckmann, J., Richnow, H.H., Luth, U., Reitner, J. and Michaelis, W. (2001) Molecular signals for anaerobic methane ocidation in Black Sea seep carbonates and a microbial mat. *Mar. Chem.* **79**: 97-112.

Tissot, B.P. and Welte, D.H. (1984) *Petroleum formation and occurrence:*. Berlin, Heidelberg, NewYork, Tokyo,: Springer-Verlag.

Treonis, A.M., Ostle, N.J., Stott, A.W., Primrose, R., Grayston, S.J. and Ineson, P. (2004) Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs. *Soil Biol. Biochem.* **36**: 533-537.

Treude, T., Orphan, V., Knittel, K., Gieseke, A., House, C. and Boetius, A. (2007) Consumption of methane and CO_2 by methanotrophic microbial mats from gas seeps of the anoxic Black Sea. *Appl. Environ. Microb.* **73**: 2271-2283.

Treude, T., Boetius, A., Knittel, K., Nauhaus, K., Elvert, M., Kruger, M. et al. (2003) Anaerobic oxidation of methane at Hydrate Ridge (OR). *Geochim. Cosmochim. Ac.* **67**: A491-A491.

Tsunogai, U., Yoshida, N., and Gamo, T. (2002) Carbon isotopic evidence of methane oxidation through sulfate reduction in sediment beneath cold seep vents on the seafloor at Nankai Trough. *Mar. Geol.* **187**: 145–160.

Valentine, D.L. (2002) Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. *Anton Leeuw. J. Microb.* **81**: 271-282.

Ward, D.M. and Winfrey, M.R. (1985) Interactions between methanogenic and sulfate-reducing bacteria in sediments. In *Advances in microbial ecology*. Jannasch, H.W. and Williams, P.J.L. (eds). New York: Plenum Press, pp. 219-286.

Webster, G., Watt, L.C., Rinna, J., Fry, J.C., Evershed, R.P., Parkes, R.J. and Weightman, A.J. (2006) A comparison of stable-isotope probing of DNA and phospholipid fatty acids to study prokaryotic functional diversity in sulfate-reducing marine sediment enrichment slurries. *Environ. Microbiol.* **8**: 1575-1589.

Welhan, J.A. and Craig, H. (1977) Methane and hydrogen in East Pacific Rise hydrothermal fluids. *Geophys. Res. Lett.* **6**: 829-832.

Werne, J., Baas, M. and Damste, J. (2002) Molecular isotopic tracing of carbon flow and trophic relationships in a methane-supported benthic microbial community. *Limnol. Oceanogr.* **47**: 1694-1701.

Werne, J.P., Haese, R.R., Zitter, T., Aloisi, G., Bouloubassi, I., Heijs, S.K. et al. (2004) Life at cold seeps: a synthesis of biogeochemical and ecological data from Kazan mud volcano, eastern Mediterranean Sea. *Chem. Geol.* **205**: 367-390.

Whiticar, M. (1993) Stable isotopes and global budgets. Atmospheric Methane—Sources, Sinks, and Role in Environmental Change, *NATO ASI Ser.: Global Environ. Change* **1**.

Whiticar, M.J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **161**: 291-314.

Whiticar, M.J., Faber, E. and Schoell, M. (1986) Biogenic methane formation in marine and freshwater environments: CO_2 reduction vs. acetate fermentation--Isotope evidence. *Geochim. Cosmochim. Ac.* **50**: 693-709.

Widdel, F. (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In *Biology of anaerobic microorganisms*. Zehnder, A.J.B. (ed). New York: John Wiley & Sons, Inc., p. 469–585.

Wilkinson, T., Topiwala, H., and Hamer, G. (1974) Interactions in a mixed bacterial population growing on methane in continuous culture. *Biotec. Bioeng.* **16**: 41-59.

Wuchter, C., Schouten, S., Boschker, H.T.S., and Sinninghe Damste, J.S. (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol. Lett.* **219**: 203-207.

Chapter II

Biogeochemical processes and microbial diversity of the Gullfaks and Tommeliten methane seeps (Northern North Sea)

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Abstract: Fluid-flow related seafloor structures and gas seeps were detected in the North Sea in the 1970s and 1980s by acoustic sub-bottom profiling and oil rig surveys. A variety of features like pockmarks, gas vents and authigenic carbonate cements were found to be associated with sites of oil and gas exploration, indicating a link between these surface structures and underlying deep hydrocarbon reservoirs. In this study we performed acoustic surveys and videographic observation at Gullfaks, Holene Trench, Tommeliten, Witch's Hole and the giant pockmarks of the UK Block 15/25, to investigate the occurrence and distribution of cold seep ecosystems in the Northern North Sea. The most active gas seep sites, i.e. Gullfaks and Tommeliten, were investigated in detail: at both sites gas bubbles escaped continuously from small holes in the seabed to the water column, reaching the upper mixed surface layer as indicated by acoustic images of the gas flares. At Gullfaks a 0.1 km² large gas emission site was detected on a flat sandy seabed, covered by filamentous sulfide-oxidizing bacteria. At Tommeliten we found a patchy distribution of small bacterial mats indicating sites of gas seepage. Here the seafloor consists of layers of sand and stiff clay, and gas emission was observed from small cracks in the seafloor. At both sites the anaerobic oxidation of methane (AOM) coupled to sulfate reduction is the major source of sulfide. Molecular analyses targeting specific lipid biomarkers and 16S rRNA gene sequences identified an active microbial community dominated by sulfide-oxidizing and sulfate-reducing bacteria (SRB) as well as methanotrophic bacteria and archaea. Carbon isotope values of specific microbial fatty acids and alcohols were highly depleted, indicating that the microbial community at both gas seeps incorporates methane or its metabolites. The microbial community composition of both shallow seeps show high similarities to the deep water seeps associated with gas hydrates such as Hydrate Ridge or Eel River basin.

1. Introduction:

The North Sea is a marginal sea of the Atlantic on the European continental shelf. Its sedimentary basin, especially the Western and Northern areas, hosts large gas and oil fields which are exploited since the 1970s. Leaking methane reservoirs are a major source for shallow gas accumulations and emission into the water column and atmosphere (Hovland and Judd, 1988, Rehder et al., 1998; Judd and Hovland 2007). In the North Sea, eruptive gas ebullition through impermeable seabed consisting of stiff glacial clays leads to the formation of small craters at the seafloor, also known as pockmarks (Hovland and Judd, 1988). These pockmarks have diameters in the range of few to several hundred meters and are widespread in gas and oil fields of the central and northern North Sea. Intensive bathymetric and videographic surveys by the British Geological Survey and oil industry have led to a good understanding of the distribution of these and other gas escape structures in the North Sea (Judd and Hovland, 2007). As part of the 5th EU framework project METROL "Methane fluxes in ocean margin sediments: microbiological and geochemical control" we have studied the distribution, biogeochemistry and microbiology of gas seepage in the North Sea, to identify potential sites of methane emission to the atmosphere, and to better understand the functioning of the associated shallow water seep ecosystems.

Although methane is abundant in the seafloor, the oceans account for only 3 to 5% of the global atmospheric methane flux (Reeburgh, 2007). Aerobic and anaerobic microbial methane consumption almost completely control the gas flux into the water column and atmosphere, except at sites of high fluid flow and free gas ebullition (Valentine and Reeburgh, 2000). The anaerobic oxidation of methane with sulfate as terminal electron acceptor is the dominant biogeochemical process in gassy sediments (Hinrichs and Boetius, 2002). Its net reaction can be described according to equation (1), but the underlying biochemistry of this process remains unknown (Widdel et al., 2007).

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

Several phylogenetic clades of archaea related to the order *Methanosarcinales* were identified as anaerobic methanotrophs by analyses of 16S rRNA gene sequences and of stable isotope signatures of specific biomarkers (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001b, Niemann et al., 2006). In most seep habitats archaea form consortia with sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* or *Desulfobulbus* groups (Knittel et al., 2003; Niemann et al., 2006; Lösekann et al., 2007). These associations are commonly attributed to obligate syntrophy, in which the archaeal partner activates and metabolizes methane, providing an intermediate that is scavenged by the sulfate-reducing partner (Nauhaus et al., 2002; 2007). Analyses of carbon isotopes in seep ecosystems have shown a tight link between methane, the microbial consortia, authigenic carbonate precipitates and higher trophic levels in the food web (Hovland et al., 1985; Ritger et al., 1987, Hinrichs and Boetius, 2002; Hovland et al., 2002; Levin, 2005). Deep water gas seeps often support an enormous biomass of free-living and symbiotic microbial life that is nourished by the oxidation of methane and the product of its anaerobic oxidation, sulfide. A prominent feature of such seeps are mat-forming chemoautotrophic bacteria using sulfide as energy source, including *Beggiatoa* (Treude et al., 2003; Joye et al., 2004), and *Arcobacter* (Omoregie et al., in review). Furthermore, authigenic carbonates related to anaerobic oxidation of methane (AOM) are found at many seeps. The precipitation of these carbonates is possibly related to an increase of pore water alkalinity due to AOM (Luff and Wallmann, 2003). Carbonate outcrops attract a variety of hardground fauna like corals, ophiurids, sponges and bivalves (Hovland and Risk, 2003, Niemann et al., 2005). Ultimately, the carbonate precipitation associated with AOM can fill and seal gas escape conduits (Hovland, 2002).

Biogeochemical research efforts on cold seeps during the last decade mainly focused on deep water systems, especially those associated with gas hydrates. As a consequence, much more is known about these systems than about shallow water seeps in estuaries and

shelf seas, despite their potential relevance for gas emission to the atmosphere. Specifically in the Northern North Sea, where the water column is frequently mixed by storms, methane emission from the seabed is likely to result in an export of this greenhouse gas to the atmosphere. However, well constrained estimates of the methane flux from the seabed to the atmosphere are still missing for the North Sea.

Here we investigated several pockmarks and potential sites of gas seepage of the Northern North Sea. Sediments were sampled from sulfide oxidizing bacterial mats which were associated with gas ebullition. Based on the concentration and carbon isotopy of specific lipid biomarkers, as well as by 16S rRNA sequence analysis, we describe the microbial communities of the two active shallow water seeps in the North Sea (Gullfaks and Tommeliten). Furthermore, their phylogenetic and biogeochemical characteristics are compared with those of known deep water cold seep communities to investigate whether shallow and deep seeps are populated by different types of methanotrophs.

2. Material and methods

Sampling sites

Figure 1 gives an overview on the cold seep sites in the North Sea visited during the METROL cruises (R/V Heincke cruises HE169, HE180, HE208 and R/V Alkor cruise AL267; also see Tab. 1). For all investigated sites detailed background information was obtained previously during extensive geological surveys including seismic and sonar monitoring of seabed and water column features, as well as by videographic exploration using towed cameras and ROVs (Hovland and Judd, 1988). Gas emissions at Tommeliten and Gullfaks have been documented for a period over 25 years (Niemann et al., 2005, Judd and Hovland, 2007).

Gullfaks is one of the four major Norwegian oil and gas fields, located in the northeastern edge of the North Sea Plateau. The water depth in this area is ca. 140 m and

deepens northeastwards towards the Norwegian trench (Hovland, 2007). During the last glacial maximum the plateau was exposed and coastal sands were deposited. Younger sediments of the Holocene have not been found in this area because tidal currents prevent deposition (Eisma and Kalf, 1987). The Gullfaks hydrocarbon reservoir is situated in a depth of nearly 3 km (Hovland and Judd, 1988). Shallow gas accumulations formed above the leaky reservoir at depths between 300 and 450 meters below the seafloor (mbsf) (Judd and Hovland, 2007).

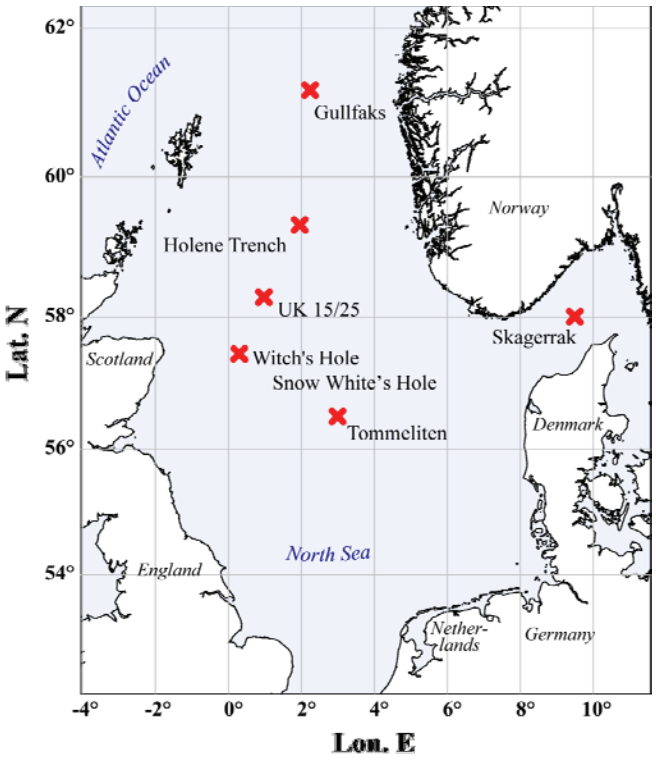

Fig. 1. Tommeliten, Gullfaks and other potential gas escape structures investigated for current gas emission during the METROL research expeditions HE 169, HE 180, HE 208 and Alkor 267.

Tommeliten lies in the greater Ekofisk area north of the central North Sea (Hovland and Judd, 1988; Hovland, 2002; Judd and Hovland, 2007) at a water depth of 75 m. This gas field is associated with salt diapirs at about 1 km bsf, and has already been fully exploited (Hovland, 2002). Seismic profiles indicate extensive gas escape pathways in the seabed above the deposit. Eruptive discharge of free gas probably formed the shallow pockmarks which lie 0.5-1 meter below the surrounding seabed level (Hovland and Sommerville, 1985). The sediments consist of sands, silt and marl (Niemann et al., 2005). Associated with gas

leakage pathways are calcareous cements, some of which extend above the seafloor and form reefs populated by diverse anthozoa and other hardground fauna (Hovland and Judd, 1988; Niemann et al., 2005). Acoustic turbidity indicated the presence of free gas in the seabed in an area of about 0.12 km^2. Sonar surveys revealed gas escape to the water column, and accordingly, numerous gas seeps associated with whitish bacterial mats were observed during ROV surveys (Hovland et al. 1993; Judd and Hovland, 2007, Niemann et al., 2005). Gas seepage was confined to about 120 individual bubble streams in an area of 6500 m^2. An emission of 47g CH_4 m^{-2} was estimated for this seepage area (Hovland et al., 1993). In the vicinity of the gas vents, elevated methane concentrations and gas bubbles were found in the seabed, at a sediment depth of 1-5 mbsf, associated with layers of carbonate precipitates and cements (Niemann et al. 2005). These observations and biogeochemical rate measurements indicate that most methane may be consumed in the seafloor, but that considerable gas escape to the water column occurs through cracks and fissures (Niemann et al., 2005). The carbon isotope signature of methane emitted from the seafloor of -45.6‰ indicates its thermogenic origin (Hovland and Sommerville, 1985; Hovland and Judd, 1988).

Seafloor observations

During the cruises HE 169, HE 180, HE 208 and AL267 (Tab. 1), several seep locations of the North Sea were visited (Fig. 1). Gas flares were detected using the sediment echo sounder system SES-2000 provided by INNOMAR (Rostock, Germany). The emitter induces two primary frequencies near 100 kHz to generate secondary bandwidths of 4 and 15 kHz. The long waves were used to visualize shallow sea floor structures and layering. Water depth and gas flares were recorded with the 15 kHz spectrum, while sediment features were observed with a 4 kHz spectrum. Several acoustic transects were evaluated to quantify the gas flares and their extensions, and to localize the flare source at the seafloor. Video observations were performed with the ROV Sprint (Alfred Wegener Institute for

Polar and Marine Research, Bremerhaven; HE169), the towed camera system of the AWI (HE180) or the MPI (Spy, Mariscope, Kiel; HE208), or via a remotely operating vehicle at Tommeliten (ROV Cherokee, MARUM, Bremen; AL267).

Table 1. Visited gas escape structures and the presence of gas flares and methane-derived carbonates. The cruises took place in June 2002 (HE169), October 2002 (HE180), May 2004 (HE208), and September 2005 (AL267). *n.a. – bottom observations were not available, #described in Judd and Hovland 2007*

Structure	Latitude	Longitude	Water depth	Cruise	gas escape	authigenic carbonates
Gullfaks seep	61°10.40'	02°14.50'	150 m	HE 169, HE 180, Al 267 HE208	yes	no
Holene Trench	59°19.60'	01°57.60'	130 - 145 m	HE 169	no	yes#
UK 15/25 pockmarks	58°17.00'	00°58.50'	155 - 170m	HE 180, HE 208	yes	yes
Witch's Hole	57°56.50'	00°23.30'	135 m	HE 208	no	yes#
Snow White's Hole	57°58.81'	00°23.30'	145 m	HE 208	yes	n.a.
Tommeliten	56°29.90'	02°59.80'	75 m	HE 169, HE 180, AL267	yes	yes
Skagerrak pockmarks	58°00.00'	09°40.00'	120 -150 m	HE 208	no	no#

Sediment sampling

At Gullfaks sediments were sampled in May 2004 (HE208) using a video-guided multiple corer system (MUC; Octopus, Kiel). The samples were recovered from an area densely covered with bacterial mats where gas ebullition was observed. The coarse sands limited MUC penetration depth to max 30 centimeters. The highly permeable sands did not allow vertical subsampling of the MUCs at high resolution, because porewater was lost during subsampling.

The gas flare mapping and videographic observation at Tommeliten indicated an area of gas emission, which consisted of a few small patches of bacterial mats with diameters < 50 centimeters, spaced apart by 10-100 m, from most of which a single stream of gas bubbles emerged. Sampling of these patchy gas vents was only possible with the ROV Cherokee to which we mounted 3x 3.8cm diameter push cores. The cores were sampled in 3 cm intervals.

Sediment porosity, sulfate and methane concentrations were determined following the METROL protocol (http://www.metrol.org/index.php?bereich=1). In situ methane concentrations were calculated using PHREEQC, Version 2, US Geological Survey, 2007. Samples for molecular, microbiological and biomarker analyses were processed as described below.

Radiotracer based *in vitro* measurements of AOM and SR were performed in the home laboratory. Sediment samples stored anoxically in wide mouth bottles with artificial, anaerobic seawater medium (28 mM Sulfate, 30 mM carbonate, 1 mM sulfide, equilibrated at a pH of 7.2; see also Widdel and Bak, 1992) were transferred into Hungate tubes, refilled with medium and brought into equilibrium with one atmosphere of methane (Krüger et al., 2005). Controls without methane addition were prepared to determine methane independent SR. Rates were determined from replicate incubations (n ≥3). After one day of equilibration, $^{35}SO_4$ (50 kBq dissolved in 10µl water) for SR and $^{14}CH_4$ (10 kBq dissolved in 50 µl water) for AOM were injected into the Hungate tubes through a butyl rubber septum. Samples were incubated for 7 days at in situ temperatures (8°C). The reactions were stopped by transferring the samples into zinc acetate and NaOH solution, respectively (Treude et al., 2003). Further processing of AOM and SR samples was carried out as described previously (Treude et al., 2003; Kallmeyer et al., 2004), respectively. Concentrations and activities of the reactants (methane or sulfate) and the activities of the products (sulfide or carbonate) were measured to determine AOM and SR rates according to the following formulas:

$$AOMrate = \frac{[CH_4] \cdot a^{14C} carbonate}{t \cdot a^{14C} methane} \quad (2),$$

$$SRR = \frac{[SO_4] \cdot aTRIS}{t \cdot aTOT \cdot m_{sed}} \cdot 1.06 \quad (3),$$

In equation (2) [CH_4] is the concentration of methane, a^{14C}methane and a^{14C}carbonate the activity of the reactant and the product of AOM (Treude et al., 2003). In equation 3, *[SO4]* is the concentration of sulfate, *aTRIS* the activity of the total reduced inorganic sulfur, *aTOT* is the total activity of sulfide and sulfate and t is the reaction time. The factor 1.06 accounts for the discrimination of the heavier radio nucleotide ^{35}S (Jørgensen and Fenchel, 1974).

Total organic carbon content (TOC) and carbon nitrogen ratios (C/N) were analyzed from freeze-dried samples. Briefly, inorganic carbon (carbonate) was removed via HCl acidification. Subsequently, 20 to 30 mg of homogenized samples were filled in zinc cartridges and organic carbon and nitrogen was measured in a CNS analyzer (Carlo Erba NA 1500 CNS analyzer).

Biomarker analyses. Lipid biomarker extraction from 10 - 17 g wet sediment was carried out as described in detail elsewhere (Elvert et al., 2003). Briefly, defined concentrations of cholestane, nonadecanol and nonadecanolic acid with known $\delta^{13}C$-values were added to the sediments prior to extraction as internal standards for hydrocarbons, alcohols and fatty acids, respectively. Total lipids were extracted from the sediment by ultrasonification with organic solvents of decreasing polarity. Esterified fatty acids were cleaved by saponification with methanolic KOH solution. From this mixture, the neutral fraction (mainly hydrocarbons and alcohols) was extracted with hexane. After subsequent acidification, fatty acids were extracted with hexane. For analysis, fatty acids were methylated using BF_3 in methanol yielding fatty acid methyl esters (FAMES).

The neutral fraction was further separated into hydrocarbons, ketones and alcohols on a SPE cartridge with solvents of increasing polarity (Niemann et al., 2005). The ketone fraction was not further analyzed. Shortly before analyses alcohols were methylated to trimethylsilyl (TMS) ethers using bis(trimethylsilyl)triflouracetamid (BSTFA). Concentration

and identity of single lipid compounds was determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis, respectively (Elvert et al., 2003, 2005; Niemann et al, 2005). Double bound positions of monoeonic fatty acids were determined analyzing their dimethyl disulfide (DMDS) adducts according to Moss and Labert-Faeir (1989). δ^{13}C-values of single lipid compounds were determined by GC-IRMS analyses according to Elvert et al. (2003). Concentration and isotopic signatures of fatty acid and alcohol were corrected for the additional carbon atoms added during derivatisation.

DNA extraction, PCR amplification and clone library construction. From both sites DNA was extracted from 2 g of wet sediment (1-3 cm depth) using bio101 soil kit (Bio101, La Jolla, California). Domain-specific primers were used to amplify almost full-length 16S rRNA genes from the extracted chromosomal DNA by PCR. For Bacteria, primers GM3F (Muyzer et al., 1995) and EUB1492 (Kane et al., 1993) were used, for Archaea, the primers ARCH20F (Massana et al., 1997) and Uni1392 (Lane et al., 1985) were used. PCRs were performed (30 cycles) and products purified as described previously (Ravenschlag et al., 1999). DNA was ligated in the pGEM-T-Easy vector (Promega, Madison, WI) and transformed into *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA) following manufacturer's recommendation.

Sequencing and phylogenetic analysis. Sequencing was performed by *Taq* cycle sequencing with a model ABI377 sequencer (Applied Biosystems). The presence of chimeric sequences in the clone libraries was determined with the CHIMERA_CHECK program of the Ribosomal Database Project II (Center for Microbial Ecology, Michigan State University, http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Sequence data were analyzed with the ARB software package (Ludwig et al., 2004). Phylogenetic trees were calculated with the ODP 204 sequences from this project together with reference sequences,

which were available in the EMBL, GenBank and DDJB databases by maximum-likelihood and neighbor-joining analysis with different sets of filters.

Cell counts and CARD-FISH (Catalyzed reporter deposition - fluorescence in situ hybridization). The fixation for total cell counts and CARD-FISH were performed on-board directly after sampling. For both methods 2 ml of the sediment were added to 9ml of 2% formaldehyde artificial seawater solution, respectively. After fixation for two hours CARD-FISH samples were washed three times with 1xPBS (10mM sodium phosphate solution, 130 mM NaCl, adjusted to a pH of 7.2 by titration with Na_2HPO_4 or NaH_2PO_4) and finally stored in 1:1 PBS:ethanol solution at -20°C until further processing. Samples for total cell counts were stored in formalin at 4°C.

For the sandy samples the total cell count/CARD-FISH protocol were optimized to separate the sands particles from the cells. Cells were dislodged from sediment grains and brought into solution with the supernatant by sonicating each samples on ice for 2 min at 50W. This procedure was repeated four times and supernatants were combined. Total cell numbers were determined from the supernatant using acridine orange direct counting (AODC; Meyer-Reil, 1983).

CARD-FISH was performed following the protocol of Pernthaler (2002). The sediment samples were brought to a final dilution of 1:2000 to 1:4000 and filtered onto 0.2µm GTTP filters (Millipore, Eschbonn, Germany). The probes used in this study (all purchased from biomers.net GmbH, Ulm, Germany) were EUB 338 I-III specific for most Bacteria (Amann et al., 1990; Daims et al., 1999), DSS658 specific for *Desulfosarcina* spp., *Desulfococcus* spp. and closely related clone sequences (Manz et al., 1998), Arch915 specific for most *Archaea* (Stahl and Amann, 1991), and probes ANME1-350 (Boetius et al., 2000), ANME2a-647, ANME2c-622 (Knittel et al., 2005) and ANME3-1249 (Niemann et al., 2006) specific for ANME-1, -2a, -2c, and -3 archaeal cells, respectively. Cell

permeabilization and probe hybridization were performed according to the author's instructions. For reference cell numbers, samples were also stained with 4'6'-diamidino-2-phenlyindole (DAPI) for 15minutes (1µg/ml) and washed with sterile filtered water and ethanol for 60 and 30 seconds, respectively. Air-dried filters were imbedded in Citifluor (Citifluor Ltd., Leicester, UK). Cells were counted using an epifluorescence microscope (Axioplan, Zeiss, Germany). At least 1000 cells in randomly chosen fields were counted per sample. To quantify aggregates of ANME-2a, -2c and -3 up to 250grids were counted under 400-fold magnification. Dual hybridizations for sulfate reducers (DSS) and archaea (ANME-2a and -2c) were performed using different fluorescence dyes (cy3 and cy5) and images were taken with a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

3. Results and discussion

Distribution of active seeps in the Northern North Sea

The presence of gas flares indicating active methane seepage from the seabed was detected at different fluid flow-related seafloor structures visited during the METROL cruises. Large and abundant gas flares were found at Tommeliten, Gullfaks, Snow White's Hole and the giant pockmarks of UK block 15/25 (Fig. 1, 2; Tab. 1).

The pockmarks of UK Block 15/25, Snow White's Hole and Witch's Hole are part of the Witch Ground formation, northwest of the Tommeliten seep area (Fig. 1, Hovland and Judd, 1988). During our cruises (HE180 and HE208), most of the 5 giant (Scanner, Scotia and Challenger formation) and two medium sized pockmarks of the UK Block 15/25 showed active seepage of methane from the deepest part of each depression (up to 17 m below the surrounding seafloor; Fig. 2c). Hovland et al. (1993) estimated the average flux at this location with 26 g CH_4 m^{-2} yr^{-1}. In the center of the pockmarks we also observed carbonate outcrops which were populated with benthic organisms, mostly sea anemones. Unfortunately we were not able to sample the surface and subsurface cements at the bottom

of the steep pockmarks because the gravity corer and multiple corer could not penetrate. The methane venting and the carbonate cements have been observed previously (Hovland and Judd, 1988) and during all expeditions to this site, indicating that the UK 15/25 pockmarks may have been continuously emitting methane to the hydrosphere for at least two decades. The gas flares at the UK 15/25 pockmarks reached up to 80 m below the sea surface. In contrast to earlier observations (Hovland and Judd 1998), the Witch's Hole structure itself was presently dormant, but we found an active pockmark southwest of it, which we named Snow White's Hole (Fig. 2d). This structure emits a large gas flare extending about 80 m from the seafloor. However sampling directly at the flare was not possible most likely because of the presence of carbonate cements. Sampling in the vicinity of the gas flare at the edge of the pockmark recovered only oxidized non-seep sediments.

The Holene Trench is an open channel of ca. 1 km width located on the Norwegian Plateau in about 120 m water depth. Previous surveys showed an acoustic turbidity in the top most 30 m of the surface sediments indicating gas charged sediments (Hovland and Judd, 1988). During our survey, two places were found where the turbidity extends to the surface of the sea floor, which could be related to active seep sites. However, during HE-169 we could not find any traces for active seepage (Fig. 2b) and only beige, oxidized clay sediments were recovered by multiple corer sampling. The western slope of the Norwegian Trench hosts several pockmark-like structures, which do not show carbonate outcrops (Hovland and Judd, 1988). Our survey during cruise HE-169 did not reveal any traces of seepage and one grab sample recovered beige, oxidized sandy-silty sediments. Previous surveys in the Skagerrak found several pockmarks with active gas escapes and seepage related fauna (Dando et al., 1994; Rise et al., 1999). During HE-208 we did not observe gas seepage from the positions reported in Dando et al. 1994 (Fig. 2f).

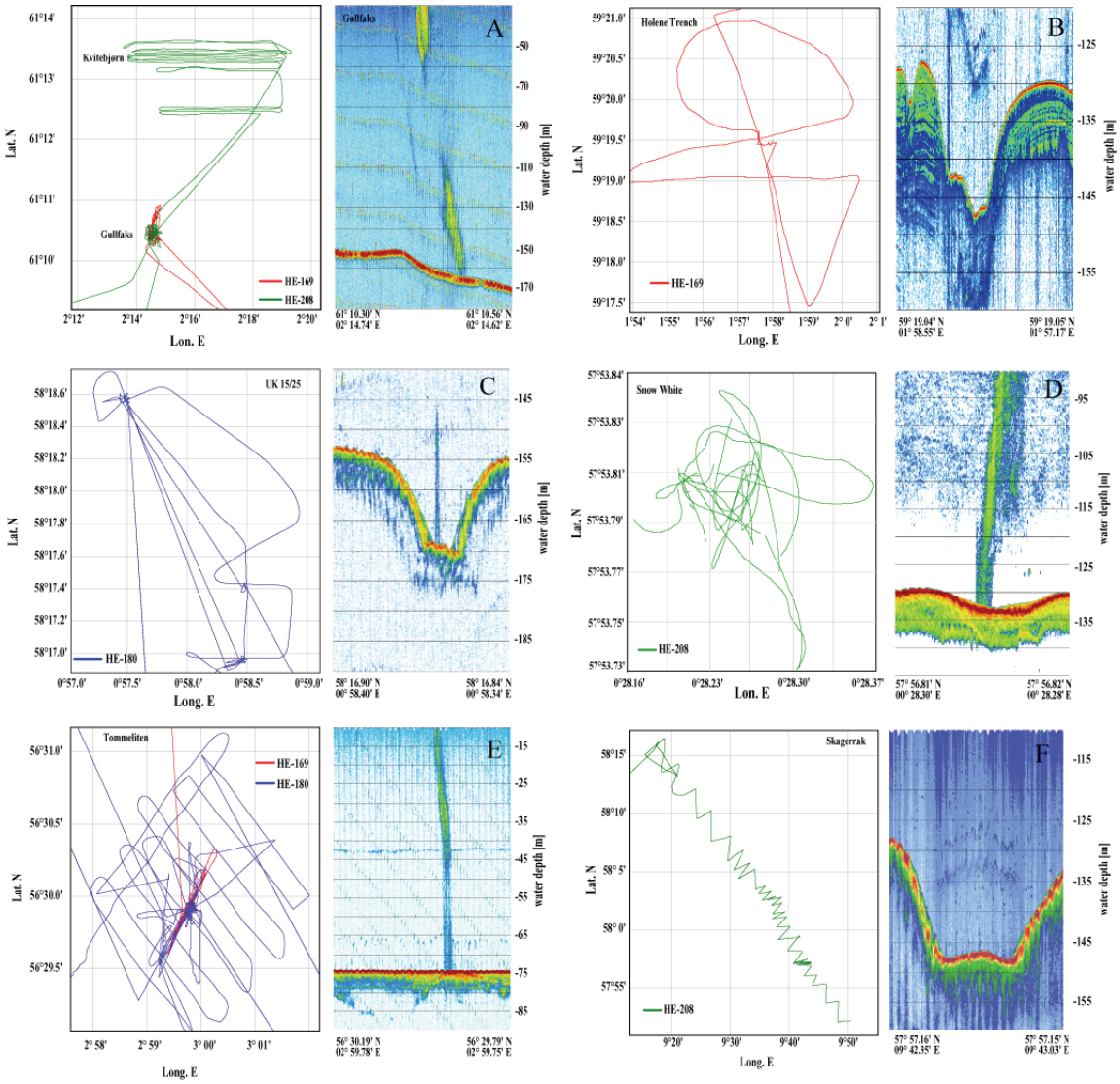

Fig. 2. Survey tracks and exemplary SES-2000 echo images showing gas flares and sea floor structures of the sites Gullfaks with the 'Heincke' seep area (A), Holene Trench (B), Scanner pockmark (UK 15/25 field) (C), Snow White's Hole (D), Tommeliten (E).and Skagerrak (F).

For further investigations of the biogeochemistry and microbiology of shallow water cold seeps in the North Sea we focused on the Tommeliten and Gullfaks sites. The results from subsurface sampling of the Tommeliten seeps with help of a vibrocorer were already reported in Niemann et al. (2005). Here we focused on the hot spots for microbial methane turnover and methane emission which were associated with bacterial mats at both sites.

Gullfaks

Seafloor observations. The Gullfaks field is located on the North Sea Plateau (~150 m water depth) and on an ancient beach (140 – 190m), respectively. The submerged beach was formed during the sea-level low of the last glacial maximum (Hovland and Judd 1988). Pockmark-like depressions were absent, which may be explained by the sediment properties of this area. Pockmark formation is believed to be limited to silty or clayish seafloor with low permeability and may not occur in highly permeable sandy seafloors (Hovland and Judd, 1988; Judd, 2003).The flat seafloor of the Gullfaks seep area is composed of coarse sand and gravel. However, ripple structures observed during the ROV dives indicate episodically high bottom water current velocities. Several gas flares were found within an area of about 0.5 km^2, which was named 'Heincke seep area' (Hovland 2007). The flares extended up to 120 meters above the seafloor, reaching the mixed water layer (Fig. 2a). Visual observations of the seafloor showed 1-2 bubble streams escaping from the sands every 5 m^2 within a smaller area of 0.1 km^2 covered by microbial mats (Fig. 3a). The macroscopic appearance resembled mats formed by giant filamentous sulfide oxidizing bacteria, such as *Beggiatoa,* which establish above zones of high sulfide flux (Nelson et al., 1986). No megafauna was observed to populate the seep site or to graze upon the bacterial mats, but many large cod-like fish were observed in this area. Considering the solubility of methane in situ at a water depth of 150m (equivalent to ~16 bar modeled with PREEQC, USGS), methane concentrations in the seabed should exceed 26 mM.

Multicorer sampling was conducted at Gullfaks in the bacterial mat field (Fig. 3a). Upon recovery, the sediment cores degassed strongly, releasing streams of methane bubbles into the overlaying water. The sediments smelled strongly of sulfide. The top 30 cm of sediment retrieved by multiple coring was unsorted, coarse to medium grained sands. Sediment porosity was on average 33%. Only the top surface layer of 1 to 5 centimeters was of beige color mixed with black particles, probably marking the oxygen penetration depth.

The color shift to blackish sediments below indicated the change of redox conditions to negative values and the presence of free sulfide and iron sulfide precipitations. Within the gassy sediment abundant ferromagnetic minerals, probably magnetite or greigite, were found, indicating a connection of methane and iron fluxes. Total organic carbon showed average values of only 0.17%, indicating that the main source for the observed high sulfide concentrations may be methane rather than deposited organic material.

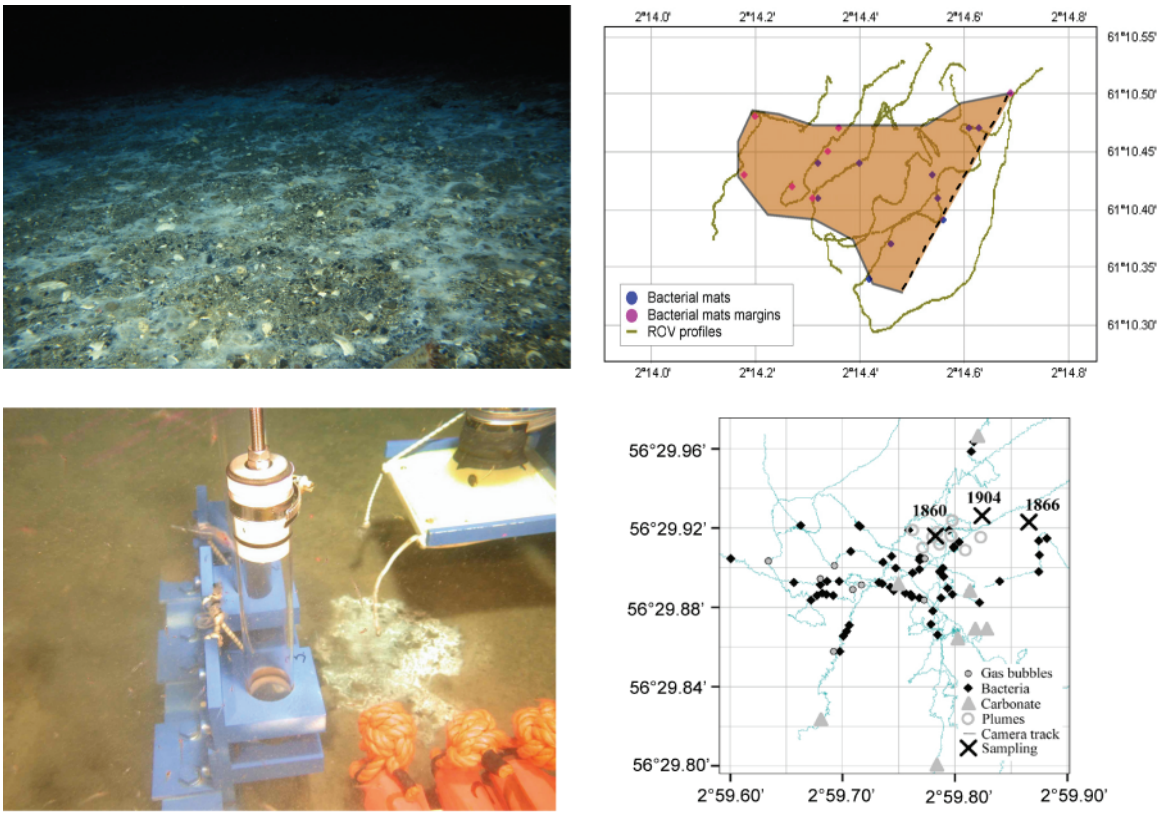

Fig. 3a. Mats of giant sulfide-oxidizing bacteria covering coarse sands and pebbles at the Heincke seep area at Gullfaks (left panel). The mats coincide with the area of gas ebullition and cover an area of about 0.1 km^2 (right panel). 3b. Bacterial mats of the Tommeliten gas seep. Left panel: Sampling of a mat patch with a diameter of about 30cm. Gas ebullition was observed during sampling. Right panel: Map showing the distribution of mats, gas flares and carbonates (after Niemann et al. 2005).

Microbial methane oxidation and sulfate reduction. From all six multicorer samples retrieved from the mat covered area, methane oxidation and sulfate reduction rate measurements were performed using replicate subsamples of the bulk sediments from the top 25 cm. Methane oxidation rates ranged from 0.006 µmol g^{-1} to 0.18 µmol g^{-1} dry weight

d^{-1} and sulfate reduction rates (SRR) from 0.05μmol g^{-1} to 0.3 μmol g^{-1} dry sediment d^{-1}. In control measurements without methane, SRR was negligible, indicating that methane was the dominant electron donor for sulfate reduction. The integrated methane oxidation rates averaged 12.5 mmol m^{-2} d^{-1} and the integrated SRR 18.5 mmol m^{-2} d^{-1}. These are comparatively high rates which fall within the range of other measurements from bacterial mat covered seep sites like Hydrate Ridge (5.1 to 99 mmol m^{-2} d^{-1}; (Treude et al., 2003) or Haakon Mosby Mud Volcano (4.5 mmol m^{-2} d^{-1}; (Niemann et al., 2006)). Using the mean methane oxidation rate from our incubations, a methane consumption of 15 t yr^{-1} can be roughly calculated for an area of 0.1 km^2. Based on quantifications of single gas bubble streams (Hovland et al., 1993) we estimated a gas flux to the water column of 76 t yr^{-1} for this area, which means that the microbial filter could consume at least 16% of the total gas flux. Of course, these are only rough estimates, as the gas streams may vary strongly in intensity and methane content.

Biomarker and carbon isotope signatures. The ^{13}C-carbon compositions of methane at Gullfaks cover a range from -44.4‰ vs. PDB (Pee Dee Belemnite) at reservoir level (2,890 m below seafloor) to -70‰ at seafloor level (Judd and Hovland, 2007). The abundance of specific biomarker lipids and their stable carbon isotope signatures were analyzed to reveal the distribution of chemoautotrophic and methanotrophic microbial communities. The lipid concentrations and isotopic signatures of the Gullfaks microbial communities (Core 766) are shown in Table 2. In comparison to highly active seep sites such as Hydrate Ridge, concentrations of lipid biomarkers at Gullfaks and Tommeliten were low.

In surface sediments of Gullfaks monounsaturated fatty acids were strongly dominant. Although these fatty acids are produced by a wide range of gram negative bacteria (Fang et al., 2005), their relative abundance can be used to trace specific microbial groups. The measured ratio for $C_{16:1\omega7c}$, $C_{18:1\omega7c}$ and $C_{16:0}$ of 68:18:14 was close to the ratio of

73:18:9 previously reported for *Beggiatoa* filaments covering cold seep sediments of the Gulf of Mexico (Zhang et al., 2005). The $\delta^{13}C$-values of the FA $C_{16:1\omega7c}$ and $C_{18:1\omega7c}$ (-59 and -41‰, respectively) extracted from the bacteria covered sands, indicate a substantial contribution of autotrophic carbon fixation as previously found for several species of giant filamentous sulfide oxidizers (Nelson and Jannasch, 1983; Nelson et al., 1986; Nelson et al., 1989). In addition to signatures of sulfide oxidizing bacteria, substantial amount of diplopterol were found. This hopanoid is synthesized by several aerobic bacteria including methanotrophs (Rohmer et al., 1984). A methanotrophic origin of this biomarker at Gullfaks is likely because of its highly depleted stable carbon composition (-84‰).

Table 2. Biomarker concentrations and their isotopic signatures in sediments from bacterial mat covered sands (Gullfaks Station 766), and sediments (Tommeliten Station 1274-K3) as well as from the subsurface SMTZ of Tommeliten (155 cm data by Niemann et al., 2005). *n.d. = not detected; n.a. = not available.*

	Gullfaks Station 766 (0-10cm)		**Tommeliten 1274-K3 (0-10cm)**		**Tommeliten Core 1904 155cm SMTZ**	
Compound	µg gdw^{-1}	$\delta^{13}C$ VPDB	µg gdw^{-1}	$\delta^{13}C$ VPDB	µg gdw^{-1}	$\delta^{13}C$ VPDB
$C_{14:0}$	1.27	-60	0.64	-30	0.15	-28
i-$C_{15:0}$	0.61	-41	0.30	-35	0.06	-43
ai-$C_{15:0}$	0.64	-42	0.43	-34	0.14	-43
$C_{15:0}$	0.40	-48	0.22	-38	0.07	-37
$C_{16:1\omega7c}$	24.04	-59	2.54	-38	0.02	-38
$C_{16:1\omega5c}$	3.79	-77	0.39	-51	0.01	n.a.
$C_{16:0}$	6.17	-46	1.93	-32	0.60	-31
10Me-$C_{16:0}$	0.45	-39	0.21	-33	0.02	n.a.
i-$C_{17:0}$	0.12	n.d.	0.06	-39	0.04	n.a.
ai-$C_{17:0}$	0.09	n.d.	0.07	-37	0.04	n.a.
cy-$C_{17:0}$	0.24	-97	0.27	-59	n.a.	n.a.
$C_{17:0}$	0.16	-36	0.08	-29	0.04	n.a.
isoprenFA-$C_{19:0}$	0.38	-34	0.13	-27	n.a.	n.a.
$C_{18:1\omega9c}$	0.91	-37	0.65	-26	0.27	n.a.
$C_{18:1\omega7c}$	6.65	-41	1.60	-45	0.06	-32
$C_{18:0}$	0.35	-37	0.32	-30	0.35	-34
Diplopterol	0.43	-84	n.d.	n.d.	n.a.	n.a.
archaeol	0.05	-115	0.05	-86	0.47	-61
sn2-hydro.arch	0.16	-117	0.31	-90	0.08	-80

Characteristic lipids for seep associated sulfate reducing proteobacteria are $C_{16:1\omega5c}$, cy-$C_{17:0\omega5,6}$, (Blumenberg et al., 2004; Elvert et al., 2003; Niemann et al., 2005, Niemann and

Elvert in press). We found a biomarker pattern with a clear dominance of $C_{16:1\omega5c}$ relative to $iC_{15:0}$ previously identified as a typical signature for populations of sulfate reducers associated to ANME-2 and -3 dominated communities (Niemann and Elvert, in press). Specifically the $\delta^{13}C$-value of $C_{16:1\omega5c}$, of -77‰ strongly indicates an incorporation of methane-derived carbon. Previously, Niemann et al. (2006) reported an association of *Desulfobulbus* related bacteria to ANME-3. We did not find the typical biomarker $C_{17:1\omega6}$ of this group, nor the related sequences in the Gullfaks sediments (Lösekann et al., 2007). Instead, substantial amounts of the ^{13}C-depleted FA cy-$C_{17:0\omega5,6}$ (-97‰,) provides evidence for a dominance of SRB of the Seep-SRB1 cluster associated with ANME-2 (Elvert et al., 2003; Niemann et al., 2006, Niemann and Elvert in press). The fatty acids i*so*- and *anteiso*-$C_{15:0}$ were less depleted with isotope compositions of -41 to -42‰. From the difference in the carbon isotope composition of these two groups of sulfate reducing bacteria, it appears likely that a diverse community of sulfate reducers is present in the Gullfaks sediments, of which only some are coupled to the anaerobic methanotrophs.

Several clades of ANME produce ^{13}C-depleted archaeol and *sn2*-hydroxyarchaeol which are used as biomarkers for AOM (Orphan et al., 2001a; Michaelis et al., 2002; Blumenberg et al., 2004). At Gullfaks, *sn2*-hydroxyarchaeol was 2.2-2.9 fold more abundant than archaeol, indicating the dominance of ANME-2 populations (Blumenberg et al., 2004; Niemann and Elvert, in press). Both compounds were highly depleted in ^{13}C, with $\delta^{13}C$-values of -115‰ and -117‰. Assuming that methane was the sole carbon source ($\delta^{13}C$-methane ~-70‰ (Judd and Hovland, 2007) for the synthesis of archaeol and sn2-hydroxyarchaeol, this is equivalent to a ^{13}C-fractionation of -45 to -47‰. This is in the range of isotope fractionation factors previously reported for anaerobic methanotrophic archaea from different marine settings (Hinrichs and Boetius, 2002; Niemann and Elvert, in press).

Microbial diversity and community composition based on 16S rRNA gene analyses. The bacterial 16S rRNA gene library from the gassy sands at Gullfaks comprised 69 gene sequences and was clearly dominated by microorganisms associated with seep ecosystems. We found different groups of sulfate-reducing bacteria and thiotrophic as well as methylotrophic bacteria (Tab. 3, Fig. 4). 15 sequences belonged to relatives of sulfur-oxidizing symbiotic *Gammaproteobacteria* usually hosted by different marine worms or mussels. No closely related sequences of filamentous sulfide oxidizers were found, although these were visually present in the sample from which DNA was extracted. Sequences related to methylotrophic bacteria (*Methylomonas* and *Methylophaga* relatives) were also abundant (6 sequences) matching our results from the biomarker studies. We found 10 sequences of *Deltaproteobacteria*, which were all related to SRB of the *Desulfosarcina/Desulfococcus* group (Seep-SRB-1), which is the partner of ANME-1 and -2 (Knittel et al., 2003). 19 clones were related to the cluster of *Cytophaga – Flavobacterium – Bacteriodetes* (CFB) which is a diverse group of bacteria commonly found in the plankton and sediment involved in the degradation of complex organic matter, but which also occur at cold seep ecosystems (Knittel et al., 2003).

The archaeal 16S rRNA sequence library was strongly dominated by *Euryarchaeota* sequences. Sixty three of 69 analyzed clones were affiliated with ANME-2a (Tab. 3, Fig. 5), the most common group of anaerobic methane oxidizers in gas hydrate bearing environments (Knittel et al., 2005). The ANME-2a clones were highly similar to sequences from other seep sites (Fig. 5), e.g. the Santa Barbara Basin Orphan et al., 2001a), Hydrate Ridge (Knittel et al., 2005), the Gulf of Mexico seeps (Mills et al., 2003), and a Pacific carbon dioxide seep (Inagaki et al., 2006). Sequences related to other groups performing AOM, such as ANME-1, -2c, -3, were not found in our library. The only other euryarchaeotal sequence belonged to the Marine Benthic Group D.

Table 3. Overview of bacterial and archaeal 16S rRNA gene libraries and number of clones retrieved for the individual phylogenetic groups. Gullfaks (HE208, MUC766, 0-10 cm); Tommeliten (Bacterial patch 4-6cm, AL267 1274 K3-2) and Tommeliten deep SMTZ sulfate methane transition zone (HE180 1904, 160cm, Niemann et al., 2005).

		Gullfaks	Tommeliten (bact. patch)	Tommeliten (deep SMTZ)
Bacteria				
Alphaproteobacteria		1	1	
Gammaproteobacteria	*Methylomonas*	4		
	Methylophaga	2		
	Rel. of sulfur-oxidizing symbionts	15		
	Thioalkalivibrio, Thioploca rel.		18	
	Oceanospirillales		1	
Deltaproteobacteria	Seep-SRB1	1	3	51
	Seep-SRB2			3
	Seep-SRB3	3		
	Seep-SRB4	1		
	Desulfobacterium aniline rel.	4	3	4
	Desulfobacterium rel.		1	2
	Desulfobulbus rel.	1	29	
	Myxobacteriales rel.		12	
	Desulfuromonas rel.	1		
Epsilonproteobacteria		3	2	
Spirochaeta			1	
Cytophaga/Flavobacterium/Bacteriodetes		19	4	3
Planctomycetales			3	4
Verrucomicrobia	*Victivalliaceae*	3	1	36
Nitrospira			1	6
Holophaga/Acidobacterium		4	10	
Nitrospina		2		
OP11		1		
Actinobacteria		2	7	
Firmicutes	*Desulfotomaculum* rel.	3	1	
Thermomicrobia			4	
Cyanobacteria			1	
unaffiliated		4	5	3
	Total bacterial clones analyzed	**69**	**107**	**117**
Archaea				
Euryarchaeota	ANME-1		17	16
	ANME-2a	63		
	ANME-2c		83	
	Marine Benthic Group D	1		1
	Methanococcoides		1	
Crenarchaeota	Marine Benthic Group B	3	1	
	Marine Group 1	2		
	Total archaeal clones analyzed	**69**	**102**	**17**

Table 4. In situ quantification of different microbial groups using CARD-FISH. The specific probes used for CARD-FISH are listed in Materials & Methods.
n.a., not analyzed; * present, but not countable

Depth (cm)	Total single cell counts (TCC) (10^9 cm^{-1})	*Bacteria* (single cells)		*Archaea* (single cells)					Aggregates		
		Bacteria % of TCC	*DSS* %. TCC	Archaea % TCC	ANME-1 % TCC	ANME-2a % TCC	ANME-2c % TCC	ANME 3 % TCC	ANME-2a-aggregates (10^6 cm^{-3})	ANME-2c-aggregates (10^6 cm^{-3})	ANME 3-aggregates (10^6 cm^{-3})
Gullfaks 766											
0-10	7.9	46	8	12	<0.1	2	2	0	5.6	8.4	n.a.
10-20	3.0	37	12	10	<0.1	1	2	0	4.8	4.8	n.a.
20-30	0.85	39	7	n.a.	0.5	0	<1	0	0	0	n.a.
Gullfaks 771											
0-10	6.7	50	18	8	<0.1	<1	<1	0	6.6	0.4	n.a.
10-20	3.6	26	29	8	<0.1	0.9	<0.5	0	0.8	0.4	n.a.
20-30	1.3	29	31	16	<0.1	0	0	0	0.6	0	n.a.
Tommeliten 1274-K1											
0-3	4	69	25	10	<1	<1	2	<0.5	1.0	0	0
Tommeliten 1274-K2											
0-3	7	74	20	4	1	2	2	1	1.1	0	0
Tommeliten 1274-K3											
0-3	6	71	28	13	2	1	0	1	1.6	0	*
3-6	4	80	28	7	n.a.	<0.1	0	5	2.7	0	*
6-10	3	91	34	4	4	<0.1	0	2	0.9	0	*

Fig. 4. Phylogenetic tree showing the affiliations of bacterial 16S rRNA clone sequences from Gullfaks and Tommeliten to selected references of the Deltaproteobacteria. The tree was calculated on a subset of nearly full length sequences by maximum-likelihood analysis in combination with filters, which considered only 50% conserved regions of the 16S rRNA of *δ*-proteobacteria to exclude the influence of highly variable positions. Partial sequences were inserted into the reconstructed tree by using parsimony criteria with global-local optimization, without allowing changes in the overall tree topology. Probe specificity is indicated by brackets. The bar represents 10% estimated sequence divergence. Sequences from this study are written in bold.

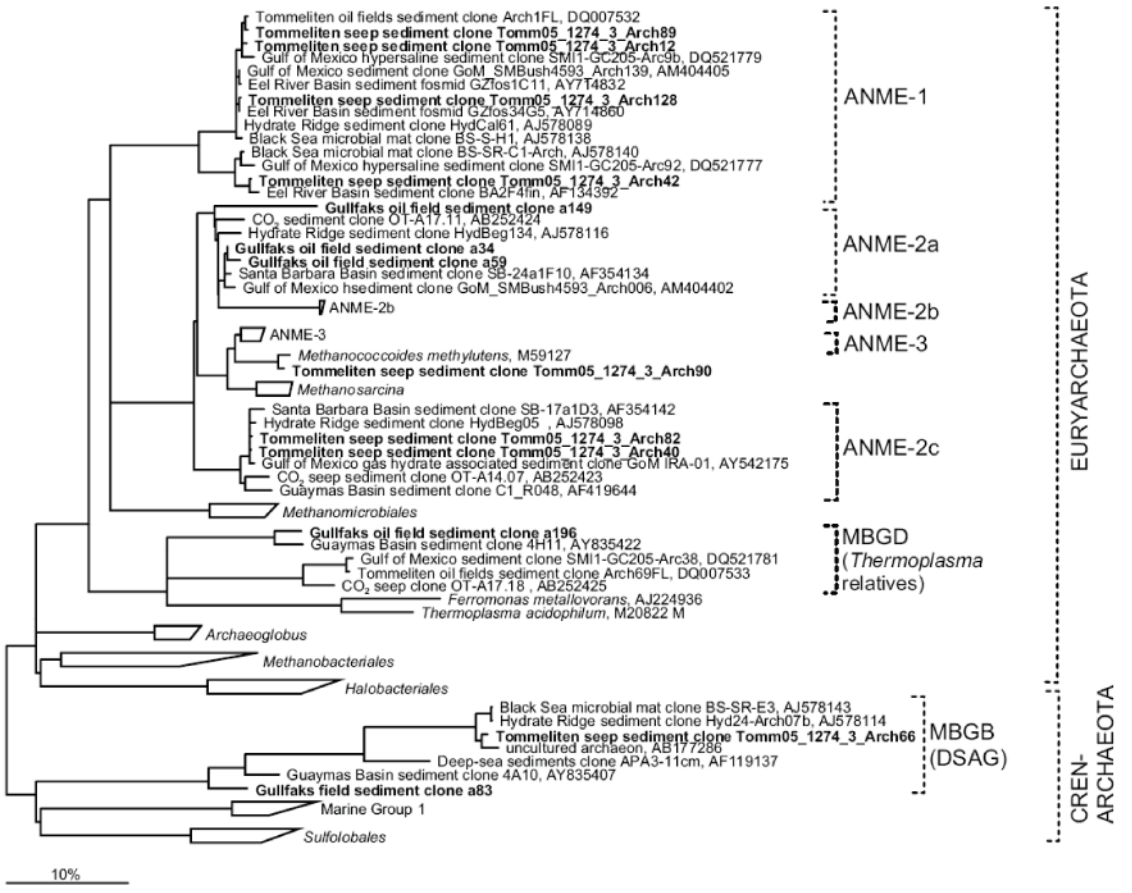

Fig. 5. Phylogenetic tree showing the affiliations of archaeal 16S rRNA gene sequences retrieved from sediments underlying the microbial mats of Gullfaks and Tommeliten to selected references of the domain *Archaea*. Besides cultivated organisms, at least one representative per phylogenetic group of all previously published clone sequences from methane-rich sites is included as references. The tree was constructed by using maximum-likelihood analysis in combination with filters excluding highly variable positions. Partial sequences were inserted into the reconstructed tree by using parsimony criteria with global-local optimization, without allowing changes in the overall tree topology. Probe specificity is indicated by brackets. The bar represents 10% estimated sequence divergence. Sequences from this study are written in bold.

Five clones belonged to the phylum *Crenarchaeota* including three clones of the Marine Benthic Group B which is regularly found at seeps (Knittel et al., 2005). The biogeochemical function of members of both archaeal marine benthic groups has not been identified yet. A study based on isotopic composition of lipid biomarkers suggests that subsurface *Crenarchaeota* of the Marine Benthic Group B could be heterotrophic, although they are commonly associated with methane sulfate transition zones (Biddle et al., 2006).

Total cell numbers in surface sediments at Gullfaks were quite high with 6.7×10^{9} and 7.9×10^{9} single cells ml^{-1} sediment in the uppermost 10 cm at station 771 and 766,

respectively (Tab. 4). These cell numbers are higher than previously reported for non-seep sandy sediments (Llobet-Brossa et al., 1998; Wieringa et al., 2000; Rusch et al., 2001, 2003) and underline the stimulating effect of methane seepage on the microbial community inhabiting sands. The only other published cell counts from methane percolated sands showed numbers similar to those found at Gullfaks (Ishii et al., 2004). At a depth of 20-30 cm cell numbers decreased considerably to 0.85×10^9 and 1.3×10^9 cells ml^{-1}, indicating that the peak of microbial activity is in the upper 10 cm. The ratio of bacterial and archaeal cells were quantified by CARD-FISH. *Bacteria* comprised 29-50% and Archaea 8-16% of the DAPI stained cells. We found relatively high numbers of ANME 2a- and ANME 2c- cell aggregates; however, they did not reach the size and abundance of deep sea sites such as Hydrate Ridge (Nauhaus et al., 2002, 2007; Knittel et al., 2005). ANME-2a cells were clustering in dense, typically spherical aggregates associated with sulfate reducing bacteria in diameters of up to 10 μm (Fig. 6). ANME-2c cells were detected in less dense, small aggregates. The highest counts of ANME aggregates (8.4×10^6 ANME-2c aggregates at station 766, 6.6×10^6 ANME-2a-aggregates at 771) and ANME single cells (1.6×10^8 cells ml^{-1}) were also found in the top 10 cm, matching the distribution of total cell numbers. The abundance of methanotrophic microorganisms strongly decreased with depth, providing evidence for a near surface peak of AOM activity. This may indicate that the highest availability of both methane and sulfate as main energy source is generally found in the surface sediment horizon. Interestingly, this horizon is likely to experience temporary flushing with oxygenated bottom waters, as indicated by the observed ripple structures on the seafloor, which are presumably caused by high bottom water currents. This should have a negative effect on the anaerobic microbial communities. However, it is possible that the high upward advection of gas may restrict the downward diffusion of oxygenated bottom waters at the seep site.

The Gullfaks seep is the first site at which abundant single cells of ANME-2a and ANME-2c were detected. Previously, only ANME-1 (Orphan et al., 2002; Niemann et al., 2005) and ANME-3 (Lösekann et al., 2007) were found as single cells. Monospecific ANME-2a or ANME-2c aggregates have been previously reported from Eckernförder Bay (Treude et al, 2005) and Eel River Basin (Orphan et al., 2002). ANME-2 cells aggregates without bacterial partner were also found to dominate specific regions of a microbial mat from the Black Sea (Treude et al., 2007).

Tommeliten seeps

Seafloor observations. The Tommeliten seep site lies at 75 m water depth and is part of the greater Ekofisk area of the Norwegian Block 1/9. In this area, the sedimentary rocks host a now exploited hydrocarbon reservoir. This reservoir is pierced by a salt diapir at about 1 km depth below the sea floor, and disturbances on seismic profiles indicate that free gas migrates to the sea floor (Hovland and Judd, 1988; Hovland, 2002). Within an area of 0.5 km^2 at the Tommeliten site we observed several gas flares, which extended from the seafloor to the sea surface (Fig. 2f). Many of these flares were concentrated in a small area of about 0.06 km^2, which we assume to be the centre of the subsurface gas escape pathway. The distribution of the gas flares in 2005 (AL 267) was similar to the observations made in 2002 (HE180; Niemann et al., 2005). Previous expeditions to the Tommeliten seepage area used remotely operated vehicles (ROVs) for bottom observations and sampling, and documented streams of single methane bubbles of thermogenic origin ($\delta^{13}C$ -45.6‰), small patches of microbial mats and methane derived authigenic carbonates outcropping at the sea floor (Hovland and Sommerville, 1985; Hovland and Judd, 1988; Trasher et al., 1996; Niemann et al., 2005).

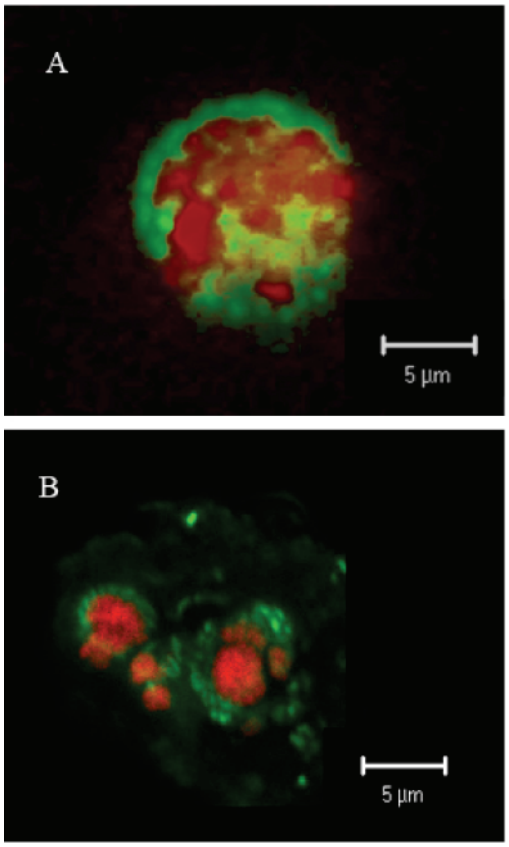

Fig. 6: Dual hybridizations with fluorescently labeled rRNA targeting oligonucleotide probes. a) Consortia of ANME 2a/DSS from Gullfaks (probes ANME2a-647 [red] and DSS658 [green]); b) Consortia of ANME2c/DSS from Tommeliten (probes ANME2c-622 [red] and DSS-658 [green])

.

Our observations by ROV in 2005 confirmed that most gas flares originated from small holes (1-5 cm diameter) in the seafloor emitting single streams of bubbles. They were associated with small patches of bacterial mats (30-50 cm in diameter, Fig. 3b). We sampled three cores from such bacterial mats. The recovered sediments below the mats were highly gassy and consisted of fine sands. In contrast, sediments a few meters away from the gas flares consisted of consolidated, hardly permeable marls enclosing layers of unsorted silt and sands above carbonate cements (Niemann et al., 2005). Accordingly, gas migration from the subsurface to the seabed may be limited to sandy horizons between the impermeable clays, and the gas bubbling may further erode the fine grain fraction. Upon recovery, the cores from these sediments continued to release methane gas as verified by GC measurements. This indicates a high in situ gas pressure in the seabed. At a water depth of 75 m the equilibrium methane concentrations in the interstitial waters in the direct vicinity of the gas ebullition sites could be around 12 mM.

Methane and sulfate turnover. The sample from a black patch from which gas escape was observed was composed of silty sediments, and contained little TOC with 0.22 to 0.32% w/w. Due to the small amount of recovered sediment, we could not measure AOM and SR rates. It can be assumed that the rates are locally very high due to the saturation with gas and the flux of sulfate from overlying bottom water into the bubble sites. However, for the subsurface sulfate methane transitions zones (SMTZ) associated with the gas-migration pathways at Tommeliten, Niemann et al. (2005) showed low AOM and SR rates of a few nmol cm^{-3} d^{-1}, resulting in ca 50 g CH4 m^{-2} yr^{-1}, or 0.3 t yr^{-1} for the whole seep area of ca. 6500 m^2. In comparison, the gas ebullition from the same site was estimated with 47 g CH_4 m^{-2} yr^{-1} (Hovland et al., 1993) suggesting a 50% efficiency of the microbial filter against methane. The higher methane consumption efficiency compared to Gullfaks could be due to the impermeable nature of the Tommeliten sediments allowing only for few gas leakage pathways.

Biomarker and carbon isotope composition. Similar to surface sediments at Gullfaks, monounsaturated fatty acids were also the dominant biomarker fraction in the surface sediments from the Tommeliten gas vents (Tab. 2). However, concentrations of these and other bacteria lipids were lower in comparison to the mat-covered sands at Gullfaks. The fatty acid distribution suggests the presence of sulfide oxidizing bacteria, but the ratio of $C_{16:1\omega7c}$ to $C_{18:1\omega7c}$ to $C_{16:0}$ (42: 32: 26) indicated a lower contribution to total bacterial biomass than in the Gullfaks sands.

In comparison to the Gullfaks seeps, the sediments from the Tommeliten gas vents contained similar amounts of archaeol but even more *sn2*-hydroxyarchaeol, indicating the dominance of ANME-2 populations. Both archaeol and *sn2*-hydroxyarchaeol were less depleted than at Gullfaks with δ^{13}C-values of -86‰ and -90‰. However, considering the heavier isotope composition of the source methane at Tommeliten ($\delta^{13}C$ CH_4 -46‰

Hovland 2002), a stable carbon isotope fractionation of the lipid biomass relative to source methane of more than 40‰ typical for AOM and comparable to that at Gullfaks was found.

The specific biomarkers for SRB associated with AOM such as $C_{16:1\omega5c}$, i-$C_{15:0}$, and cy-$C_{17:0}$ were similar in ratio but less abundant than at Gullfaks. The δ^{13}C-values of $C_{16:1\omega5c}$ and cy-$C_{17:0}$ were relatively depleted with -51‰ and -59‰, respectively, whereas *ai*-$C_{15:0}$ showed a substantially less depleted δ^{13}C-value of -34‰. Interestingly, the surface seep sediments at Tommeliten resembled the biomarker signatures in the authigenic carbonate outcrops, but differed substantially from subsurface sediments (Niemann et al., 2005). In the deep SMTZ Niemann et al. (2005) found a dominance of ANME-1 communities as indicated by the dominance of archaeol over hydroxyarchaeol and a typical fatty acid pattern typical for sulfate reducing partners of ANME-1 (Blumenberg et al. 2004; Niemann and Elvert, in press; Niemann et al., 2005). These results correlated with 16S rRNA and FISH analyses (Niemann et al., 2005). We conclude that ANME-1 could be better adapted to the low energy conditions characteristic for deep sulfate methane transition zones, whereas ANME-2 dominates the advection driven highly active surface zones as already observed in some deep water seeps (Knittel et al., 2005).

Microbial diversity and community composition based on 16S rRNA gene analyses.

Similar to Gullfaks, the bacterial 16S rRNA gene sequence library (Tab. 3.) and the selected sequences in the phylogenetic tree (Fig. 4) obtained from sediments of Tommeliten showed a relatively high diversity of sulfate reducing bacteria including relatives of the uncultured Seep-SRB1 (ANME-2 partners), Seep-SRB2 (Eel2), and of *Desulfobacterium anilini*. Seep-SRB2 organisms have been retrieved from nearly all seep sediments (e.g. Knittel et al., 2003; Lösekann et al., 2007; Mills et al., 2005; Orphan et al., 2001b). However, their function remains unknown since no isolates are available. *Desulfobacterium anilini* relatives have been shown to oxidize different aromatic hydrocarbons such as naphthalene or xylene and could

also have a function in hydrocarbon degradation at this site (for an overview see Widdel et al., 2007). Also a high number of clones related to *Desulfobulbus* were found. In cold seep sediments of the Haakon Mosby mud volcano (Barents Sea), the SRB community was dominated by such relatives of *Desulfobulbus,* which formed aggregates with anaerobic methanotrophs of the ANME-3 cluster (Niemann et al., 2006, Lösekann et al., 2007). Single ANME-3 cells and a few aggregates occurred in the Tommeliten cold seep sediments, but their abundance was too low to analyze the potential bacterial partners.

As predicted by the biomarker signatures, the archaeal gene sequence library of Tommeliten contained mainly relatives of ANME-1 and ANME-2c (Fig. 5). These were most closely related to sequences retrieved from gas hydrate bearing sediments such as the Eel River Basin, Hydrate Ridge, and the Gulf of Mexico. ANME-2a sequences were not represented in the clone library, although ANME-2a aggregates were detected by CARD-FISH (see below).

Cell counts in the cores 1274 K1 to K3 ranged from 4.1 to 5.8×10^{9} cells ml^{-1} in the top layer of sediment and hence were comparable to the cell numbers at Gullfaks. With depth, cell numbers decreased to 3.0×10^{9} cell ml^{-1} (6-10cm). Quantification with CARD-FISH indicated the presence of ANME-2a, ANME-2c and low numbers of ANME-3 single cells and aggregates. Sulfate reducing bacteria were highly abundant comprising up to one third of total single cells.

Hence, the microbial communities in gassy sediments below thiotrophic mats of both seep sites in the North Sea were dominated by anaerobic methanotrophs of the ANME-2 cluster, and their partner sulfate reducing bacteria. At Tommeliten, the AOM community in the mat covered surface sediments resembled more that of the Gullfaks seep and of the Tommeliten authigenic carbonates than that of the subsurface SMTZ community (Niemann et al., 2005). The microbial communities also comprised sequences from other microorganisms typically occurring in methane rich deep-water seep ecosystems such as the

crenarchaeotal Marine Benthic Group B. Gene libraries and CARD-FISH counts indicated also differences between Gullfaks and Tommeliten AOM communities – such as the dominance of the ANME 2a cluster in Gullfaks cold seep sediments and the dominance of ANME 2c at Tommeliten, which may be a result of the different geological and hydrological features of both sites.

The North Sea seeps in comparison to deep water cold seeps

At the shallow water seeps of the Northern North Sea, methane emission from deep reservoirs has been observed for more than 20 years. The habitats investigated here are characterized by locally high advection of gaseous methane and ebullition of gas bubbles to the hydrosphere. Both Tommeliten and Gullfaks likely contribute to methane emission to the atmosphere as indicated by gas flares reaching to the upper mixed water layers. It would be an important future task to attempt quantification of gas emission to the atmosphere by monitoring flares and methane concentrations in the flares and the overlying surface waters.

In the interstitial porewaters of the Tommeliten and Gullfaks seeps, dissolved methane can reach concentrations of 12 mM and 25 mM, respectively. The highly permeable sands at Gullfaks allow the migration of gas bubbles through the sediment, leading to a relatively large seep area populated by methanotrophs and thiotrophs. The high upward advection of gas may restrict the downward diffusion of oxygenated bottom waters below a few cm, protecting the anaerobic methanotrophic communities, which showed very high activities in the top 10 cm. In contrast, the compact silty sands of Tommeliten allow gas migration only through small cracks in the seafloor sediments, restricting the distribution of methanotrophs and thiotrophs to small patches around the gas vents. Phylogenetic analysis of *Deltaproteobacteria* and *Euryarchaeota* at Gullfaks and Tommeliten indicate a high similarity of these to sequences from deep water seep sites, such as Hydrate Ridge (Boetius

et al., 2000; Knittel et al., 2003; Knittel et al., 2005), Eel River Basin (Orphan et al., 2001b) or Guyamas Basin (Teske et al., 2002).

The concentrations of most of the AOM specific biomarker were about one order of magnitude lower at the investigated sites compared to deep water seep ecosystems such as Hydrate Ridge (Elvert et al., 2003) and Haakon Mosby Mud Volcano (Niemann et al., 2006). This finding was reflected in the low number of ANME aggregates of around 10^6 cm^{-3}. In deep water cold seeps such as Hydrate Ridge, Haakon Mosby and Eel River Basin, ANME aggregate numbers reach 10^8, comprising a large fraction of the total microbial biomass (>90%) (Knittel et al., 2005). A reason for this difference in ANME biomass could be the higher availability of dissolved methane in deep water seeps due to the increased solubility of methane at high hydrostatic pressures. Previous experiments indicate that AOM rates and the energy yield available for growth are higher at elevated methane concentrations (Nauhaus et al., 2002; 2007), which may support higher biomasses of AOM consortia. A second explanation for lower aggregate sizes in the North Sea may be the high bottom water currents causing relocation of particles, as well as sporadic oxygen influx into the sandy sediments. The only shallow water seeps known with higher biomass of ANME are the conspicuous methanotrophic microbial reefs of the Black Sea (Blumenberg et al., 2004; Treude et al., 2005). These lie in permanently anoxic waters and are hence protected from grazing.

4. Conclusions

Several gas seepages were mapped during the METROL cruises with R/V Heincke and Alkor. From these, we chose the two most active and accessible seepage areas, Tommeliten and Gullfaks, for detailed surveys using geochemical and molecular tools. At Gullfaks a seepage area of about 0.1 km^2 was characterized by a high density of gas vents and extensive coverage by thiotrophic bacterial mats. At Tommeliten gas vents were less

dense and the bacterial mat covered areas were limited to small patches around small holes in the seafloor emitting gas. The different permeability of the seabed at both seep sites could explain the observed differences in the efficiency of the microbial filter against methane. From both sites considerable amounts of methane are emitted, some of which may reach the atmosphere as indicated by large gas flares reaching the upper mixed water layers. Specific biomarker and carbon isotope compositions, as well as 16S rDNA gene sequences and fluorescence in situ hybridization of specific microbial groups indicated that the bacterial mat covered sediments were populated by active communities of ANME-2 and their sulfate reducing partner bacteria. Archaeal biomarkers were about 40 to 50‰ depleted in ^{13}C relative to the carbon source methane. Specific fatty acids of sulfate reducers involved in AOM were also considerably depleted in ^{13}C indicating that they partially assimilate methane derived carbon. The 16S rRNA based gene libraries of both sites mostly included sequences from known groups of deep water cold seep microorganisms, indicating that water depth or other oceanographic conditions may not be limiting the dispersal of these groups. In contrast, distinct differences were found between the microbial community in the mat covered surface sediments (dominated by ANME-2) to those of the subsurface sulfate methane transition zone at Tommeliten (dominated by ANME-1), suggesting that different energy availabilities may select for different methanotrophic communities.

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References

Amann, R.I., Krumholz, L. and Stahl, D.A.: Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol., 172(2), 762-770, 1990.

Biddle, J.F., Lipp, J.S., Lever, M.A., Lloyd, K.G., Sørensen, K.B., Anderson, R., Fredricks, H.F., Elvert, M., Kelly, T.J., Schrag, D.P., Sogin, M.L., Brenchley, J.E., Teske, A., House, C.H., and Hinrichs, K.U.: Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. Proc. Natl. Acad. Sci., 103(10), 3846-3851, 2006.

Blumenberg, M., Seifert, R., Reitner, J., Pape, T. and Michaelis, W.: Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. Proc. Natl. Acad. Sci., 101(30), 11111-11116, 2004.

Boetius, A. Ravenschlag, K., Schubert, C.J., Rickert, D. Widdel, F., Gieseke, A., Amann, R., Jørgensen, B.B., Witte, U. and Pfannkuche, O.: A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature, 407, 623-626, 2000.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H. and Wagner, M.: The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol., 22(3), 434-444, 1999.

Dando, P.R., Bussmann, I., Niven, S.J., O'Hara, S.C.M, Schmaljohann, R. and Taylor, L.J.: A methane seep area in the Skagerrak, the habitat of the pogonophore Siboglinum poseidoni and the bivalve mollusc Thyasira sarsi. Mar. Ecol. Prog. Ser., 107. 157–167, 1994.

Eisma, D. and Kalf, J.: Dispersal, concentration and deposition of suspended matter in the North Sea. J. Geol. Soc. London, 144(1), 161-178, 1987.

Elvert, M., Boetius, A., Knittel, K. and Jørgensen, B.B.: Characterization of specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. Geomicrobiol. J., 20(4), 403-419, 2003.

Elvert, M., Hopmans, E. C., Treude, T., Boetius, A. and Suess, E.: Spatial variations of methanotrophic consortia at cold methane seeps: implications from a high-resolution molecular and isotopic approach. Geobiology, 3(3), 195-209, 2005.

Fang, J., Shizuka, A., Kato, C. and Schouten, S.: Microbial diversity of cold-seep sediments in Sagami Bay, Japan, as determined by 16S rRNA gene and lipid analyses. FEMS Microbiol. Ecol., 57(3), 429–441, 2005.

Hinrichs, K.-U., Hayes, J.M., Silva, S.P., Brewer, P.G. and DeLong, E.F.: Methane-consuming archaebacteria in marine sediments. Nature, 398, 802-805, 1999.

Hinrichs, K.-U. and Boetius, A.: The anaerobic oxidation of methane: New insights in microbial ecology and biogeochemistry. In: Wefer, G., Billett, D. and Hebbeln, D. (eds.), Ocean Margin Systems. Springer-Verlag, Berlin Heidelberg, pp. 457-477, 2002.

Hovland, M.: On the self-sealing nature of marine seeps. Cont. Shelf. Res., 22(16), 2387-2394, 2002.

Hovland, M.: Discovery of prolific natural methane seeps at Gullfaks, northern North Sea. Geo-Mar. Lett., 27(2), 197-201, 2007.

Hovland, M., Gardner, J.V. and Judd, A.G.: The significance of pockmarks to understanding fluid flow processes and geohazards. Geofluids, 2(2), 127-136, 2002.

Hovland, M. and Judd, A.G.: Seabed pockmarks and seepages. Graham and Trotham, London, 293 pp., 1988.

Hovland, M., Judd, A.G., Burke Jr., R.A.,. The global flux of methane from shallow submarine sediments. Chemosphere, 26(1-4), 559-578, 1993.

Hovland, M. and Risk, M.: Do Norwegian deep-water coral reefs rely on seeping fluids? Mar. Geol., 198(1-2), 83-96, 2003.

Hovland, M. and Sommerville, J.H.: Characteristics of two natural gas seepages in the North Sea. Mar. Petrol. Geol., (2): 319-326, 1985.

Inagaki, F., Kuypers, M. M. M., Tsunogai, U., Ishibashi, J., Nakamura, K. Treude, T., Ohkubo, S., Nakaseama, M., Gena, K., Chiba, H., Hirayama, H., Nunoura, T., Takai, K., Jørgensen, B. B., Horikoshi, K. and Boetius, A.: Microbial community in a sediment-hosted CO2 lake of the southern Okinawa Trough hydrothermal system. Proc. Natl. Acad. Sci., 103(38), 14164-14169, 2006.

Ishii, K., Mussmann, M., MacGregor, B.J. and Amann, R.: An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. FEMS Microbiol. Ecol., 50(3), 203-212, 2004

Jørgensen, B.B. and Fenchel, T.: The sulfur cycle of a marine sediment model system. Mar. Biol., 24(3), 189-201., 1974.

Joye, S.B., Boetius, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J., Lugo S.: The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. Chem. Geol., 205, 219-238, 2004.

Judd, A.G.: A global importance and context of methane escape from the seabed. Geo-Mar. Lett., 23, 147-154, 2003.

Judd, A. and Hovland, M.: Seabed Fluid Flow: The impact on geology, biology and the marine environment. Cambridge University Press, 492 pp., 2007.

Kallmeyer, J., Ferdelman, T.G., Weber, A., Fossing, H. and Jørgensen, B.B.: A cold chromium distillation procedure for radiolabeled sulfide applied to sulfate reduction measurements. Limnol. Oceanogr. Meth., 2, 171-180, 2004.

Kane, M.D., Poulsen, L.K. and Stahl, D.A.: Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microb., 59, 682-686, 1993.

Knittel, K., Boetius, A., Lemke, A., Eilers, H., Lochte, K., Pfannkuche, O., Linke, P. and Amann, R.: Activity, distribution, and diversity of sulfate reducers and other bacteria

in sediments above gas hydrate (Cascadia margin, Oregon). Geomicrobiol. J., 20(4), 269-294, 2003.

Knittel, K., Lösekann, T., Boetius, A., Kort, R. and Amann, R.: Diversity and distribution of methanotrophic archaea at cold seeps. Appl. Environ. Microb., 71(1), 467-479, 2005.

Lane, D.J. Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. and Pace, N. R.: Rapid Determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci., 82(20), 6955-6959, 1985.

Levin, L.A.: Ecology of cold seep sediments: Interactions of fauna with flow, chemistry and microbes. In: R.N. Gibson, R.J.A. Atkinson and J.D.M. Gordon (eds.), Oceanography and Marine Biology: An Annual Review. Taylor & Francis. 1-46, 2005.

Llobet-Brossa, E., Rossello-Mora, R. and Amann, R.: Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microb., 64(7) 2691-2696, 1998.

Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B. Niemann, H., Boetius, A. and Amann, R.: Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. Appl. Environ. Microb., 73(10), 3348-3362, 2007.

Ludwig, W. Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.-H.: ARB: A software environment for sequence data. Nucl. Acids Res., 32(4), 1363-1371, 2004.

Luff, R. and Wallmann, K.: Fluid flow, methane fluxes, carbonate precipitation and biogeochemical turnover in gas hydrate-bearing sediments at Hydrate Ridge, Cascadia Margin: Numerical modeling and mass balances. Geochim. Cosmochim. Ac., 67(18), 3403-3421, 2003.

Manz, W., Eisenbrecher, M., Neu, T.R. and Szewzyk, U.: Abundance and spatial organization of gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol., 25, 43-61, 1998.

Massana, R., Murray, A., Preston, C. and DeLong, E.: Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. Appl. Environ. Microb., 63(1), 50-56, 1997.

Meyer-Reil, L.-A.: Benthic response to sedimentation events during autumn to spring at a shallow water station in the Western Kiel Bight. Mar. Biol., 77(3), 247-256, 1983.

Mills, H.J., Hodges, C., Wilson, K., MacDonald, I.R. and Sobecky, P.A.: Microbial diversity in sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico. FEMS Microbiol. Ecol., 46(1), 39-52, 2003.

Mills, H.J., Martinez, R.J., Story, S. and Sobecky, P.A.: Characterization of microbial community structure in Gulf of Mexico gas hydrates: Comparative analysis of DNA- and RNA-derived clone libraries. Appl. Environ. Microb., 71(6): 3235-3247, 2005.

Moss, C.W. and Lambert-Fair, M.A.: Location of double bonds in monounsaturated fatty acids of Campylobacter cryaerophila with dimethyl disulfide derivatives and

combined gas chromatography-mass spectrometry. J. Clin. Microbiol., 27(7), 1467-1470, 1989.

Muyzer, G., Teske, A., Wirsen, C.O. and Jannasch, H.W.: Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol., 164(3), 165-172, 1995.

Nauhaus, K., Boetius, A., Krüger, M. and Widdel, F.: In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediments from a marine gas hydrate area. Environ. Microbiol., 4(5), 296-305, 2002.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A. and Widdel, F.: In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. Environ. Microbiol., 9(1), 187-196, 2007.

Nelson, D.C. and Jannasch, H.W.: Chemoautotrophic growth of a marine Beggiatoa in sulfide-gradient cultures. Arch. Microbiol., 136(4), 262-269, 1983.

Nelson, D.C., Jørgensen, B.B. and Revsbech, N.P.:. Growth pattern and yield of a chemoautotrophic Beggiatoa sp. in oxygen-sulfide microgradients. Appl. Environ. Microb., 52(2): 225-233, 1986.

Nelson, D.C., Wirsen, C.O. and Jannasch, H.W.: Characterization of large, autotrophic Beggiatoa spp. abundant at hydrothermal vents of the Guaymas Basin. Appl. Environ. Microb., 55(11): 2909-2917, 1989.

Niemann, H. and Elvert, M., Diagnostic lipid biomarker and stable carbon isotope signatures of microbial communities mediating the anaerobic oxidation of methane with sulphate. Chem. Geol., in press: doi: 10.1016/j.orggeochem.2007.11.006.

Niemann, H., Elvert, M., Hovland, M., Orcutt, B., Judd, A., Suck, I., Gutt, J., Joye, S., Damm, E., K. Finster, K. and Boetius, A.: Methane emission and consumption at a North Sea gas seep (Tommeliten area). Biogeosciences, 2(4): 335-351, 2005.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K., Amann, R., Sauter, E. J., Schlüter, M., Klages, M., Foucher, J. P. and Boetius, A.: Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. Nature, 443(7113): 854-858, 2006.

Omoregie, E., Niemann, H., Masterlerz, V. de Lange, G., Stadnitskaia, A., Foucher, J.P. and Boetius: A. Anaerobic oxidation of methane and sulfate reduction at cold seeps in the Eastern Mediterranean sea Mar. Geol., in review.

Orphan, V.J., Hinrichs, K.-U., Ussler, W., Paull, C. K., Taylor, L. T., Sylva, S. P., Hayes, J. M. and Delong, E. F.: Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. Appl. Environ. Microb., 67(4): 1922-1934, 2001a.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F.: Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science, 293(5529): 484-487, 2001b.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F.: Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. Proc. Natl. Acad. Sci., 99(11): 7663-7668. 2002.

Pernthaler, A., Pernthaler, J. and Amann, R.: Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl Environ. Microb., 68(6): 3094-3101, 2002.

Ravenschlag, K., Sahm, K., Pernthaler, J. and Amann, R.: High bacterial diversity in permanently cold marine sediments. Appl. Environ. Microb., 65(9): 3982-3989, 1999.

Reeburgh, W.: Oceanic methane biogeochemistry. Chem. Rev., 107(2): 486-513, 2007.

Rehder, G., Keir, R.S., Suess, E. and Pohlmann, T.: The multiple sources and patterns of methane in North Sea waters. Aquat. Geochem., 4(3): 403-427, 1998.

Rise, L., Saettem, J., Fanavoll, S., Thorsnes, T., Ottesen, D. and Boe, R.: Sea-bed pockmarks related to fluid migration from Mesozoic bedrock strata in the Skagerrak offshore Norway. Mar. Petrol. Geol., 16(7): 619-631, 1999.

Ritger, S., Carson, B. and Suess, E.: Methane-derived authigenic carbonates formed by subduction-induced pore-water expulsion along the Oregon/ Washington margin. Geol. Soc. Am. Bull., 98(2): 147-156, 1987.

Rohmer, M., Bouvier-Nave, P. and Ourisson, G.: Distribution of hopanoid triterpenes in prokaryotes. Microbiology, 30(55): 1137-1150, 1984.

Rusch, A., Forster, S. and Huettel, M.: Bacteria, diatoms and detritus in an intertidal sandflat subject to advective transport across the water-sediment interface. Biogeochemistry, 55(1): 1-27, 2001.

Rusch, A., Huettel, M., Reimers, C.E., Taghon, G.L. and Fuller, C.M.: Activity and distribution of bacterial populations in Middle Atlantic Bight shelf sands. FEMS Microbiol. Ecol., 44(1): 89-100, 2003.

Stahl, D.A. and Amann, R.: Development and application of nucleic acid probes. In: E. Stackebrandt and M. Goodfellow (eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley&Sons Ltd., Chichester, UK, 205-248, 1991.

Teske, A., Hinrichs, K. U., Edgcomb, V., de Vera Gomez, A., Kysela, D., Sylva, S. P., Sogin, M. L. and Jannasch, H. W. Microbial diversity of hydrothermal sediments in the Guaymas Basin: Evidence for anaerobic methanotrophic communities. Appl. Environ. Microb., 68(4): 1994-2007, 2002.

Trasher, J., Fleet, A.J., Hay, S.J., Hovland, M. and Düpenbecker, S.: Understanding geology as the key to using seepage in exploration: spectrum of seepage styles. In: D. Schumacher and M.A. Abrams (eds.), Hydrocarbon migration and its near-surface expression. AAPG Memoir, 223-241, 1996.

Treude, T., Boetius, A., Knittel, K., Nauhaus, K., Elvert, M., Krüger, M., Lösekann, T., Wallmann, K., Jørgensen, B. B., Widdel, F. and Amman, R.: Anaerobic oxidation of methane at Hydrate Ridge (OR). Geochim. Cosmochim. Ac., 67(18): 491-491, 2003.

Treude, T., Knittel, K., Blumenberg, M., Seifert, R. and Boetius, A.: Subsurface microbial methanotrophic mats in the Black Sea. Appl. Environ. Microb., 71(10): 6375-6378, 2005.

Treude, T., Krüger, M., Boetius, A. and Jørgensen, B. B.: Environmental control on anaerobic oxidation of methane in the gassy sediments of Eckernfoerde Bay (German Baltic). Limnol. Oceanogr. 50(6): 1771-1786, 2005.

Valentine, D.L. and Reeburgh, W.S.: New perspectives on anaerobic methane oxidation. Environ. Microbiol., 2(5): 477-487, 2000.

Widdel, F. and Bak, F.: Gram-negative mesophilic sulfate-reducing bacteria. In: T.H. Balows A, Dworkin M, Harder W, Schleifer KH (ed.), The prokaryotes. Springer, Berlin Heidelberg New York, 3352-3378, 1992.

Widdel, F., Musat, F., Knittel, K. and Galushko, A.: Anaerobic degradation of hydrocarbons with sulphate as electron acceptor. In: L.L. Barton and W.A. Hamilton (eds), Sulphate-reducing bacteria: environmental and engineered systems. Cambridge University Press, Cambridge, United Kingdom. 265-304, 2007.

Wieringa, E.B.A., Overmann, J. and Cypionka, H.: Detection of abundant sulphate-reducing bacteria in marine oxic sediment layers by a combined cultivation and molecular approach. Environ. Microbiol., 2(4): 417-427, 2000.

Zhang, C.L. Huang, Z., Cantu, J., Pancost, R. D., Brigmon, R. L., Lyons, T. W. and Sassen, R.: Lipid biomarkers and carbon isotope signatures of a microbial (Beggiatoa) mat associated with gas hydrates in the Gulf of Mexico. Appl. Environ. Microb., 71(4): 2106-2112, 2005.

Chapter III

Short-Term Responses of Anaerobic Methane-Oxidizing Microbial Communities from Submarine Cold Seeps to Variations in Methane and Sulfate

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Abstract: A major role in global methane fluxes has been attributed to the process of anaerobic oxidation of methane, which is performed by consortia of methanotrophic archaea and sulfate reducing bacteria. An important question remains how these very slow growing microorganisms with generation times of 3-7 months respond to natural variations in methane fluxes at cold seeps. Here, we used an experimental flow-through column system filled with cold seep sediments naturally enriched in methanotrophic communities, to test their response to short-term variations in methane and sulfate fluxes. At stable methane and sulfate concentrations of ~2 mM and 28 mM, respectively, we measured constant rates of anaerobic oxidation of methane (AOM) and sulfide production (SR) during up to 160 days of incubation. When percolated with methane free media, the anaerobic methanotrophs ceased to oxidize methane and to produce sulfide. After a starvation phase of 40 days, the addition of methane restored former AOM and SR rates immediately. At methane concentrations between 0-2.3 mM we measured a linear correlation between methane availability, AOM and SR. At constant fluid flow rates of 30 m yr^{-1}, ca. 50% of the methane was consumed by the ANME population. In contrast, reducing the sulfate concentration from 28 to 1 mM, only a 35% decrease in AOM and SR was observed. The marine anaerobic methanotrophs (ANME) appear to be capable to consume substantial amounts of methane rising from the subsurface seabed to the hydrosphere over a wide range of fluxes of methane and sulfate.

1. Introduction

Between 5 to 10% of the organic matter deposited on the seafloor is converted to methane by a sequence of microbial processes in which methane production is the terminal

degradation step (Canfield, 1993, Canfield et al., 2005). The dissolved concentrations of methane in the ocean range from a few nM in seawater to about hundred mM in hydrate-bearing subsurface sediments (Reeburgh, 2007). With about 4×10^{15} m^3 methane, submarine gas hydrates bind more methane than all other reservoirs on earth (Milkov, 2004). However methane emission is rather low, contributing an estimated 3-5% of the atmospheric methane budget. This is due to the consumption of methane by anaerobic and aerobic microorganisms in the seabed which represent an effective filter against this potential greenhouse gas (Reeburgh, 1996; 2007).

The principles and regulation of aerobic methane consumption are well understood (reviews: Hanson and Hanson, 1996; Trotsenko and Khmelenina, 2005). Due to the limited penetration of oxygen into the seabed, AOM is considered the globally more relevant sink for methane in the ocean (Hinrichs and Boetius, 2002; and literature therein). The net reaction for AOM with sulfate is:

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

This process is performed by consortia of methanotrophic archaea and sulfate reducing bacteria (Boetius et al., 2000; Orphan et al., 2001). Unfortunately, the detailed reaction pathways have not been fully understood.

Sedimentary methane oxidation rates range from a few pmol cm^{-3} day^{-1} in diffusion driven deep sulfate methane transition zones (e.g., Blake Ridge; Wellsbury et al., 2000) up to 3 μmol cm^{-3} day^{-1} at cold seeps of Hydrate Ridge (Treude et al., 2003), and up to 10 μmol cm^{-3-} day^{-1} in the methanotrophic microbial mats of the Black Sea (Treude et al., 2007). In vitro incubation studies with enriched ANME communities showed that AOM rates predominantly depend on methane concentrations, and suggested extraordinary high apparent methane half-saturation constants (k_M-values) in the range of several mM (Nauhaus et al., 2002; 2005; 2007).

First budgets for total methane fluxes, including microbial oxidation, at cold seep systems were calculated for Hydrate Ridge (Cascadia margin; Boetius and Suess 2004) and the Haakon Mosby Mud Volcano (Barents Sea; Niemann et al., 2006). At fluid flow rates of 10-250 cm yr^{-1}, microbial methane oxidation removed about 50% of total methane flux in the sulfate penetrated surface sediments. In the transition from high to low fluid flow rates, the efficiency of the microbial filter increased at both sites, until no methane emission from the seabed was measured and all methane was consumed within the seabed (e.g. *Acharax* fields at Hydrate Ridge, *Pogonophora* fields, HMMV). Less is known about the control of AOM by sulfate fluxes. The apparent kinetics of AOM with regard to concentrations of methane and sulfate in the natural range are not yet known, but field data indicate that AOM decreases at sulfate concentrations < 1mM. The most significant problem in constraining budgets of methane emission at cold seeps is the lack of quantitative in situ methods to measure gaseous and dissolved methane emission to the hydrosphere, as well as subsurface transport processes of methane and sulfate. In addition, cold seeps show an extreme spatial and temporal variation in gas ebullition and fluid flow (Tryon et al., 2002; Sauter et al., 2006). It is not known how the slow growing methanotrophs respond to variations in methane and sulfate supply.

Here we used continuous flow-through incubation of sediments from different cold seep ecosystems to test the response of ANME communities to short-term (2-40 days) variations in methane and sulfate fluxes. Flow-through reactors have been used previously for the study of growth patterns of ANME communities (Girguis et al., 2003; 2005). Our main questions were 1) how does the availability of methane and sulfate influence AOM, 2) what is the efficiency of the ANME communities in consuming methane at high fluid flow rates, 3) does the community retain its activity after starvation periods.

2. Material and Methods:

Sediments from the Gullfaks (Heincke seep area, Hovland, 2007) in the northern North Sea (61°10.44' N, 2°14.65' E, 150 m water depth) were sampled on Heincke cruise 208 in May 2004 using TV-guided multicorer. The sandy sediments were widely covered with mats of sulfide oxidizing bacteria which indicate the area of strong methane based sulfate reduction below the mats. The recovered sediments were highly permeable, and consisted of medium to coarse grained sands. For the incubations we sampled the blackish sediment horizon between 2 and 15 cm, omitting the oxic top layer. Methane consumption rates, measured in in vitro incubations (using ^{14}C-labeled methane according to Krüger et al., 2005), were on average 0.15 μmol g^{-1} day^{-1}. Molecular analyses showed that the methanotrophic community was dominated by consortia of ANME-2a and -2c and their sulfate reducing partner bacteria of the *Desulfosarciana/ Desulfococcus cluster* (Wegener et al., 2008).

Hydrate Ridge sediment (44°34.20' N, 125°08.77' E; 776 m water depth) was retrieved on Sonne cruise SO165-2 in 2002 via push core sampling. The seafloor was covered with *Beggiatoa* indicating a high flux of sulfide from AOM (Treude et al., 2003).

Black Sea sediment was obtained from the Dniepr basin (44°46.41' N, 31°58.20' E, 326 m water depth) on R/V Poseidon cruise 317/3 in 2004 by pushcoring with the submersible JAGO. Samples were taken from the direct vicinity of a methane seep; degassing of methane during recovery and authigenic carbonate precipitates indicated a high AOM activity in the recovered sediments (Treude et al., 2005).

After recovery, all sediments were immediately transferred to gas tight Duran bottles and supplied with sulfate reducer medium (Widdel and Bak, 1992) as well as a methane headspace. Seawater medium was repeatedly replaced with new medium when sulfide

concentrations exceeded 20 mM. All further handling of sediment was performed in an anaerobic glove box.

The flow-through setup. Sediments were filled into glass columns onto glass frits. (Ochs Glasgerätebau, Bovenden, Germany) (Fig.1). In the first setup columns with a diameter of 40 mm were filled with sediments from Gullfaks to a height of 120 mm (total volume 151 ml, ca. 250 gram dry weight (gdw)). In the second setup, columns with a diameter of 40 mm were filled with different sediments to a height of 50 mm (total volume 63 ml), including inoculates from Gullfaks (sandy sediments, 95 gdw), Black Sea and Hydrate Ridge (clay sediments, 25 gdw). All concentrations and rates were normalized to dry weight of sediment.

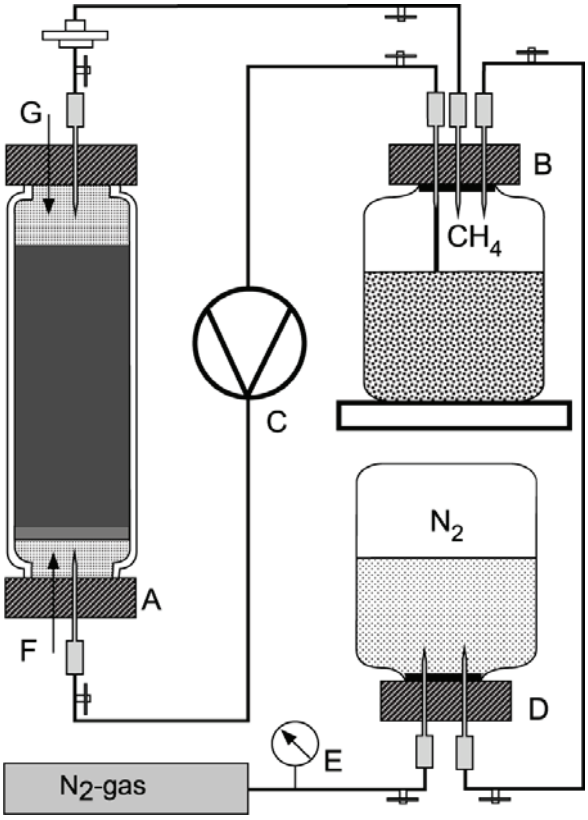

Fig. 1. Flow-through system with the sediment column (A), medium reservoir (B) and the peristaltic pump (C). System pressure is stabilized via a second medium reservoir (D) which is pressurized by nitrogen (F). Sampling was done in inflow (F) and outflow (G).

The columns were closed with 2.5cm thick butyl rubber stoppers and GL45 screw caps. Medical needles and tubing with lowest gas transmissibility (Viton®; DuPont Performance Elastomers, Willmington, US) connected the columns with the reservoir of 2 l (1 l in the short column experiment setup) artificial sea water medium. Oxygen transmissibility of the tubing was qualitatively tested with Resazurin ($C_{12}H_6NO_4Na$, 1mg/l) labeled seawater media and was not visible. The setup was operated as a closed system with medium recycling through a large reservoir. A high precision peristaltic pump (IP-N®, Ismatec SA, Glattbrugg, Switzerland) circulated the seawater media between the reservoir and the sediment column. In all experiments a flow rate of 0.025 ml min^{-1} (36 ml d^{-1}) was adjusted.

Experimental procedure. The filled flow-through cells were mounted into the tubing system and sediment was allowed to settle for two days. The sediments were then percolated for at least 20 days with methane saturated media before starting the measurements. Samples were taken directly from in- and outflow of the columns to determine concentrations of methane and sulfide. Sulfide concentrations were determined by the copper sulfate method (Cord-Ruwisch, 1985). Briefly, 0.1 ml of the aliquot was added to 4ml copper sulfate solution (5mmol $CuSO_4$ in 0.05N HCl). The liquids absorption of monochromatic light (wavelength of 480nm) was measured immediately on a spectrometer. Absolute concentrations were determined by the calibration with sulfide standard solutions and blanks. Three replicate measurements were performed for each sample. An error value of 5% was reported for the copper sulfide method (Cord-Ruwisch, 1985). From the concentrations, sulfide production rates per g dry weight are calculated according to Eq. 1,

$$SRR = \left(H_2S_{out} - H_2S_{in}\right) * \frac{F_{day}}{dw} \qquad [\mu mol\ gdw^{-1}d^{-1}] \qquad (1)$$

with the concentrations of H_2S (μM) in the out- and inflow, the volume of percolated media per day (ml day^{-1}, F_{day}) and the dry weight (gdw). We assume that sulfide production is equivalent to sulfate reduction (SR). However, in all experiments we observed a consistent offset between sulfide production and methane oxidation in the presence of methane. The difference between sulfide production and methane oxidation is most likely due to a contamination of the methane gas with another electron donor (e.g. carbon monoxide), because background sulfide production without methane was as low as 0.01 μmol gdw^{-1} day^{-1}. This low background cannot explain the offset between SR and AOM of ca. 0.07 μmol gdw^{-1} day^{-1} arising from saturation of the medium with methane.

For methane concentrations, subsamples of 0.5ml media were added into gas tight 6ml exetainers filled with 0.5ml NaOH. Methane concentrations were determined from the 100μl headspace triplicates using a GC-FID (Hewlett Packard 5890A, equipped with Porapak-Q column, 6ft, 0.125', Agilent Technologies, Sta. Clara, CA), which was calibrated with methane standards. The AOM rate is calculated according to Eq. 2

$$AOM = CH_{4\,in} - CH_{4\,out} * \frac{F_{day}}{dw} \text{ [μmol gdw}^{-1}\text{d}^{-1}\text{]} \qquad (2)$$

with the methane concentrations of the in- and the outflow ($CH_{4\ in/\ out}$), the flow rate per day F_{day} as well as the dry weight *dw* of the sediment in the column. Sulfate concentrations were measured using nonsuppressed ion chromatography according to according to Ferdelman et al. (1997).

3. Results and Discussion

The first task in the series of experiments was to reach stable conditions in the two different flow-through setups with long and short columns, both with regard to between column comparisons and temporal evolution. In the experiment using five replicate long

columns, Gullfaks sediments were percolated for more than 100 days with constant methane concentrations of around 1.6 mM. The flow rate was 36 ml d^{-1}, (32 m yr^{-1}), and the passage time for the medium entering at the bottom of the column (inflow) to the outflow was 36 hours. A fluid flow velocity of 30 m yr^{-1} is at the higher end of transport rates at cold seep ecosystems and was previously observed e.g. in active settings like those above gas hydrate at stability limits (Linke et al., 1994; Torres et al., 2002).

Fig 2 shows the results for one replicate column of Gullfaks seep sediments. Both sulfide production and methane oxidation were relatively stable. Methane oxidation was on average 0.16 ± 0.04 µmol per gram dry weight (gdw) $^{-1}$. The offset between sulfide production and methane oxidation in the experiments of ca 30% was consistently observed when medium was saturated with methane, which was most likely contaminated with another electron donor such as carbon monoxide or higher hydrocarbons.

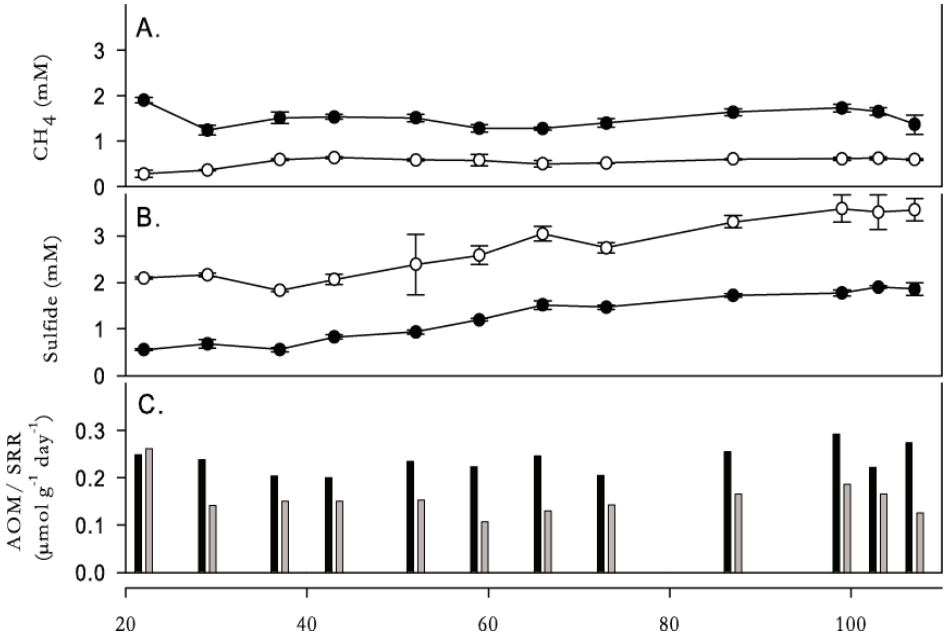

Fig. 2. Comparison of methane oxidation and sulfide production in a long term continuous flow through experiment. The data shown are from one of five similar replicates (Gullfaks seep sediments). a) The development of methane and b) sulfide (inflow concentration (filled circles) and outflow concentrations (open circles) as well as c) the calculated methane oxidation (grey bars) and sulfide production rates (black bars).

In the short column setup, inoculates from the Black Sea, from Hydrate Ridge and Gullfaks were tested in parallel incubations. The sulfate reduction and methane oxidation rates stayed constant for a period of 160 days. We measured average methane consumption rates of 0.42 (±0.15), 0.34 (±0.15) and 0.08 (±0.03) μmol gdw^{-1} d^{-1} and sulfate reduction rates of 0.58 (0.18), 0.47 (±0.016) and 0.07 (±0.03) μmol gdw^{-1} d^{-1} for Black Sea, Hydrate Ridge and Gullfaks, respectively. The AOM rates match well with measurements directly obtained after sampling for the sediment horizons used as inoculate for the flow through columns (data not shown).

At the given methane and sulfate concentrations, we did not observe a significant increase of metabolic activity over time, which would have indicated population growth (Nauhaus et al., 2007). We can exclude that a lack of nutrients has limited growth since they were added to the medium in substantial amounts (Widdel and Bak, 1992). However, similar observations of constant AOM rates over long incubation times were published by Girguis et al. (2003, 2005). In their investigation, seep sediments were percolated with methane-saturated seawater at atmospheric pressure for 24 weeks, however no growth-related increase AOM activity was observed. Reasons for this stagnation of population size may be energy limitation by methane supply at atmospheric pressure. In high pressure batch incubations (~1.4 MPa CH_4), Nauhaus et al. (2007) increased the microbial activity by the factor of ten within almost two years. (growth rate of 0.021 $week^{-1}$). Hence, growth of anaerobic methanotrophs is extremely slow, probably with generation times of >7 months at atmospheric pressure (Girguis et al., 2005; Nauhaus et al., 2007).

In conclusion, we could show that flow through columns can be used as a stable set up for short (days to weeks) and long term experiments (months) for physiological experiments using seep sediments naturally enriched in ANME populations from a variety of locations with different sediment grain sizes. The total mass balance calculated either via accumulated rate measurements (methane oxidation), or via the difference in sulfide

concentrations in the reservoir at beginning and end of the experiments match rather well (ca 15 μmol H_2S gdw^{-1} sediment in 83 days).

Reaction of SR and methane consumption to a methane pulse.

The results of a 40 days methane pulse experiment are presented in Fig. 3. We only show the results of one column (C1) filled with sediments from Gullfaks, the other 4 replicate columns with Gullfaks sediments gave similar results. The columns were run for 120 days at 2 mM methane and 28 mM sulfate, before methane was removed for 36 days (Fig 3a). After

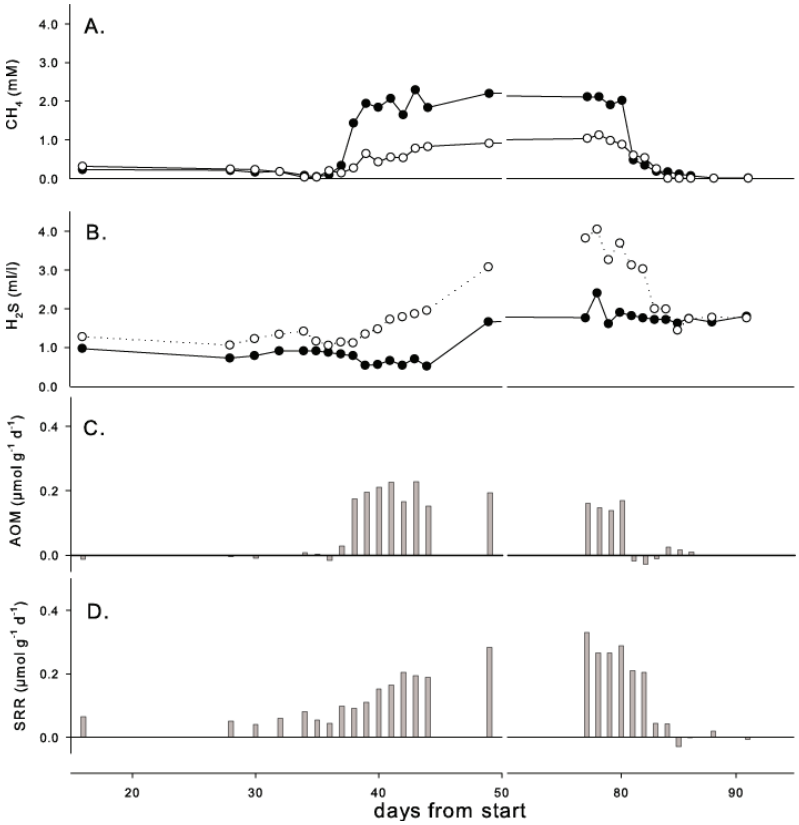

Fig. 3. Changing activities of a methanotrophic community in response to a methane pulse. a) Methane concentrations, b) sulfide concentrations. Filled circles represent the measurements at the inflow and open circles those at the outflow; c) the resulting methane oxidation rates and d) sulfide production.

36 days of starvation, methane concentration in the medium was increased to 2 mM within 2 days. The outflow showed increasing sulfide concentrations immediately after methane

was percolated through the column (day 38, Fig. 3b). Immediately after methane was reset to 2 mM, AOM (Fig 3c) and SR (Fig. 3d) returned to similar rates as before the starvation phase, with 0.19±0.03 μmol gdw^{-1} d^{-1} and 0.24±0.05 μmol gdw^{-1} d^{-1}, respectively. During the 36 days starvation phase, background SR dropped to rates as low as ~0.04 μmol g^{-1} day^{-1}. After 40 days of exposure to methane, the medium was again degassed. Within 6 days, the methane concentration in the outflow fell below 50 μM and both methane oxidation and sulfate reduction rates decreased almost completely to zero. This proves the direct coupling between methane oxidation and sulfide production in AOM. However, it remains unknown whether the electron transfer from methane to sulfide is carried out within one or between two organisms.

Previous investigations found that anaerobic methanotrophs produce large amounts of methyl-coenzyme M reductase (MCR), which is most likely the enzyme responsible for the first step in methane oxidation. For example, this MCR constituted 7% of the total environmental protein extract in methanotrophic mats of the Black Sea (Krüger et al., 2003). It may be a good strategy to maintain a high amount of functional proteins in the extremely slow growing cells, to utilize a wide range of methane concentrations. For example, in our experiment, the present methanotrophic population utilized the same fraction of methane over 2 orders of magnitude in substrate availability without any delay.

Furthermore, the results of the methane pulse experiment support previous observations on the longevity of seep methanotrophs kept under anoxic conditions at in situ temperature without substrate. Even after storage of months to years, immediate sulfide formation can be observed directly after methane addition, reaching similar rates as in the field, at the time of sampling. The ability of anaerobic methanotrophs to survive long starvation periods could be an important advantage, especially with regard to the high spatial and temporal variability of methane fluxes at seeps, and also when considering their slow growth.

Sulfate reduction and methane oxidation at different methane and sulfate concentrations.

At the low energy yield of AOM, efficient use of the natural range of methane and sulfate concentrations is critical to the anaerobic methanotrophs. In nature, AOM is often limited to a narrow zone where methane and sulfate overlap (SMTZ), in which both reactants show very low concentrations. At seeps, methane concentrations and fluxes may be extremely high, but very often sulfate is depleted within the top few cm, and its penetration from the overlying bottom water can be suppressed by high upward fluxes of sulfate free subsurface fluids (Niemann et al., 2006). Previous environmental observations suggested a strong dependence of AOM rates on the fluxes of sulfate (Treude et al., 2003). To examine the kinetic effect of AOM reactant availability, we incubated two replicate columns from Gullfaks with a series of different methane and sulfate concentrations. After an adjustment time of ≥ 8 days for each concentration, three to five measurements of methane and sulfide concentrations were performed within about ten days. Figure 4 and 5 show AOM and SR rates at different concentrations of methane and sulfate and constant flow velocities of 30 m yr^{-1}.

In Fig. 4 the metabolic activities without methane and at different methane concentrations between 0.3 and 2.3 mM are plotted. In the absence of methane, a background sulfide production of about 0.02 μmol gdw^{-1} d^{-1} was determined. Sulfide production increased to 0.17 μmol gdw^{-1} d^{-1} at 1.35 mM CH_4 and to 0.21 μmol gdw^{-1} day^{-1} at methane concentrations of 2.3 M. Methane oxidation followed the trend of sulfate reduction with rates of 0.02, 0.10, 0.17 μmol gdw^{-1} day^{-1} at 0.33 mM, 1.35 mM and 2.3 mM CH_4, respectively. The relation between methane concentration and methane consumption as well as sulfide production was linear in this range. The 20-30% offset between SR and AOM is explained as above.

A methane saturation effect (indicated by a declining slope) was not observed in our experiments, which suggests AOM K_M-values beyond 2 mM. Nauhaus et al. (2002) observed a linear relationship between methane oxidation and sulfide production below methane pressures of 0.1 MPa (about 1.5 mM), and suggested a high methane K_M in the range of several mM for AOM and methane-fueled SR. In comparison, for hydrogenotrophic sulfate reduction, half saturation constants for H_2 are as low as 141 Pa (~1 µmol; Lovley et al., 1982), but the energy yield of this process is orders of magnitudes higher than in AOM. The high half saturation constant for methane in AOM may be due to predicted reverse operation of the methanogenic methyl-coenzyme M reductase, producing a methyl radical as a first step in AOM (Krüger et al., 2003; Hallam et al., 2004; Shima and Thauer, 2005).

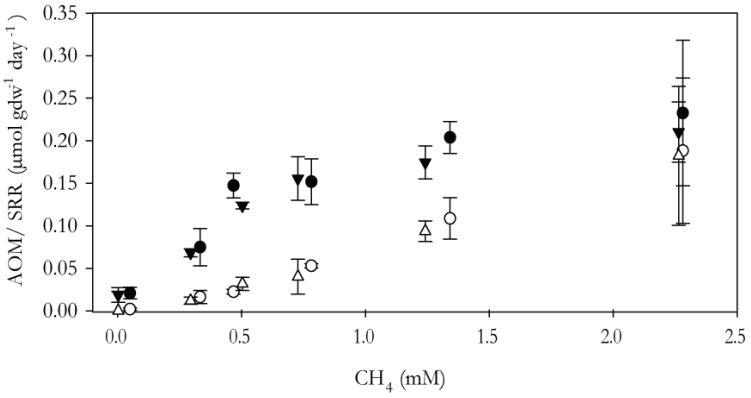

Fig. 4. Effect of different methane concentrations on rates of methane oxidation (open symbols) and sulfate reduction (filled symbols). Data are shown for two replicate columns marked as circles (C1) and triangles (C2)

The influence of sulfate concentration on the oxidation of methane was examined by percolating two other replicate columns with reduced seawater medium containing 28, 3, 2 and 1 mM sulfate (Fig. 5). Within the tested low sulfate concentrations from 3 to 1 mM a weak decline of metabolic rates was determined. However the scattering within the data was quite strong. We conclude that the half saturation for sulfate in methanotrophy is below the examined concentrations, around 0.5 mM. In comparison, organoclastic sulfate reducers show half saturation constants between 70 µM (*Desulfovibrio salexigens*) and 200 µM (*Desulfobacter postgatei*) (Ingvorsen and Joergensen, 1984; Ingvorsen et al., 1984). These sulfate reducers have different strategies to achieve low kM-values; e.g. *Desulfovibrio desulfuricans*

shows intracellular sulfate enrichment up to the factor of 5000 compared to its environment (Cypionka, 1989). Hence, the anaerobic methanotrophs from cold seep ecosystems investigated here appear to have a relatively high kM for sulfate. It remains unknown if the methanotrophic populations of the ubiquitous sulfate methane transition zones in the seabed are better adapted to low sulfate concentrations than their relatives inhabiting the cold seeps.

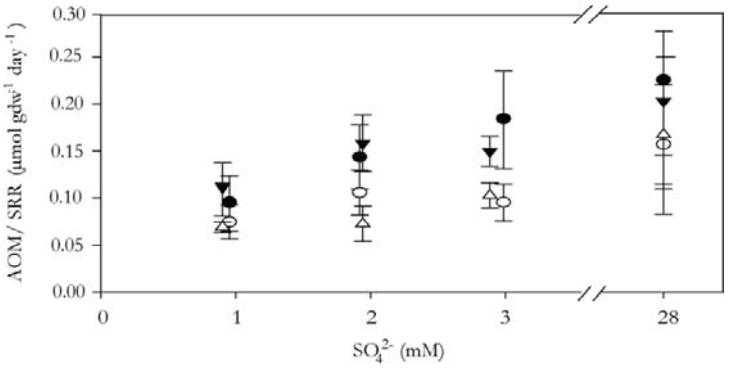

Fig. 5. Effect of different sulfide concentrations on rates of methane oxidation (open symbols) and sulfide production (filled symbols). Data are shown for two replicate columns marked as circles (C1) and triangles (C2)

4. Conclusions:

At a constant methane supply of about 2 mM, different marine methanotrophic communities enclosed in continuous flow through columns resulted in a stable rate of anaerobic oxidation of methane during 160 days. A tight link between methane oxidation and sulfate reduction was clearly shown by providing pulses of methane to environmental methanotrophic communities. An interruption of the methane supply led to an immediate decline of sulfate reduction. After percolation with methane free media for more than 40 days, former methane oxidation and sulfate reduction rates were reached immediately without a lag phase. Hence, the methanotrophic populations seem to be able to survive relatively long starving periods. Rates of anaerobic oxidation of methane were strongly regulated by methane concentrations. Between 0.3 and 2.3 mM CH_4 we found an almost linear increase of methane oxidation and sulfide production. This suggests half saturations

(k_M values) for methane of several mM in AOM. Sulfate concentrations below 3 mM decreased methane oxidation rates, the k_M for sulfate is estimated at around 0.5 mM. Apparently, the high level of functional proteins maintained by the anaerobic methanotrophs allows for immediate responses to a wide range of concentrations of both electron donor and acceptor in the anaerobic oxidation of methane.

References

Boetius, A., and Suess, E.: Hydrate Ridge: a natural laboratory for the study of microbial life fueled by methane from near-surface gas hydrates. Chem. Geol., 205: 291-310, 2004.

Boetius, A. Ravenschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jørgensen, B.B., Witte, U. and Pfannkuche, O.: A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature, 407: 623-626, 2000.

Canfield, D. (1993) Organic matter oxidation in marine sediments. Interactions of C, N, P and S biogeochemical cycles and global change. NATO ASI Series, 14: 33–363.

Canfield, D., Kristensen, E. and Thamdrup, B. Aquatic Geomicrobiology. San Diego, California: Elsevier, 656pp, 2005.

Cord-Ruwisch, R.: A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. Microbiol. Meth., 4(1): 33-36, 1985.

Cypionka, H.: Characterization of sulfate transport in Desulfovibrio desulfuricans. Arch. Microbiol., 152(3): 237-243, 1989.

Ferdelman, T. G., Lee, C., Pantoja, S., Harder, J., Bebout, B. M. and Fossing, H.: Sulfate reduction and methanogenesis in a Thioploca-dominated sediment off the coast of Chile. Geochim. Cosmochim Ac., 61(15): 3065-3079, 1997.

Girguis, P. R., Cozen, A. E. and DeLong, E. F.: Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. Appl. Environ. Microb., 71(7): 3725-3733, 2005.

Girguis, P. R., Orphan, V., Hallam, S. and DeLong, E. F.: Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. Appl. Environ. Microb., 69(9): 5472-5482, 2003.

Hallam, S. J., Putnam, N., Preston, C. M., Detter, J. C., Rokhsar, D., Richardson, P. M. and DeLong, E. F.: Reverse methanogenesis: Testing the hypothesis with environmental genomics. Science 305(5689): 1457-1462, 2004.

Hanson, R. S. and Hanson, T. E.: Methanotrophic bacteria. Microbiol. Rev., 60: 439-471, 1996.

Hovland, M.: Discovery of prolific natural methane seeps at Gullfaks, northern North Sea. Geo-Mar. Lett. 27: 197-201, 2007.

Ingvorsen, K. and Joergensen, B.B.: Kinetics of sulfate uptake by freshwater and marine species of Desulfovibrio. Arch .Microbiol., 139: 61-66, 1984.

Ingvorsen, K., Zehnder, A.J.B. and Jørgensen, B.B.: Kinetics of sulfate and acetate uptake by Desulfobacter postgatei. Appl. Environ. Microb., 47: 403-408, 1984.

Krüger, M., Anke Meyerdieks, A., Glöckner, F.O., Amann, R., Widdel, F., Kube, M., Reinhardt, R., Kahnt, J., Böcher, R., Thauer, R. K. And Shima, S.: A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. Nature, 426: 878-881, 2003.

Krüger, M. Treude, T., Wolters, H., Nauhaus, K. and Boetius, A.: Microbial methane turnover in different marine habitats. Palaeogeogr. Palaeocl., 227(1-3): 6-17, 2005.

Linke, P., Suess, E., Torres, M., Martens, V., Rugh, W. D., Ziebis, W., Kulm, L. D.: In situ measurement of fluid flow from cold seeps at active continental margins. Deep-Sea Res., 41: 721–739, 1994

Lovley, D. R., Dwyer, D. F. and Klug, M. J.: Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol., 43(6): 1379-1379, 1982.

Milkov, A.V.: Global estimates of hydrate-bound gas in marine sediments: how much is really out there? Earth-Sci. Rev., 66(3-4): 183-197, 2004.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A. and Widdel, F.: In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. Environ. Microbiol., 9(1): 187-196, 2007.

Nauhaus, K., Boetius, A., Krüger, M. and Widdel, F.: In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediments from a marine gas hydrate area. Environ. Microbiol., 4(5): 296-305, 2002.

Nauhaus, K., Treude, T., Boetius, A. and Kruger, M.: Environmental regulation of the anaerobic oxidation of methane: a comparison of ANME-I and ANME-II communities. Environ. Microbiol., 7(1): 98-106, 2005.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K., Amann, R., Sauter, E. J., Schlüter, M., Klages, M., Foucher, J. P. and Boetius, A.: Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. Nature, 443(7113): 854-858, 2006.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F. Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science, 293(5529): 484-487, 2001.

Reeburgh, W. S.: Oceanic methane biogeochemistry. Chem. Rev., 107(2): 486-513, 2007.

Reeburgh, W.S.: 'Soft spots' in the global methane budget. In: M.E. Lidstrom and F.R. Tabita (Editors), Microbial Growth on C1 Compounds. Kluwer, Dordrecht, pp. 334–342, 1996.

Shima, S., and Thauer, R. (2005) Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic Archaea. Curr. Opin. Microbiol., 8: 643-648.

Sauter, E., Muyakshin, S., Charlou, J., Schlüter, M., Boetius, A., Jerosch, K. et al. Methane discharge from a deep-sea submarine mud volcano into the upper water column by gas hydrate-coated methane bubbles. Earth Planet. Sc. Lett., 243: 354-365, 2006.

Torres, M. E., McManus, J., Hammond, D. E., de Angelis, M. A., Heeschen, K.U., Colbert, S. L., Tryon, M. D., Brown, K. M. and Suess, E.: Fluid and chemical fluxes in and out of sediments hosting methane hydrate deposits on Hydrate Ridge, OR, I: Hydrological provinces. Earth Planet. Sc. Lett. 201: 525-540, 2002.

Treude, T., Boetius, A., Knittel, K., Wallmann, K. and Jorgensen, B. B.: Anaerobic oxidation of methane above gas hydrates at Hydrate Ridge, NE Pacific Ocean. Mar. Ecol.-Prog. Ser., 264: 1-14, 2003.

Treude, T., Knittel, K., Blumenberg, M., Seifert, R. and Boetius, A.: Subsurface microbial methanotrophic mats in the Black Sea. Appl. Environ. Microb., 71(10): 6375-6378, 2005.

Treude, T., Orphan, V., Knittel, K., Gieseke, A., House, C. and Boetius, A. Consumption of methane and CO2 by methanotrophic microbial mats from gas seeps of the anoxic Black Sea. Appl. Environ. Microb. 73: 2271-2283, 2007.

Trotsenko, Y.A. and Khmelenina, V.N.: Aerobic methanotrophic bacteria of cold ecosystems. FEMS Microbiol. Ecol., 53(1): 15-26, 2005.

Tryon, M.D., Brown, K.M., and Torres, M.E.: Fluid and chemical flux in and out of sediments hosting methane hydrate deposits on Hydrate Ridge, OR, II: Hydrological processes. Earth Planet. Sc. Lett., 201: 541-557, 2002

Wegener, G., Shovitri, M., Knittel, K., Niemann, H., Hovland, M. and Boetius, A.: Biogeochemical processes and microbial diversity of the Gullfaks and Tommeliten methane seeps (Northern North Sea). Biogeosciences Discuss., 5: 971-1015, www.biogeosciences-discuss.net/5/971/2008/, 2008.

Wellsbury, P., Goodman, K., Cragg, A. and Parkes, R.J.: 36. The geomicrobiology of deep marine sediments from Blake Ridge containing methane hydrate (site 994, 995 and 997) Proceedings of the Ocean Drilling Program. Scientific results. 164: 379-391, 2000.

Widdel, F. and Bak, F., Gram-negative mesophilic sulfate-reducing bacteria. In: T.H. Balows A, Dworkin M, Harder W, Schleifer KH (Editor), The prokaryotes. Springer, Berlin Heidelberg New York, pp. 3352-3378, 1992.

Chapter IV

Assimilation of Methane and Inorganic Carbon by Microbial Communities Mediating the Anaerobic Oxidation of Methane

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Abstract: The anaerobic oxidation of methane (AOM) is a major sink for methane on Earth and is performed by consortia of methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB). Here we present a comparative study using *in vitro* stable isotope probing to examine methane and carbon dioxide assimilation into microbial biomass. Three sediment types comprising different methane-oxidizing communities (ANME-1 and -2 mixture from the Black Sea, ANME-2a from Hydrate Ridge and ANME-2c from the Gullfaks oil field) were incubated in replicate flow-through systems with methane-enriched anaerobic seawater medium for 5-6 months amended with either $^{13}CH_4$ or $H^{13}CO_3^-$. In all three sediment types methane was anaerobically oxidized in a 1:1 stoichiometric ratio compared to sulfate reduction. Similar amounts of $^{13}CH_4$ or $^{13}CO_2$ were assimilated into characteristic archaeal lipids, indicating a direct assimilation of both carbon sources into ANME biomass. Specific bacterial fatty acids assigned to the partner SRB were almost exclusively labeled by $^{13}CO_2$, but only in the presence of methane as energy source and not during control incubations without methane. This indicates an autotrophic growth of the ANME-associated SRB and supports previous hypotheses of an electron shuttle between the consortium partners. Carbon assimilation efficiencies of the methanotrophic consortia were low, with only 0.25-1.3 mol% of the methane oxidized.

Introduction:

Methane is an important greenhouse gas. In spite of the high production rates and large reservoirs of methane in marine sediments, the oceans contribute only little to the atmospheric budget (Judd et al., 2002; Reeburgh, 2007). This is because the microbially mediated anaerobic oxidation of methane (AOM), with sulfate as the terminal electron

acceptor, removes the largest fraction of methane before it can reach the hydrosphere and atmosphere (Martens and Berner, 1974; Reeburgh, 1976; Iversen and Jørgensen, 1985; Hinrichs and Boetius, 2002).

Marine AOM is mediated by archaea in consortium with sulfate reducing bacteria (SRB) (Boetius et al., 2000; Hinrichs et al., 2000; Orphan et al., 2001a; Knittel et al., 2005). The three known phylogenetic groups of marine anaerobic methanotrophs (ANME-1, -2, and -3) are distantly related to methanogens of the order *Methanosarcinales* and *Methanomicrobiales* (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2002; Knittel et al., 2005; Niemann et al., 2006; Lösekann et al., 2007). The known sulfate reducing partners of the of ANME-1 and -2 groups belong to the *Desulfosarcina/Desulfococcus* (DSS) group (Boetius et al., 2000; Orphan et al., 2001b; Michaelis et al., 2002; Knittel et al., 2003), whereas ANME-3 was recently found in association with SRB related to the *Desulfobulbus* cluster (Niemann et al., 2006; Lösekann et al., 2007). These consortia of anaerobic methanotrophic microorganisms dominate microbial biomass and biogeochemical processes at shallow and deep water cold seeps, in gas hydrate environments, and in ubiquitous methane sulfate transition zones of the sediments (Knittel et al., 2005).

Today, none of the methanotrophic consortia have been obtained in pure culture, and physiological studies have relied on *in vitro* experimentation with sediment samples naturally enriched in methanotrophic communities (Nauhaus et al. 2002, 2005, 2007; Girguis et al. 2003, 2005; Blumenberg et al. 2005; Moran et al., 2008). In addition to their 16S rRNA gene-based phylogenetic classification, each of these groups can be recognized according to profiles of specific ^{13}C-depleted lipid biomarkers (Hinrichs and Boetius 2002; Blumenberg et al. 2004; Niemann and Elvert in press). A direct evidence for this link was provided by the combination of fluorescence *in situ* hybridization with secondary ion mass spectrometry (FISH-SIMS; Orphan et al. 2001a). To further investigate the relation between concentrations, ratios and $\delta^{13}C$-values of specific microbial lipids to the distribution of

active methanotrophic communities, three approaches have been used previously: (1) the correlation of lipids with cell counts in field samples (Boetius et al. 2000; Elvert et al., 2003, 2005; Orcutt et al., 2005; Niemann et al. 2006), (2) the *in vitro* growth of ANME consortia with subsequent lipid analysis of the biomass yield (Nauhaus et al. 2007); (3) lipid analysis coupled to stable isotope probing of an active methanotrophic microbial mat (Blumenberg et al. 2005). Among the archaeal lipids, ^{13}C-depleted archaeol and hydroxyarchaeol as well as crocetane and penthamethylicosane have been repeatedly shown to correlate with the presence of different active ANME consortia (Blumenberg et al. 2004; Nauhaus et al. 2007; Niemann and Elvert, in press). Among the bacterial lipids, several types of ester linked fatty acids (e.g. $C_{16:1\omega5c}$; cy(cyclopropane)$C_{17:0\omega5,6}$) and abundances of monoalkyl and dialkyl glycerol ethers have been suggested as specific biomarkers for partner SRB of methanotrophic consortia (Hinrichs et al., 2000; Elvert et al. 2003; Blumenberg et al. 2004; Nauhaus et al. 2007; Niemann and Elvert, in press).

In the present study, we aimed at investigating the assimilation of methane and inorganic carbon as by members of methanotrophic communities using stable isotope probing of their lipid biomarkers. For this *in vitro* experiment, we used flow-through reactors, which showed constantly high rates of sulfate-driven AOM when filled with sediment slurries naturally enriched in methanotrophic communities from three different cold seep sites in the Black Sea (BS), at Hydrate Ridge (HR) and Gullfaks (GF). The main advantage of flow-through reactors is the option to monitor the concentration of the reactants and end products of AOM (i.e. sulfide and bicarbonate), dilute products of AOM in a large medium pool, and to provide relatively high growth rates at comparatively low methane partial pressures (Girguis et al. 2005). Based on results from previous experiments with highly enriched ANME-2/DSS and ANME-1/DSS communities (Nauhaus et al. 2002, 2005, 2007), we tested the following hypotheses: (1) ANME cell biomass is formed by assimilation of both methane and CO_2 (2) the syntrophic sulfate reducing partners are

autotrophs; (3) different AOM communities show different lipid patterns, but the stoichiometry of methane and inorganic carbon assimilation is similar.

Results

Biogeochemical rate measurements

Replicate sediment samples naturally enriched in AOM communities from cold seeps of the BS, HR and GF were incubated at methane concentrations of 1.8-2.2 mM in flow-through reactors. The experiments started with a sulfide inflow concentration of about 1 mM. During incubation, the sulfide concentration did not exceed 4 mM in all six flow-through reactors. All inoculates showed relatively stable rates of sulfate reduction and methane oxidation, calculated from the difference from inflow and outflow sulfide and methane concentrations. In incubations with ^{13}C-methane, average rates of SR and AOM were 0.58 and 0.42 μmol per gram dry weight (gdw^{-1}) (BS), 0.47 and 0.34 μmol gdw^{-1} (HR), and 0.07 and 0.08 μmol gdw^{-1} (GF), respectively. Similar SR and AOM rates were calculated for the bicarbonate labeling experiment using unlabeled methane with 0.58 and 0.53 μmol gdw^{-1} (BS), 0.73 and 0.57 μmol gdw^{-1} (HR) and 0.06 and 0.07 μmol gdw^{-1} (GF), respectively.

Concentrations and carbon isotopic compositions of fatty acids and isoprenoidal glycerol diethers

Table 1 shows the average concentrations of selected bacterial and archaeal biomarkers in the three different sediment slurries used for the flow reactors. No substantial changes of lipid concentrations were observed. At BS, saturated fatty acids (FAs) comprised almost 80% of the FAs ($C_{14:0}$-$C_{18:0}$), followed by their ω7-derivatives with 17%. At HR and GF, saturated FAs comprised 21% and 47%, respectively, and monounsaturated FAs accounted for 45% and 35%, respectively. In all three inoculates substantial amounts of $C_{16:1\omega5}$, *ai*- and *i*-$C_{15:0}$ FAs acids as well as archaeol and hydroxyarchaeol were present with characteristically ^{13}C-depleted carbon isotopic compositions. Table 1 shows the δ^{13}C-values

of these compounds at the beginning of the experiment, as well as after inoculation with ^{13}C-labeled methane and ^{13}C-labeled bicarbonate.

Table 1. Concentrations, relative distribution and carbon isotopic compositions of selected compounds at the beginning of the experiment and after ^{13}C-labeling with methane (159 days) and inorganic carbon (IC) (176 days); T1 and T2 not shown here; # data from T2 (T3 δ^{13}C-values not available). δ^{13}C values vs. VPDB .

	Black Sea					**Hydrate Ridge**					**Gullfaks**				
	Concentration		Start	^{13}C at T3		Concentration		Start	δ^{13}C at T3		Concentration		Start	δ^{13}C at T3	
	µg gdw^{-1}	%	δ^{13}C	CH_4	IC	µg gdw^{-1}	%	δ^{13}C	CH_4	IC	µg gdw^{-1}	%	δ^{13}C	CH_4	IC
Bacterial FA															
C14:0	22.5	19.4	-26	-28	-17	1.75	5.2	-39	-17	141	0.15	6.3	-64	-24	1359
i-C15:0	3.1	2.7	-39	-33	-6	0.99	3.0	-54	-48	-7	0.08	3.3	-63	-35	450
ai-C15:0	5.9	5.1	-48	-33	20	1.57	4.7	-61	-43	-1	0.14	5.9	-63	-30	259
C16:1ω7c	8.7	7.5	-42	-18	201	7.11	21.3	-35	-27	151	0.38	15.9	-47	-31	653
C16:1ω5c	3.1	2.7	-54	-18	208	3.18	9.5	-63	9	354	0.36	15.1	-91	-13	2006
C16:0	46.9	40.1	-28	-21	-19	4.22	12.6	-39	-21	65	0.55	23.0	-25	-22	668
cyC17:0ω5,6	2.1	1.8	-54	-57		0.44	1.3	-99	-77	58	0.02	0.8	-88		
C18:1ω7c	8.7	7.5	-64	-39	140	3.91	11.7	-41	-51	-8	0.09	3.8	-27	-24	648
C18:0	11.9	10.3	-27	-28	-25	1.28	3.8	-37	-21	-30	0.43	18.0	-25	-27	53
Archaeal L.															
Archaeol	1.0	0.9	-102	-47	-45	3.33	10.0	-108	-28	-2	0.05	2.1	-115	-30#	543
*Sn*2-hydroxyarch	1.8	1.6	-105	-54	-47	5.65	16.9	-121	-70	-41	0.14	5.9	-124	-83#	517

Although lipid concentrations did not change substantially during the incubation, the uptake of ^{13}C-labeled substrate was significant. Those lipid biomarkers that were most strongly depleted in ^{13}C prior to the experiment, i.e., archaeol and hydroxyarchaeol for ANME and $C_{16:1\omega5c}$, $cyC_{17:0\omega5,6}$, and $C_{16:1\omega7c}$ for SRB, were most strongly labeled with ^{13}C in the presence of methane as energy source (Fig. 1, Tab. 1). Interestingly, archaeal biomarkers showed an incorporation of both ^{13}C labeled carbon sources, while bacteria FAS showed only a substantial assimilation of $^{13}CO_2$.

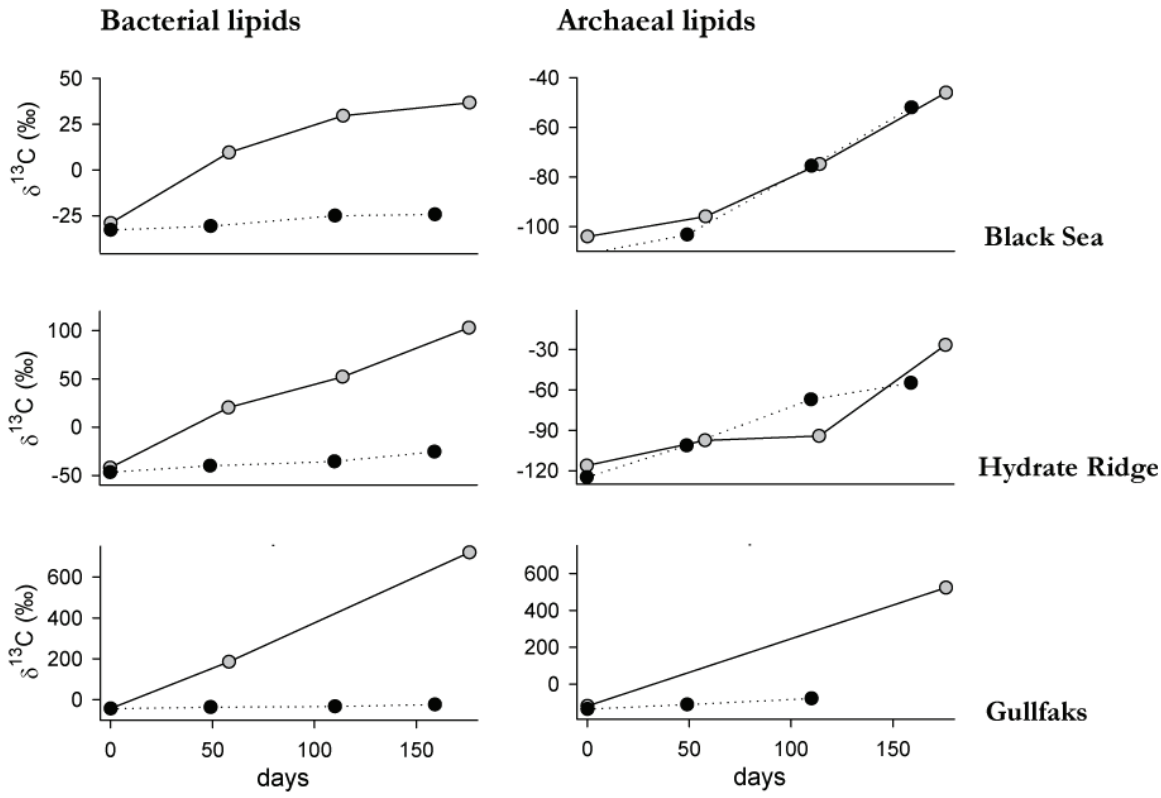

Figure 1. Development of $\delta^{13}C$ (in ‰) of bacterial fatty acids and archaeal glycerol diethers, expressed as weighted averages, in experiments with ^{13}C-labeled methane (●) and bicarbonate (○).

Uptake of inorganic carbon and methane into bacterial and archaeal biomass

The incubation with ^{13}C-bicarbonate was carried out for 176 days with subsampling for lipid extractions after 58 and 114 days. The replicate incubation with ^{13}C-methane ran for 159 days and was sampled at day 49 and 110. In the ^{13}C-methane labeling experiment we added ~12% $^{13}CH_4$ to reach a methane $\delta^{13}C$-value of about +11000‰. During the course of the incubation, oxidation of labeled $^{13}CH_4$ led to an accumulation of ^{13}C in DIC with $\delta^{13}C$-

values reaching +900‰ at the end of the experiment. This may have caused a small proportion of indirect labeling of lipids by methane-derived DIC. For the ^{13}C-inorganic carbon labeling experiment, 11% of labeled bicarbonate was added, resulting via carbonate equilibrium in a δ^{13}C of ~+10000‰ in the whole carbonate pool including CO_2. Figure 1 shows the development of the mean isotopic compositions of bacterial and archaeal lipids over time in the incubations with ^{13}C-methane and ^{13}C-bicarbonate. The carbon isotopic compositions and concentrations of individual lipids (Table 1) were used to calculate the weighted mean isotopy of both archaeal and bacterial lipids $\delta^{13}C_{\Theta}$ as follows:

$$\delta^{13}C_{\Theta} = \sum_{i=1}^{n} \left(Conc_{LDn} / conc_{LDsum} \right) * \delta^{13}C_{LDn} \qquad (1)$$

Here, $conc_{LDn}$ is the concentration of the individual lipids, $\delta^{13}C_{LDn}$ the isotopic composition of these compounds and $conc_{LDsum}$ the summed concentration of the quantified lipids, i.e. glycerol diethers (archaea) and fatty acids (bacteria), respectively.

A continuous carbon isotopic change to more positive δ^{13}C-values was measured in all incubations, indicating a transfer of added ^{13}C into lipid biosynthesis by the methanotrophic communities. However, in the three inoculates amended with ^{13}C-methane, only minor changes in δ^{13}C of bacterial FAs were observed ($\Delta\delta^{13}C_{\Theta FAs\ T0\text{-}T3}$ +9‰ (BS); +21‰ (HR) and +24‰ (GF)). In contrast, ^{13}C-bicarbonate labeling led to a much stronger ^{13}C-incorporation into FAs. Isotopic changes between the start and end of the incubation were $\Delta\delta^{13}C_{\Theta FAs\ T0\text{-}T3}$ +65‰ for BS, +145‰ for HR and +763‰ for the GF inoculate.

The $\Delta\delta^{13}C_{\Theta T0\text{-}T3}$ of archaeal lipids showed a different pattern. In the BS slurry, labeling with ^{13}C-methane or ^{13}C-bicarbonate resulted in similar isotopic changes of +59‰ and +58‰, respectively. Likewise, methane and bicarbonate labeling led to a similar ^{13}C incorporation ($\Delta\delta^{13}$C +70‰ and +91‰, respectively) in HR slurries. These values indicate that the archaeal communities assimilate both carbon sources in a ratio of about 1:1. From the GF slurries, only two measurements of archaeol derivatives were successful, because

overall concentrations were very low (Table 1). However, these results differ from those found for BS and HR slurries, showing incorporation of ^{13}C-methane to ^{13}C-bicarbonate with a ratio of about 1:10. The reason for the much higher bicarbonate incorporation in GF slurries is not clear, but could be related to differences in the specific types of anaerobic methanotrophs present (Table 3).

Figure 2 (left panel) shows the relative change in carbon isotopic compositions ($\Delta\delta^{13}$C) of individual biomarker lipids from the start to the end of the experiment for all

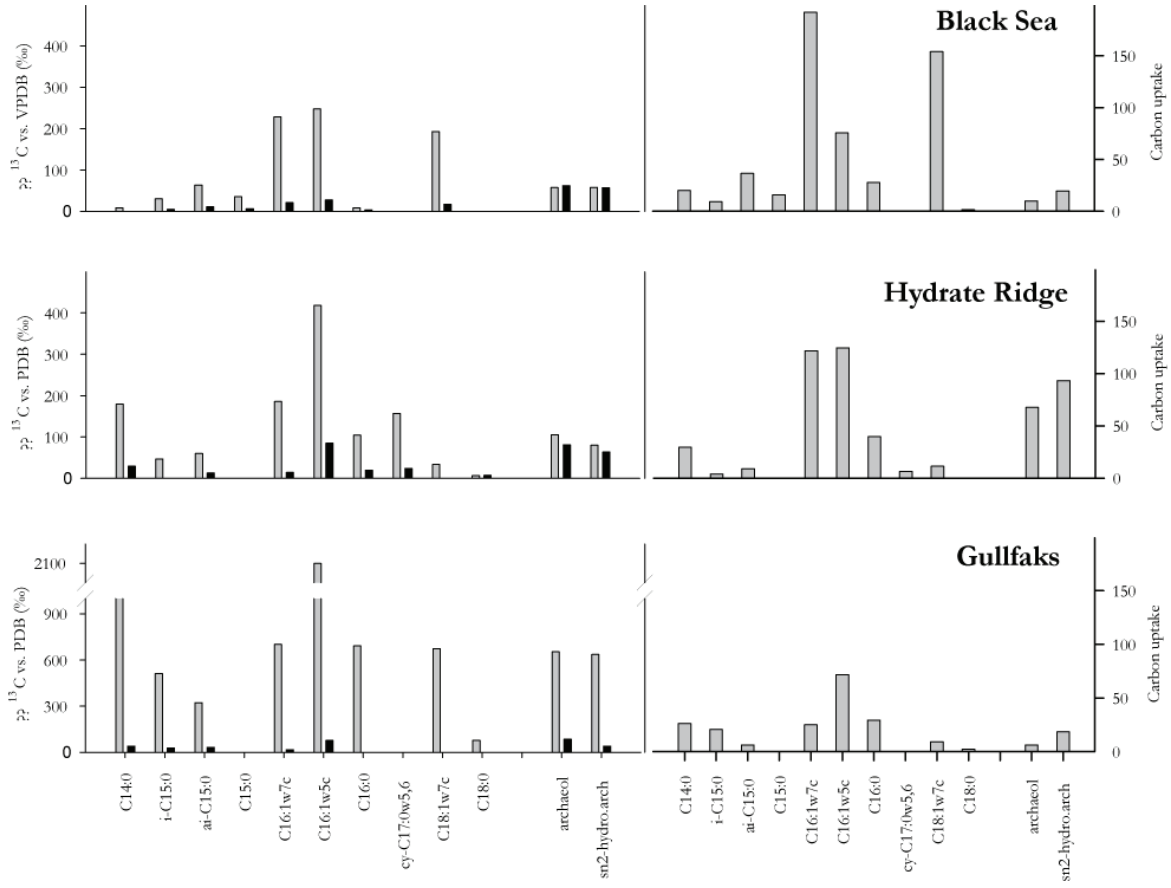

Figure 2. Left panel: Carbon isotopic change of individual fatty acids and archaeal glycerol diethers after incubation with ^{13}C-labeled bicarbonate for 176 days (grey) and ^{13}C-labeled methane for 159 days (black) in Black Sea, Hydrate Ridge and Gullfaks sediment slurries. Right panel: Carbon assimilation into biomarker lipids based on carbon isotopic changes during the course of the experiment.

replicate incubations amended with ^{13}C-methane or ^{13}C-bicarbonate. The strongest label incorporation resulted from the ^{13}C-bicarbonate amendments. However, the incorporation of label was different for each FA. Highest changes in the carbon isotopy were found for

$C_{16:1\omega5c}$ with $\Delta\delta^{13}C$-values of +247‰ (BS), +418‰ (HR) and +2097‰ (GF). But also $C_{16:1\omega7c}$ (HR, GF, BS), $C_{14:0}$ (HR, GF) and $cyC_{17:0\omega5,6}$ (HR) were highly labeled. Except for $C_{14:0}$, these FAs are usually associated with ANME-2 dominated microbial communities (Hinrichs et al., 2000; Orphan et al., 2001a; Elvert et al., 2003; Niemann and Elvert, in press). Intermediate changes were found for $C_{16:0}$, but also for i-$C_{15:0}$ and ai-$C_{15:0}$. In all three samples, the lowest carbon isotopic change was found for $C_{18:0}$.

Since carbon was mainly assimilated from the inorganic carbon pool (q.v. Table 1, Fig. 1), the uptake into individual FAs during incubation times (ΔC_{LD}) (Fig. 2, right panels) were calculated as follows:

$$\Delta C_{LD} = \frac{\Delta\delta^{13}C_{LD}}{\delta^{13}C_{Carb}} \cdot conc_{LD} \qquad (2)$$

where $\Delta\delta^{13}C_{LD}$ is the isotopic change of the individual lipid as a result of carbonate labeling, $\delta^{13}C_{DIC}$ the isotopic composition of inorganic carbon in the medium (~ +10,000‰) and $conc_{LD}$ the concentration of the individual lipids. Considering the above results, we assumed a mixed carbon assimilation of 1:1 of CO_2 and methane for the archaeal lipids, and hence multiplied ΔC_{LD} by two for the calculation of carbon assimilation by ANME.

Samples from GF and HR showed a very similar carbon assimilation pattern. Highest carbon uptake was measured for $C_{16:1w5c}$ (71 and 125 ng C gdw^{-1}), followed by $C_{16:1w7c}$ (25 and 121 ng C gdw^{-1}). Intermediate uptake was found for the saturated fatty acids $C_{14:0}$ and $C_{16:0}$. In the BS inoculate, highest uptake was found for $C_{16:1w7c}$ (192 ng gdw^{-1}) and $C_{18:1w7c}$ (154 ng gdw^{-1}), followed by $C_{16:1w5c}$ (75 ng gdw^{-1}). In all sediments, almost no carbon uptake was found for $C_{18:0}$. The calculated carbon uptake into archaeol and hydroxyarchaeol was overall lower with 10 and 20 ng gdw^{-1} (BS), 68 and 93 ng gdw^{-1} (HR) and 8 and 19 ng gdw^{-1} (GF).

Dependence of inorganic carbon assimilation on methane as energy source.

The dependence of inorganic carbon assimilation into microbial lipids on methane as energy source was tested in an additional batch experiment. Under a methane atmosphere of 3.1 mM, high SR rates of 0.8 μmol gdw^{-1} d^{-1} were measured for BS and HR sediments. In contrast, low SR rates (0.07 and 0.02 μmol gdw^{-1} d^{-1}, respectively) were determined in methane-free control samples. Due to material limitation, a less active GF inoculate was used, which reached SR rates of only 0.05 μmol gdw^{-1} d^{-1} with methane, and 0.02 μmol gdw^{-1} d^{-1} in the control experiment with N_2/CO_2.

Table 2 shows the carbon isotopic change of selected fatty acids and archaeol derivatives after 50 days of incubation with a ^{13}C enriched carbonate pool ($\delta^{13}C$ ~ + 10,000‰). In all three incubations with methane, high isotopic shifts in AOM-specific

Table 2. Assimilation of inorganic carbon by methanotrophic communities with and without methane as energy source. Sediment slurries from Black Sea, Hydrate Ridge and Gullfaks were incubated for 50 days with labeled bicarbonate $\delta^{13}C$ ~+10000‰.

	Black Sea		**Hydrate Ridge**		**Gullfaks**	
	$\Delta\delta^{13}C$ (‰)		$\Delta\delta^{13}C$ (‰)		$\Delta\delta^{13}C$(‰)	
	+ CH4	Control	+ CH4	control	+ CH4	Control
Bacterial lipids						
C14:0	6	-1	446	6	212	77
i-C15:0	7	4	9	10	151	13
Ai-C15:0	25	1	23	-4	51	29
C16:1ω7c	116	7	155	69	59	3
C16:1ω5c	226	10	836	7	614	45
C16:0	13	-1	150	45	52	7
cyC17:0ω5,6	76	-2	203	11	159	14
isoprenFA19:0	21	1	-5			
C18:1ω7c	163	2	61	44	11	-1
C18:0	18	-3	8	6	11	16
Archaeal lipids						
Archaeol	37	34	106	38	40	5
sn2-hydroxyarch	16	23	99	24	1	6

fatty acids were observed, indicating *de novo* synthesis of microbial lipids. Especially $C_{16:1\omega5c}$ showed a high carbon uptake with $\Delta\delta^{13}C$ +225‰ (BS), +836‰ (HR) and +614‰ (GF). Other highly labeled compounds were $cyC_{17:0\omega5,6}$, $C_{14:0}$, $C_{16:1\omega7c}$ and $C_{16:1\omega7c}$. In the control experiment without methane, the incorporation of ^{13}C-label was orders of magnitudes lower

and not specific for AOM biomarkers. The archaeal community of HR and GF took up considerably more ^{13}C-labeled CO_2 into archaeol when methane was supplied as energy source. However, no effect of methane supply on assimilation of ^{13}C-labeled CO_2 into *sn2*-hydroxyarchaeol was detected in the GF community. Inorganic carbon assimilation by the archaeal community of the BS sediments was not affected by methane supply.

Discussion

Patterns of microbial lipids and their carbon isotopic compositions in cold seep sediments from Black Sea, Hydrate Ridge and Gullfaks

Cold seep sediments generally show a characteristic pattern of extremely ^{13}C-depleted specific archaeal and bacterial lipids, which originate from methanotrophic archaea and sulfate reducing bacteria involved in AOM (Hinrichs et al. 1999, 2000; Elvert et al. 1999, 2000; Boetius et al. 2000; Thiel et al. 2001).

Previous phylogenetic studies based on 16S rRNA gene analysis showed a dominance of ANME-2a/DSS consortia in seep sediments of HR (Boetius et al., 2000; Knittel et al., 2005) and of ANME-2c/DSS for GF (Wegener et al., 2008, this thesis, chapter II). The biomarker patterns found at both sites resembles those at other ANME-2/DSS dominated seep areas (Blumenberg et al., 2004, Elvert et al., 2005; Niemann and Elvert, in press). Specifically the ratios >3 of the FAs $C_{16:1\omega5c}$ relative to i-$C_{15:0}$ and the presence of substantial amounts of cy$C_{17:0\omega5,6}$ are in good agreements with a dominance of ANME-2 partner SRBs of the DSS cluster (Niemann and Elvert, in press). Similarly, a ratio of >1.5 of the archaeal lipids *sn2*-hydroxyarchaeol relative to archaeol are indicative for ANME-2 archaea (Blumenberg et al., 2004; Niemann and Elvert in press). Prior to the labeling experiments, the weighted average carbon isotope compositions of the dominant FAs in sediments of HR and GF were comparatively low with δ^{13}C-values of -43‰ (HR) and -51‰

(GF). Typical biomarkers of bacteria associated with AOM communities such as the FAs *i*-$C_{15:0}$, *ai*-$C_{15:0}$, $C_{16:1\omega5}$ and $cyC_{17:0\omega5,6}$ as well as the archaeal compounds archaeol and *sn2*-hydroxyarchaeol showed highly depleted $\delta^{13}C$-values (Tab. 1). Also more common lipids such as the ubiquitous FAs $C_{16:1\omega7c}$ and $C_{18:1\omega7c}$ showed relatively negative $\delta^{13}C$-values, indicating a substantial uptake of methane-derived carbon by the microbial community.

The BS biomarker pattern strongly deviated from that of the other two sites. Saturated fatty acids like $C_{16:0}$, $C_{14:0}$ and $C_{18:0}$ accounted for almost 70% of the extracted fatty acids. Monounsaturated fatty acids represent only about 20% of the fatty acid fraction. The Seep-SRB specific FAs *a*- and *ai*-C15:0 for $C_{16:1\omega5c}$ and $cyC_{17:0\omega5,6}$ account for 0% of the total FAs analyzed. With a weighted mean FA $\delta^{13}C$-value of -32‰, the FA fraction was only slightly depleted compared to marine non-seep FAs. The sediments of the Dniepr cold seeps used here showed a higher proportion of ANME-2 over ANME-1, and a large ratio of diverse unidentified bacteria (Arnds, unpublished data). A comparably high contribution of ANME-2 and associated SRB partner is also reflected in the biomarker pattern as shown by high rations of sn2-hydroxyarchaeol relative to archaeol (1.8) and the presence of substantial amounts of $cyC_{17:0\omega5,6}$. (Blumenberg et al., 2004; Niemann and Elvert, in press).

Carbon assimilation by microorganisms performing AOM

The very low $\delta^{13}C$-values of lipids from archaea and SRB involved in AOM have been explained by the assimilation of ^{13}C-depleted methane carbon associated with a strong carbon isotopic fractionation during methane uptake into biomass (Hinrichs et al., 1999; Elvert et al. 1999; Pancost et al. 2000; Thiel et al. 2001; Orphan et al., 2001a). Archaeol and hydroxyarchaeol of anaerobic methanotrophs are depleted in ^{13}C by around -50‰ relative to methane as carbon source (Hinrichs and Boetius, 2002; Niemann and Elvert, in press). Similar isotopic relationships were found for the investigated microbial communities from HR and GF (Table. 1; $\delta^{13}C$-CH_4 at HR -64.5‰ to -67.5‰ (Kastner et al., 1998); $\delta^{13}C$-CH_4 at

GF: -73.9‰ (Hovland, 2007)). Assuming an archaeal biomass composition of $C_4H_8O_2N$ (Harder and van Dijken, 1975), Nauhaus et al. (2007) suggested an approximately equal incorporation of methane and CO_2 by archaeal methanotrophs, simply because microbial biomass is more oxidized relative to methane according to eq. 3.

$$17CH_4 + 15CO_2 + 8NH_4^+ \rightarrow 8C_4H_8O_2N + 14H_2O + 8H^+ \quad (3).$$

The observed change in the isotopic composition by labeling both carbon sources, methane and inorganic carbon, could be explained by the stoichiometry shown in this formula. Additionally an assimilation of both carbon sources would reduce the energy demands of CO_2 reduction, which would be in particular favorable for ANMEs as they operate at minimal energy yields (Hoehler et al., 1994). However, it is also possible that the ANME assimilate only CO_2 similar to the phylogenetically related autotrophic methanogens (Sprott, 1993). Although our flow-through setup effectively dilutes ^{13}C-labeled bicarbonate produced from ^{13}C-methane oxidation, the oxidation of methane and the assimilation of methane-derived CO_2 could be coupled within the cell.

Like their archaeal partners, SRB involved in AOM have strongly depleted lipid biomarkers (Table 1), suggesting that they directly assimilate methane or indirectly as a methane-derived carbon intermediate. Hinrichs and Boetius (2002) pointed out that specific archaeal and bacterial lipid biomarker show relatively constant isotopic offsets to methane and porewater DIC, suggesting autotrophic carbon fixation as possible explanation for the carbon isotopic difference between archaeal and SRB biomarker lipids. Published δ^{13}C-values of seep porewater DIC are rare, however the isotope compositions of authigenic carbonates corresponds to the isotopic composition of pore water DIC while precipitation. The offset between carbonate precipitates and the biomass or lipid biomarker of the SEEP-SRBs is about -40 to -50‰ (Hinrichs et al., 2000; Orphan et al., 2002; Hinrichs and Boetius, 2002), which is in agreement with carbon fractionation by autotrophic SRB using the acetyl-

CoA-carbon monoxide dehydrogenase pathway (Preuß et al. 1989; Londry and Des Marais, 2003; Londry et al., 2004). Our data from stable isotope probing using ^{13}C-labelled methane and bicarbonate confirm that CO_2 is the main carbon source of the partner SRB involved in AOM and that direct methane uptake or uptake of methane-derived organic intermediates is not significant in lipid biosynthesis of syntrophic SRB involved in AOM.

Carbon assimilation efficiency of methanotrophic communities

As indicated above, we can assume an autotrophic CO_2 uptake by the partner SRB and a mixed uptake of methane and CO_2 by the methanotrophic archaea. Hence, we can use carbon isotope compositional changes of lipid biomarkers to calculate yield and growth efficiency of the methanotrophic communities according to eq. 2.

For the relation between specific lipid biomarker and cell biomass, we used data from a two-year pressure incubation with HR sediments by Nauhaus et al. (2007). During their experiment, a yield of 23 mg C of consortia biomass corresponded to a yield of 0.7 mg of the FA $C_{16:1w5c}$. For our HR sediment incubations of 176 days with labeled bicarbonate, we calculated an increase of 0.13 μg $C_{16:1\omega5c}$ per gram sediment. Using the biomass/lipid ratios from Nauhaus et al. (2007), this corresponds to an increase in methanotrophic consortia biomass of 4.1 mg C gdw^{-1}. A similar calculation resulted in values of 0.5 for $C_{14:0}$, 0.3 for archaeol, and 1.3 μmol C gdw^{-1} for hydroxyarchaeol, as carbon biomass yield of the methanotrophic community. Similarly, we calculated carbon incorporation yields between 0.1 and 1.4 for BS and 0.1 to 1.1 μmol gdw^{-1} for GF inoculate. These estimates have to be considered as rough approximations, as lipid/biomass ratio may be variable across different populations and environmental conditions.

During six months of incubation of HR sediments, 60 μmol C gdw^{-1} of methane were oxidized, of which, based on the yield of $C_{16:1\omega5c}$, 0.4 μmol C gdw^{-1} were assimilated into biomass, corresponding to a carbon assimilation efficiency of 0.6%. This value is

slightly lower than that found by Nauhaus et al. (2007). The higher methane pressure applied by these authors has likely resulted in higher growth yields due to more favorable thermodynamic conditions. Using the same approach, we calculated a carbon uptake efficiency of 1.3% for GF and only 0.3% for the BS inoculate. As previously suggested (e.g., Bian et al. 2001), the remarkably low growth rate and carbon assimilation efficiency of ANMEs and their syntrophic partners likely reflects the low energy yield of AOM, especially when considering the high energy demand of autotrophic growth.

Metabolic relations between methanotrophic archaea and sulfate reducing bacteria

Previous studies combining lipid biomarker analyses and microscopy suggested a syntrophic interaction between the archaeal methanotrophs and the SRB (Boetius et al., 2000; Orphan et al. 2001a; Niemann et al., 2006; Nauhaus et al. 2007). No known cultivated sulfate reducing bacteria or sulfate reducing archaea (i.e., *Archaeoglobus fulgidus*) are capable of using methane as sole electron donor. The coupling of methane oxidation to sulfate reduction likely involves a transfer of electrons from methanotrophic archaea to SRB. Here we found evidence for an AOM-dependent uptake of inorganic carbon into fatty acids of partner SRB. In contrast, we found no evidence of a significant assimilation of methane, or a direct intermediate thereof, by the SRB. This finding excludes methane-derived intermediates such as formate, acetate, methanol, or methylamines as carbon source, as well as heterotrophic growth on ANME biomass. However, our experiments do not exclude that these potential intermediates are used as energy sources, although it seems unlikely that such intermediates would only serve in catabolic and not in anabolic processes. As an energy source for the energetically expensive autotrophic fixation of CO_2, an electron transfer by membrane bound redox shuttles or a potential transduction via cytochromes seems most likely. Hydrogen transfer was previously excluded by other physiological experiments (Nauhaus et al. 2002; 2005; Moran et al., 2008). Based on chemical equilibrium calculations,

Widdel and Rabus (2001) postulated an electric potential for electron shuttles of -0.25 to -0.29 Volts. They proposed potential candidates such as the cofactor methanophenazine (estimated mid-point potential close to -0.255 V). The direct transport via cell membrane connections would avoid the diffusion loss of electron acceptors. Electron transfer from cells to solid minerals is known from iron reducing bacteria such as *Geobacter* (Bond et al., 2002; Bond and Lovley, 2003). These organisms use cell membrane cytochromes and nanowires to connect between cell and mineral (Mehta et al., 2005). Similar mechanisms may be used in the coupling of methane oxidation and sulfate reduction. Experiments using electrodes as alternatively electron acceptor might be promising in examining the metabolic connection within the AOM consortia.

Conclusions:

Anaerobic methanotrophic communities from three different cold seep sites incorporated methane-derived carbon into lipid membranes, when provided with methane as the sole energy source. We estimated that 0.25-1.3% of the total methane oxidized was assimilated into bulk methanotrophic biomass. Archaeal lipids showed a direct uptake of ^{13}C-labeled methane in a 1:1 ratio to CO_2. In the presence of methane as sole energy source, fatty acids from the syntrophic SRB were labeled with $^{13}CO_2$ but not with ^{13}C-methane, indicating an autotrophic life style of the SRB. We did not find evidence for a direct uptake of methane-derived carbon by SRB. This supports previous hypotheses of an electron transfer via redox active electron shuttles between the archaeal and bacterial partners in AOM.

Experimental procedures

Set up of flow through reactors

Preincubated sediment slurries from three different cold seep habitats, namely BS (1), HR (2) and GF (3) (for details, see Table 3) were used in the flow-through incubations. The BS sediment was sampled with the submersible JAGO in the vicinity of an active carbonate chimney. The sediments from HR as well as GF were retrieved by multiple corer sampling from

Table. 3: Origin and characterization of the inoculated seep sediments from the Black Sea, Hydrate Ridge and Gullfaks oil field.

	Black Sea	**Hydrate Ridge**	**Gullfaks**
Expedition	R/V Poseidon Pos217/3	R/V Sonne cruise SO165-2	R/V Heincke HE208
Sampling	2004	2002	2004
Location	44°46.413' N 031°58.201' E	44°34.203' N 125°08.771' W	61°28.860' N 002°40.500' E
Area	Dniepr	NE-Pacific	Northern North Sea
Water depth	326m	776m	150m
Sediment type	clay with authigenic carbonates	clay	medium grained sand
Methanotrophic community	Mixed ANME-1/2	ANME-2a dominated	ANME-2c dominated
Reference	Arnds (unpublished)	Knittel et al., 2005	Wegener et al., 2008

mats of thiotrophic bacteria (*Beggiatoa* sp.). All three seep habitats were sulfide rich (>5 mM) and characterized by high methane-driven sulfate reduction. The clayish sediments from the BS were not covered by thiotrophic mats because bottom waters of the BS are devoid of O_2 and NO_3^- (Murray et al., 1991).

Sediment samples from three different seep habitats were transferred to Duran bottles and kept under an anoxic methane atmosphere on a rolling table at temperatures of 4°C until use in flow through incubations. All processing of sediment slurries was performed in an anaerobic glove box to avoid contact with oxygen. The columns were filled

with sediment slurries diluted ~ 1:2 with sulfate reducer medium (Widdel and Bak, 1992), except for the coarse sandy sediments from GF which were used at in situ density.

A semi-continuous flow-through system was set up according to Figure 3. Sediment slurries were filled in glass cylinders onto glass frits. Columns were filled completely with anaerobic seawater medium (0.5 mM H_2S, 28 mM SO_4^{2-} and 30 mM HCO_3^-, enriched with sulfate reducer vitamins, rare elements; according to Widdel and Bak, 1992). Anaerobic medium was used for the circulation between reservoirs and replicate sediment columns. This setup preserved stable conditions and ensured the dilution of reaction products, primarily methane based bicarbonate, in a large DIC pool. For the methane enrichment, the headspace of the reservoir was filled with 0.15 MPa methane:CO_2 mixture (96:4), keeping a

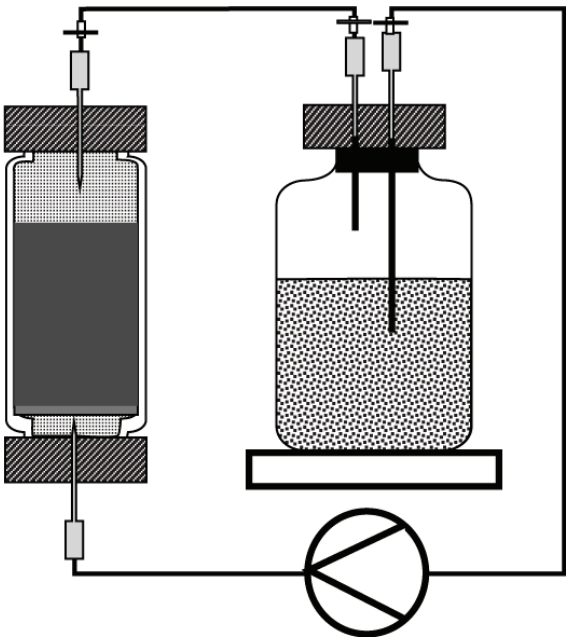

Figure 3. Simplified flow-through setup used for the carbon incorporation experiments with the sediment column (left) supplied with circulating medium from a reservoir (right).

methane concentration of about 2mM in the column inflow and a pH of 7.2. Gas-tight material (Viton®, DuPont Performance Elastomers, Willmington, US; and Tygon® HC, Saint-Gobain Performance Plastics Corporation, Akron, US) was used for all tubing to avoid contamination with oxygen or a loss of methane or CO_2. Resazurin ($NaC_{12}H_6NO_4$) was added to the medium as oxygen indicator. Metabolic activity was measured as changes

in the methane and sulfide concentrations of the in- and outflowing medium, respectively. Methane concentrations were measured from NaOH fixed water samples (0.5 ml) by gas chromatography (Hewlett Packard, 5890A with flame ionization detector, details Treude et al., 2003). Sulfide was determined using copper sulfate method from 0.1 ml triplicates (Cord-Ruwisch, 1985).

Labeling with ^{13}C-methane and -bicarbonate

Two replicate flow-through columns were set up from each of the three sediment slurries. In one experiment, the inorganic carbon pool of the reservoir medium was labeled with 11% ^{13}C-bicarbonate (calculated $\delta^{13}C$ ~+10000‰). In the other experiment, the methane atmosphere of the reservoir headspace was labeled with about 12% ^{13}C-methane ($\delta^{13}C$ ~+11000‰; measured on GC-IRMS - Thermo Finnigan, Delta V Advantage). The flow through columns of both experiments were subsampled three times within a time span of 176 ($\delta^{13}C$-HCO_3^- incubations) and 159 days ($\delta^{13}C$-methane incubations) for lipid biomarker analyses. The loss of sediment in each column due to subsampling (~ 8 g / 20g dry weight per sampling) was taken into account in the calculation of metabolic activities.

As a control experiment, the assimilation of $^{13}CO_2$ was compared in a batch incubation of a subsample of the three sediment slurries with 10% ^{13}C-labeled bicarbonate in the total bicarbonate pool and in the presence of methane (0.25 MPa ~ 3.1 mM) or N_2/CO_2 in the headspace of the reaction vessels. The experiment was carried out incubating duplicates of 7 g (BS and HR) and 20 g sediment (GF) dry weight in 100 ml of sulfate reducer medium (Widdel and Bak, 1992) in 250-ml Duran bottles filled closed with butyl stoppers.

Extraction and derivatization of *lipids*

Lipid extraction was carried out according to previously described methods (Elvert et al., 2003; Niemann et al., 2005). Briefly, total lipid extracts (TLE) were obtained by

suspending and sonicating wet sediments in organic solvents of decreasing polarity. Internal standards (*n*-nonadecanol and *n*-nonadecanoic acid) of known concentration and carbon isotopic composition were added prior to extraction. TLEs were saponified with methanolic KOH-solution (6%). After extraction of the neutral fraction from this mixture, FAs were methylated using BF_3 (14%) in methanol, yielding fatty acid methyl esters (FAMEs). Double bound positions of monoeoic FAs were determined by analyzing the dimethyl disulfide (DMDS) adducts of FAs according to a previous published method (Moss and Lambert-Fair, 1989).

Lipid analyses

FAs and archaeal glycerol diethers were quantified by gas chromatography-flame ionization detection (GC-FID), identified by gas chromatography-mass spectrometry (GC-MS, ThermoFinnigan, San Jose, CA), and their stable carbon isotopic compositions were determined by gas chromatography-combustion isotope ratio mass spectrometry (GC-IRMS). Instrument specifications and operation modes of the GC-FID and GC-MS systems can be found in Elvert et al. (2003) with modifications made by Niemann et al. (2006). For GC-IRMS measurements, a Finnigan Delta plus IRMS was used, connected via a Finnigan Combustion Interface III to a HP 6890 Series GC, equipped with a VF-5ms column (30 m, ID 0.25mm, film thickness 0.25 μm; Varian, Palo Alto, US). Selected samples were measured twice to control reproducibility. Standard deviations for replicate lipid analysis were on average 10%. Stable carbon isotope ratios of lipid compounds are given in the δ-notation against Vienna PeeDee Belemnite (V-PDB) with a precision of 1‰.

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References

Bian, L., Hinrichs, K.-U., Xie, T., Brassell, S.C., Iversen, N., Fossing, H. et al. (2001) Algal and archaeal polyisoprenoids in a recent marine sediment: Molecular isotopic evidence for anaerobic oxidation of methane, *Geochem Geophys Geosyst* **2**(1).

Blumenberg, M., Seifert, R., Nauhaus, K., Pape, T. and Michaelis, W. (2005) In vitro study of lipid Biosynthesis in an anaerobically methane-oxidizing microbial mat. *Appl Environ Microb* **71**: 4345-4351.

Blumenberg, M., Seifert, R., Reitner, J., Pape, T., and Michaelis, W. (2004) Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. *Proc Natl Acad Sci* **101**: 11111-11116.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623-626.

Bond, D.R. and Lovley, D.R. (2003) Electricity production by Geobacter sulfurreducens attached to electrodes. *Appl Environ Microb* **69**: 1548-1555.

Bond, D.R., Holmes, D.E., Tender, L.M., and Lovley, D.R. (2002) Electrode-reducing microorganisms that harvest energy from marine sediments. *Science* **295**: 483-485.

Cord-Ruwisch, R. (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *J Microbiol Meth* **4**(1): 33-36.

Elvert, M., Hopmans, E.C., Treude, T., Boetius, A., Suess, E., (2005). Spatial variations of methanotrophic consortia at cold methane seeps: implications from a high-resolution molecular and isotopic approach. *Geobiology* **3**: 195-209.

Elvert, M., Boetius, A., Knittel, K., and Jørgensen, B.B. (2003) Characterization of Specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing bacteria involved in anaerobic oxidation of methane. *Geomicrobiol J* **20**: 403-419.

Elvert, M., Suess, E. and Whiticar, M.J. (1999) Anaerobic methane oxidation associated with marine gas hydrates: superlight C-isotopes from saturated and unsaturated C 20 and C 25 irregular isoprenoids. *Naturwissenschaften* **86**(6): 295-300.

Elvert, M., Suess, E., Greinert, J., and Whiticar, M.J. (2000) Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone *Org Geochem* **31**(11): 1175-1187.

Girguis, P.R., Cozen, A.E. and DeLong, E.F. (2005) Growth and Population Dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Appl Environ Microb* **71**(7): 3725-3733.

Girguis, P.R., Orphan, V., Hallam, S. and DeLong, E.F. (2003) Growth and Methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. *Appl Environ Microb* **69**(9): 5472-5482.

Harder, W., and van Dijken, J.P. (1975) Theoretical considerations on the relation between energy production and growth of methane-utilizing bacteria. In *Microbial Production and Utilization of Gases*. Schlegel, H.G., Gottschalk, G., and Pfennig, N. (eds). Göttingen, Germany: Goltze, p. 403–418.

Hinrichs, K.-U. and Boetius, A. (2002) The anaerobic oxidation of methane: New insights in microbial ecology and biogeochemistry. In *Ocean Margin Systems*. Wefer, G., Billett, D., Hebbeln, D., Jørgensen, B.B., Schlüter, M., and van Weering, T. (eds). Berlin Heidelberg: Springer-Verlag, pp. 457-477.

Hinrichs, K.-U., Hayes, J.M., Silva, S.P., Brewer, P.G., and DeLong, E.F. (1999) Methane-consuming archaebacteria in marine sediments. *Nature* **398**: 802-805.

Hinrichs, K.-U., Summons, R.E., Orphan, V., Sylva, S.P., and Hayes, J.M. (2000) Molecular and isotopic analysis of anaerobic methane-oxidizing communities in marine sediments. *Org Geochem* **31**: 1685-1701.

Hoehler, T.M., Alperin, M.J., Albert, D.B. and Martens, C.S. (1994) Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium. *Global Biogeochem Cycles* **8**(4): 451-464.

Iversen, N., and Jorgensen, B.B. (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnol Oceanogr* **30**: 944-955.

Judd, A. G., Hovland, M., Dimitrov, L. I., Garcia Gil, S. and Jukes V. (2002) The geological methane budget at Continental Margins and its influence on climate change. *Geofluids* **2**(2), 109-126.

Kastner, M., Kvenvolden, K.A. and Lorenson, T.D. (1998) Chemistry, isotopic composition and origin of a methane-hydrogen sulfide hydrate at the Cascadia subduction zone. *Earth Planet Sc Lett* **156**(3-4): 173-183,

Knittel, K., Boetius, A., Lemke, A., Eilers, H., Lochte, K., Pfannkuche, O. et al. (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia margin, Oregon). *Geomicrobiol J* **20**: 269-294

Knittel, K., Lösekann, T., Boetius, A., Kort, R., and Amann, R. (2005) Diversity and distribution of methanotrophic archaea at cold seeps. *Appl Environ Microb* **71**: 467-479..

Londry, K.L., and Des Marais, D.J. (2003) Stable carbon isotope fractionation by sulfate-reducing bacteria. *Appl Environ Microb* **69**: 2942-2949.

Londry, K.L., Jahnke, L.L. and Des Marais, D.J. (2004) Stable carbon isotope Ratios of lipid biomarkers of sulfate-reducing bacteria. *Appl Environ Microb* **70**: 745-751.

Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B., Niemann, H., Boetius, A. and Amann, R. (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. *Appl Environ Microb* **73**: 3348-3362.

Martens, C.S. and Berner, R.A. (1974) Methane production in the interstitial waters of sulfate-depleted marine sediments. *Science* **185**: 1167 - 1169.

Mehta, T., Coppi, M.V., Childers, S.E. and Lovley, D.R. (2005) Outer Membrane c-Type Cytochromes Required for Fe(III) and Mn(IV) Oxide Reduction in Geobacter sulfurreducens. *Appl Environ Microb* **71**: 8634-8641.

Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M. et al. (2002) Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science* **297**: 1013-1015.

Moran, J.J., Beal, E.J., Vrentas, J.M., Orphan, V.J., Freeman, K.H. and House, C.H. (2008) Methyl sulfides as intermediates in the anaerobic oxidation of methane. *Environ Microbiol* **10**(1): 162-173.

Moss, C.W. and Lambert-Fair, M.A. (1989) Location of double bonds in monounsaturated fatty acids of Campylobacter cryaerophila with dimethyl disulfide derivatives and combined gas chromatography-mass spectrometry. *J Clin Microbiol* **27**: 1467-1470.

Murray, J.W., Top, Z. and Ozsoy, E. (1991) Hydrographic Properties and Ventilation of the Black Sea. *Deep-Sea Res DESRAY* **38**: 663-S689.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A., and Widdel, F. (2007) In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environ Microbiol* **9**(1): 187-196.

Nauhaus, K., Boetius, A., Krüger, M., and Widdel, F. (2002) In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediments from a marine gas hydrate area. *Environ Microbiol* **4**(5): 296-305.

Nauhaus, K., Treude, T., Boetius, A. and Krüger, M. (2005) Environmental regulation of the anaerobic oxidation of methane: a comparison of ANME-I and ANME-II communities. *Environ Microbiol* **7**(1): 98-106.

Niemann, H., and Elvert, M. (in press) Diagnostic lipid biomarker and stable carbon isotope signatures of microbial communities mediating the anaerobic oxidation of methane with sulphate. *Organic Geochemistry*, doi: 10.1016/j.orggeochem.2007.11.006

Niemann, H., Elvert, M., Hovland, M., Orcutt, B., Judd, A., Suck, I. et al. (2005) Methane emission and consumption at a North Sea gas seep (Tommeliten area). *Biogeosciences* **2**: 335-351.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K. et al. (2006) Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* **443**: 854-858.

Orcutt, B., Boetius, A., Elvert, M., Samarkin, V. and Joye, S.B. (2005) Molecular biogeochemistry of sulfate reduction, methanogenesis and the anaerobic oxidation of methane at Gulf of Mexico cold seeps. *Geochim Cosmochim Ac* **69**: 4267-4281.

Orphan, V.J., Hinrichs, K.-U., Ussler, W., III, Paull, C.K., Taylor, L.T., Sylva, S.P. et al. (2001b) Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Appl Environ Microb* **67**: 1922-1934.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., and DeLong, E.F. (2001a) Methane-Consuming Archaea Revealed by Directly Coupled Isotopic and Phylogenetic Analysis. *Science* **293**: 484-487.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., and DeLong, E.F. (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc Natl Acad Sci* **99**(11): 7663-7668.

Pancost, R., Damsté, J., de Lint, S., van der Maarel, M. and Gottschal, J. (2000) Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic archaea and bacteria. *Appl Environ Microb* **66**(3): 1126.

Preuß, A., Schauder, R. and Fuchs, G. (1989) Carbon isotope fractionation by autotrophic bacteria with three different CO2 fixation pathways. *Z. Naturforsch.* **C 44**, 397-402.

Reeburgh, W.S. (1976) Methane consumption in Cariaco Trench waters and sediments. *Earth Planet Sc Lett* **28**(3): 337-344.

Reeburgh, W. (2007) Oceanic methane biogeochemistry. *Chem Rev* **107**: 486-513.

Sprott, G., Ekiel, I. and Patel, G. (1993) Metabolic pathways in Methanococcus jannaschii and other methanogenic bacteria. *Appl Environ Microb* **59**(4): 1092-1098.

Stadnitskaia, A., Muyzer, G., Abbas, B. Coolen, M.J.L., opmans, E.C, Baas, M. et al. (2005) Biomarker and 16S rDNA evidence for anaerobic oxidation of methane and related carbonate precipitation in deep-sea mud volcanoes of the Sorokin Trough, Black Sea. *Mar Geol* **217** (1-2):67-96.

Teske, A., Hinrichs, K.U., Edgcomb, V., de Vera Gomez, A.., Kysela, D., Sylva, S.P. et al., (2002) Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. *Appl Environ Microb* **68**(4): 1994-2007.

Thiel, V., Peckmann, J., Richnow, H.H., Luth, U., Reitner, J. and Michaelis, W. (2001) Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and a microbial mat. *Mar Chem* **79**: 97-112.

Treude, T., A. Boetius, Knittel, K., Wallmann, K., Jorgensen, B.B. (2003) Anaerobic oxidation of methane above gas hydrates at Hydrate Ridge, NE Pacific Ocean. *Mar Ecol Prog Ser* **264**: 1-14.

Treude, T., Knittel, K., Blumenberg, M., Seifert, R., and Boetius, A. (2005) Subsurface microbial methanotrophic mats in the Black Sea. *Appl Environ Microb* **71**: 6375-6378.

Wegener, G., Shovitri, M. Knittel, K., Niemann, H. Hovland, M. and Boetius, A. (2008) methane seeps (Northern North Sea) Biogeochemical processes and microbial diversity of the Gullfaks and Tommeliten. *Biogeosciences discuss.* 5, 971–1015, www.biogeosciences-discuss.net/5/971/2008/.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes*. Balows A, T.H., Dworkin M, Harder W, Schleifer KH (ed). Berlin Heidelberg New York: Springer, pp. 3352-3378.

Widdel, F., and Rabus, R. (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotech* **12**: 259–276.

Chapter V

Concluding Remarks and Perspectives

Gunter Wegener

Conclusions

My doctoral thesis investigated anaerobic oxidation of methane (AOM) with a focus on the microbial ecology of shallow methane seep habitats, the physiology of AOM and the determination of carbon sources used by the organisms performing AOM.

In the first research manuscript of this thesis I presented our results on gas emissions, biogeochemistry and molecular ecology of several seepage areas in the North Sea, which we studied during 5 research cruises. Prior research on the ecology of methane rich habitats mainly focused on deep-sea cold seeps, such as Eel River Basin (Hinrichs et al., 1999; Orphan et al., 2002; Levin, 2005), Hydrate Ridge (Boetius et al., 2000; Knittel et al., 2003; Knittel et al., 2005) and Haakon Mosby Mud Volcano (Niemann et al., 2006; Lösekann et al., 2007), or seepage sites in the Gulf of Mexico (Orcutt et al., 2004; Orcutt et al., 2005). Studies on shallow water seep sites are rare (e.g., Santa Barbara hydrocarbon seeps (Montagna et al., 1986), and Skagerrak seeps (Dando et al., 1994)).

In the North Sea shallow gas accumulations, mostly sourced by leakage from deep thermogenic reservoirs, are common. Above several of these shallow gas accumulations we detected gas flares in the water column. Gas hydrate formation is not possible in the shallow sea because of low gas solubility at low pressure, therefore, gas flux in the North Sea seepage sites is due to quickly rising bubbles. Hence, the microbial barrier to methane loss to hydrosphere is less effective in these areas compared to deep sea seep areas, where free gas is rare. From rate measurements and bubble stream counts, we estimate that less than 20% of the rising methane was oxidized within the sediment of Gullfaks. This methane consumption is even less efficient than that estimated for the Beggiatoa fields of Haakon Mosby (~40%; Niemann et al., 2006), where methane oxidation efficiency is reduced by high pore water velocities, which prevent the intrusion of sulfate into the sediment. This highlights the particular relevance of shallow seep sites to the marine and global methane budget.

The active centers of the gas emissions at Gullfaks and Tommeliten are characterized by coverage with mats of sulfide oxidizing bacteria. At Gullfaks, we found those filamentous bacteria covering an area of about 0.1 km^2, representing the largest currently known active seep of the North Sea. At Tommeliten, methane leakage was limited to small patches (<0.3 m^2), which cover sand filled faults in the otherwise impermeable sediment. Chemosynthetic macrofauna, (e.g., bacterial symbiont hosting tube worms and bivalves), which are characteristic of deep sea vents, were absent at the North Sea seep sites. This might be due to the tidally forced, high bottom water currents, which prevent settlement of benthic organisms.

The lipid biomarker patterns of Gullfaks and Tommeliten were indicative of typical seep microorganisms: aerobic methanotrophs, anaerobic methanotrophs plus their partner SRBs and sulfide oxidizers. The very light average carbon isotope values of the lipid biomarker at both sites emphasized the importance of methane as an energy and carbon source in the seep sediments. Alternative carbon energy sources were rare at Gullfaks and Tommeliten, since we found low organic carbon sands at both seep sites. Also, we found a rather constant offset between the $\delta^{13}C$ carbon composition of methane and archaeal biomarkers and between inorganic carbon and biomarkers of sulfate reducing bacteria. This offset represents 40‰ to 50‰ in each case and is due to the carbon uptake patterns of those organisms, which were examined and discussed in chapter IV.

At both seepage sites, 16S rRNA based archaeal gene sequences were dominated by methanotrophs (ANME-2c at Gullfaks and ANME-2a at Tommeliten), which were highly similar to those from deepwater seepage sites. Bacterial 16S rRNA gene libraries from both sites contained sequences of the different Seep-SRB clusters. At Tommeliten many sequences were attributed to *Desulfobulbu*s, though we found only a few single cells of their typically associated partner ANME. In summary, the lower pressure, seasonal cycles, and on-average higher temperature of the North Sea did not appear to affect microbial seep

communities of Tommeliten and Gullfaks – i.e. a unique phylogenetic group of coastal or shallow-water methane oxidizers was not detected. In fact, sequences were highly similar to those observed in distant deep sea seep sites such as the Eel River Basin (coast of California), Hydrate Ridge (coast of Oregon), Black Sea and Gulf of Mexico. Without having statistical analysis, it seems that geographical distance is of minor importance for the distribution pattern of the ANME strains. Rather, other geochemical factors appear to be the crucial factor regulating the distribution of the different methanotrophs. For example, the methanotrophic community pattern in the Tommeliten bacterial patches was completely different from the Tommeliten subsurface SMTZ. Because of the limited sediment sampling and the small sample sizes we obtained in this project, an extensive geochemical characterization was not possible. For further studies comprehensive geochemical analysis should be performed to outline the factors selecting for the different ANME groups.

In the second research project, I set up an anaerobic flow-through system, in which physiological parameters of methanotrophy were examined. At steady conditions of ~1.5 atm CH_4, methane oxidation and sulfate reduction were constant and at high levels, although the reactants were not depleted at any time. We argue that threshold methane concentrations, probably in the range of several atmospheres, are necessary to stimulate detectable growth (with doubling times < 1 year). Similar behavior was found for aerobic soil methanotrophs, however at different scales of methane. Below a methane concentration of 7000 pm, linear methane consumption was measured, indicating a non growing community. At higher methane concentrations methane consumption increased exponentially, showing exponential growth (Bender and Conrad, 1995).

A nearly linear relationship between methane concentration (up to 2.3 mM) and methane consumption was observed for our inoculates, indicating extraordinarily high half saturation (K_M) values above the tested concentrations. In contrast, the K_M value for H_2 in hydrogenothrophic sulfate reduction is orders of magnitudes lower with 141 Pa (~1 μM;

Lovley et al., 1982). The high K_M values are due to enzymes catalysis of methane activation. These enzymes are very similar to those which efficiently catalyze the final step of methanogenesis (Krüger et al., 2003). Hence, catalyses with this enzyme package in the reverse direction must be of restricted efficiency.

Future growth and half saturation experiments should be performed over wide methane ranges (the upper limit is determined by gas hydrate stability) to cover environmentally possible conditions. Recently, a high pressure flow-through setup was developed which successfully reproduced high methane consumption rates (Deusner, unpublished). This setup combines the advantages of large methane pressure ranges with constant low AOM product concentrations. Further kinetic studies, especially those examining the K_M values of methane should be performed using this setup. Preferentially, enrichments with a clear dominance of a single ANME strain should be tested, since different strains have shown different growth characteristics (Holler, unpublished).

In the third research project we focused on the role of methane and CO_2 as carbon sources for microorganisms performing AOM. With a combination of stable isotope probing and lipid biomarker isotope analyses, even low-level carbon assimilation into biomass was detectable. This was necessary as prior growth experiments indicated extremely low carbon assimilation rates during AOM (Girguis et al., 2003, 2005; Niemann et al., 2006).

We inoculated sediments from three well-described seep areas (Hydrate Ridge, Black Sea, Gullfaks) with ^{13}C labeled methane or ^{13}C labeled bicarbonate for approx. 6 months. For all inoculates we observed that lipids attributed to sulfate reducing bacteria were almost exclusively labeled by inorganic carbon. In an additional experiment we showed clearly that the inorganic carbon fixation by the SRB is strictly methane oxidation dependent. In contrast, methanotrophic archaea showed similar carbon assimilation from

both carbon sources. These results suggest that methanotrophic archaea combines both carbon sources to form biomass whereas the partner SRB is autotrophic.

The observed carbon assimilation patterns explain the carbon isotope relationship between carbon sources and lipid derived biomarker found in active AOM zones. For lipids derived from SRB, constant offsets relative to carbonate are due to inorganic carbonate assimilation. The carbon in archaeal lipids derives to about 50‰ from methane, which is, in natural sediments, expressed in extremely ^{13}C depleted carbon compositions.

We argue that the reductive acetyl-CoA-carbon monoxide dehydrogenase pathway (CODH; also known as Wood-Ljungdahl pathway; Morton et al., 1991) is the anabolic pathway of the consortial SRB. The CODH pathway catalyzes carbon fixation in many other sulfate reducing bacteria (Preuß et al., 1989) and also shows the highest carbon fractionation known for autotrophy (Londry and Des Marais, 2003; Londry et al., 2004).

It is likely that ANMEs also use the CODH-pathway, as this is true for most other Euryarchaeota, especially methanogens (Bhatnagar et al., 1991; Thauer, 1998; Lindahl and Chang, 2001). In metagenome surveys Meyerdierks et al. (2005) found several ORFs (open reading frames) encoding for the CODH pathway. If this is true, carbon monoxide uptake and direct incorporation of acetate into the cell biomass must also be possible as previously demonstrated for methanogens (Sprott et al., 1993). Facultative uptake of acetate may also explain the less depleted carbon isotope compositions of archaeal biomarkers in less active methane seep areas.

The formation of biomass using the CODH pathway could explain the observed direct incorporation of methane into ANME biomass. In methanogens carbon fixation using CODH it involves the formation of methyl groups by CO_2 reduction (fig. 1). Methanotrophic archaea might able to shuttle methyl groups directly from their catabolic pathway which presumably starts with the methylization of methane catalyzed by a methyl-CoA-reductase (MCR) catalyzed radical reaction (Krüger et al., 2003).

Future work on AOM physiology should include labeling of alternative carbon sources such as acetate, the end product, and carbon monoxide, the intermediate in the CODH pathway. The comparison of deuterium labeling of water and methane might also raise interesting results. Knowledge about the origin of hydrogen in biomass might help to reconstruct carbon assimilation in ANMEs. The analyses of carbon stable isotope probing experiments with Nano-SIMS (secondary ion mass spectrometry) coupled with in situ hybridization (ISH) would allow the tracking of carbon flows on the spatial resolution of cells (discussed in the perspectives below).

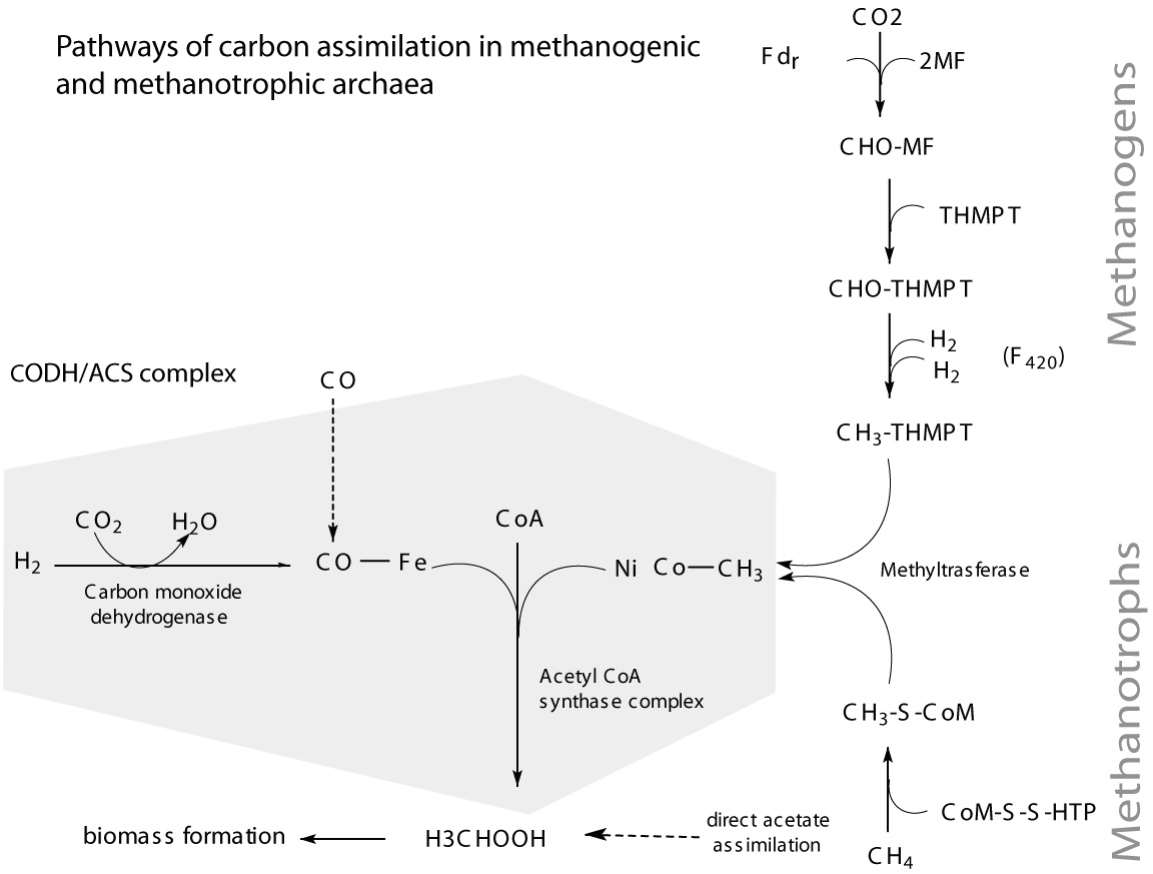

Fig. 1. Carbon fixation in methanogenic and methanotrophic archaea using the CODH (carbon monoxide dehydrogenase) pathway (after Hallam et al., 2004; Lessner et al., 2006; Ragsdale, 2007). Acetate is synthesized in CODH/ACS complexes from carbon monoxide and a methyl group. In methanogens the methyl group is formed by the reduction of CO_2 (Lessner et al., 2006). Methanotrophic archaea may channel methyl group from CH_4 as suggested by Hallam et al. (2004). The dashed arrows mark the uptake possibilities of alternative carbon sources.
(Fdr, reduced ferredoxin; MF, methanofuran; THMPT, Tetrahydromethan-opterin; HSCoM, coenzyme M , F420, H_2 dehydrogenase complex; MP, methanophenazine)

We calculated carbon assimilation efficiency into microbial biomass of 0.3% to 1.3% (mol% organic carbon/ mol carbon oxidized) in AOM. This is consistent with earlier studies of Nauhaus et al. (2007) who calculated, based on enrichment attempts, carbon uptake efficiency of about 1%. The extremely low carbon uptake efficiencies of AOM demonstrate the challenge of enriching anaerobic methanotrophs. In comparison, slow growing methanogens show carbon uptake efficiencies of ~5% (acetogenic M; Weimer, 1978) to ~7.5% (CO_2-reduction; Lupton and Zeikos, 1984).

Our results indicate that the transfer of organic intermediates such as formate, acetate or methylamine between ANME and SRB is unlikely, as the use of those compounds in catabolism, but not anabolism, would be energetically counterproductive. Additionally, previous experiments also excluded hydrogen as an intermediate (SRB do not instantly oxidize hydrogen (Nauhaus et al., 2002), and methane consumption was not inhibited by hydrogen addition (results not shown)). Furthermore, extracellular redox shuttles have been tested, but did not influence methanotrophy (Basen and Holler, unpublished). Hence, direct electron transfer seems to be the most likely mechanism for the connection between sulfate reducers and methanotrophic archaea. Terminal electron transfer to solid metal phases is known in the iron reducer *Geobacter,* which uses outer membrane cytochromes (Mehta et al., 2005) or nanowires (Reguera et al., 2005) to directly transfer electrons to mineral surfaces. Reguera and coworkers speculate that electron transfer through nanowires is also possible for cell-cell interactions, which could include AOM. Electron transfer between methanotrophic archaea and sulfate reducing bacteria would present novel way of tight syntrophy between organisms. Experiments examining extracellular electron transfer are in progress. Basen and coworkers try to describe the potential terminal electron transfer from ANMEs to bacterial sulfate reducers by charged electrodes (see below).

Perspectives: Stable isotope probing in AOM research.

Research on AOM has progressed significant within the last few years. However, the metabolic relationship within the consortia, carbon flows into the consortia, and the accompanied stable isotope pattern of the consortial biomass are not fully understood. Stable isotope probing has strong potential to contribute to this topic. Some interesting experimental possibilities are cited here and described in detail below:

- Acetate labeling to test an alternative carbon source for organisms performing AOM
- Hydrogen labeling of water and methane to distinguish archaeal carbon sources in methanotrophic aggregates
- Combination of stable isotope labeling and electron transfer to electrodes to describe the electron transfer gap
- Nano-SIMS, ISH and SIP (stable isotope probing) combination to describe carbon source assimilation on phylogenetic levels

Acetate incorporation into archaeal biomass.

Acetate is not the primary energy source for methanotrophic archaea, but might be assimilated as an alternative carbon source if present. Acetate incorporation would potentially save energy for those organisms living on substrates with extremely low energy yields. Heterotrophic growth is widespread in the Kingdom *Euryarchaeota*, even in CO_2-reducing methanogens. Acetate uptake might also explain the different carbon isotope patterns of the different ANME groups. As a carbon source acetate might explain the preferential growth of one or other archaeal groups of archaea in methanotrophic environments. To unravel this question I recommend lipid biomarker studies that compare ^{13}C based carbon uptake on acetate, methane and carbonate.

Hydrogen labeling of water and methane to test autotrophy.

In our carbon source labeling study, we demonstrated mixed incorporation of methane and carbonate into the archaeal biomass at a ratio of 1:1. This is consistent with a redox state equilibration of both compounds to organic biomass (Nauhaus et al., 2007). However, the measured carbon source assimilation might also be an artifact of autotrophic CO_2 fixation, since DIC is a product of AOM.

To determine the pathways of carbon fixation, parallel incubations of anaerobic methanotrophs while labeling deuterium of methane and water might be a promising approach. If the archaea incorporate methane-derived methyl groups into biomass (as indicated by our results, and illustrated in fig. 1), then a deuterium labeling signal in the biomass should be predominantly found while D-labeling of methane. If, in contrast, the archaeal methanotroph fixes only CO_2, the deuterium labeling of methane will not be recovered in the archaeal biomass (lipids).

Closing the electron transfer gap. Combining stable isotope labeling with electron transfer to electrodes

Numerous studies have proposed that AOM is performed in a tight syntrophic coupling of archaeal methane oxidation and bacterial sulfate reduction. However, the electron sharing mechanism is not understood; in fact, most theoretically possible energy-sharing mechanisms have been excluded (Nauhaus et al., 2002; Wegener unpublished). Hence, extra-cellular electron transfer, performed via cytochromes or nanowires is the most likely mechanism to couple the metabolism of ANME and SRB. Extra-cellular electron transfer has been described in the last decade for several organisms, including *Geobacter spp.*, which generates ATP by using solid phase iron oxides as the terminal electron acceptor. These organisms create microanodes which transfer the electrons from the cell directly to the minerals. It is also possible to harvest energy from *Geobacteraceae* (fuel cell) or let these

organisms grow providing a positively charged electrode as the sole electron acceptor (Bond et al., 2002). A similar mechanism is conceivable for methanotrophic consortia. The incubation of AOM enrichments without sulfate but with a cathode providing an electric potential, suitable for electron transfer, is a promising approach to investigate this theory. Currently a setup is in use to test this theory. This could also be combined with lipid isotope probing experiments in combination with lipid biomarker analyses or NanoSIMS (discussed below) to show activity and carbon uptake of archaeal methanotrophs decoupled from sulfate reducing bacteria.

New technologies: The combination of Nano-SIMS, ISH and SIP

The coupling of in situ oligonucleotide hybridization and secondary ion mass spectrometry (SIMS) has been demonstrated as a powerful tool for resolving the origin and spatial distribution of isotopic signals on the AOM-aggregate level (Orphan et al., 2001). The recent further development to Nano-SIMS enhances the resolution of this method to the cell and sub-cell level. In combination with in situ oligonucleotide labeling this can be a new powerful tool, to link identity and carbon or nutrient uptake into microorganisms (Kuypers and Jørgensen, 2007). Instead of a fluorescent dye, oligonuclides are labeled with rare halogen ions or radionucleotides, whose mass can be detected via SIMS. Although a name for this new method combination does not exist yet, this approach has a huge potential for helping to understand carbon flows within the organism. Coupling of SIMS with 16S rRNA-based oligonucleotide methods can resolve carbon or other elemental uptake by cells at defined phylogenetic levels as recently shown in pioneering work by Li et al. (2008). For mixed cell cultures Li and coworkers showed preferred uptake of glucose (^{13}C-labeled) and ammonium (^{15}N-labeled) into *Escherichia coli* cells

In AOM research the final goals of this new method are: determination of carbon sources in different, phylogenetically defined methanotrophic organisms, clarification of

metabolic relationship within the consortia and finally, the determination of factors for the different environmental ANME/SRB associations.

References:

Bender, M. and Conrad, R. (1995) Effect of methane concentrations and soil conditions on the induction of methane oxidation activity. *Soil Biol. Biochem.***27:**1517–1527.

Bhatnagar, L., Jain, M. and Zeikus, J. (1991) Methanogenic bacteria. *Variation in autotrophic Life,* Academic Press, NY: 251–270.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623-626.

Dando, P.R., Bussmann, I., Niven, S.J., O'Hara, S.C.M., Schmaljohann, R. and Taylor, L.J. (1994) A methane seep area in the Skagerrak, the habitat of the pogonophore Siboglinum poseidoni and the bivalve mollusc Thyasira sarsi. *Mar. Ecol. Prog. Ser.* **107**: 157–167.

Girguis, P.R., Cozen, A.E. and DeLong, E.F. (2005) Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Appl. Environ. Microb.* **71**: 3725-3733.

Girguis, P.R., Orphan, V., Hallam, S. and DeLong, E.F. (2003) Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. *Appl. Environ. Microb.* **69**: 5472-5482.

Hallam, S.J., Putnam, N., Preston, C.M., Detter, J.C., Rokhsar, D., Richardson, P.M. and DeLong, E.F. (2004) Reverse methanogenesis: Testing the hypothesis with environmental genomics. *Science* **305**: 1457-1462.

Hinrichs, K.-U., Hayes, J.M., Silva, S.P., Brewer, P.G. and DeLong, E.F. (1999) Methane-consuming archaebacteria in marine sediments. *Nature* **398**: 802-805.

Knittel, K., Lösekann, T., Boetius, A., Kort, R. and Amann, R. (2005) Diversity and distribution of methanotrophic archaea at cold seeps. *Appl. Environ. Microb.* **71**: 467-479.

Knittel, K., Boetius, A., Lemke, A., Eilers, H., Lochte, K., Pfannkuche, O. et al. (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia margin, Oregon). *Geomicrobiol. J.* **20**: 269-294.

Krüger, M., Meyerdieks, A., Glöckner, F.O., Amann, R., Widdel, F., Kube, M. et al. (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878-881.

Kuypers, M.M.M. and Jørgensen, B.B. (2007) The future of single-cell environmental microbiology. *Environ. Microbiol.* **9**: 6-7.

Lessner, D.J., Li, L., Li, Q., Rejtar, T., Andreev, V.P., Reichlen, M. et al. (2006) An unconventional pathway for reduction of CO_2 to methane in CO-grown

Methanosarcina acetivorans revealed by proteomics. *Proc. Natl. Acad. Sci.* **103**: 17921-17926.

Levin, L.A. (2005) Ecology of cold seep sediments: Interactions of fauna with flow, chemistry and microbes. In *Oceanography and Marine Biology: An Annual Review*. Gibson, R.N., Atkinson, R.J.A. and Gordon, J.D.M. (eds): Taylor & Francis, pp. 1-46.

Li, T., Wu, T.-D., Mazeas, L., Toffin, L., Guerquin-Kern, J.-L., Leblon, G. and Bouchez, T. (2008) Simultaneous analysis of microbial identity and function using NanoSIMS. *Environ. Microbiol.* **10**: 580-588.

Lindahl, P. and Chang, B. (2001) The evolution of acetyl-CoA synthase. *Origins Life Evol. B.* **31**: 403-434.

Londry, K.L. and Des Marais, D.J. (2003) Stable carbon isotope fractionation by sulfate-reducing bacteria. *Appl. Environ. Microbiol.* **69**: 2942-2949.

Londry, K.L., Jahnke, L.L. and Des Marais, D.J. (2004) Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. *Appl. Environ. Microb.* **70**: 745-751.

Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B., Niemann, H., Boetius, A. and Amann, R. (2007) Diversity and abundance of aerobic and anaerobic methane oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. *Appl. Environ. Microb.* **73**: 3348-3362.

Lovley, D.R., Dwyer, D.F. and Klug, M.J. (1982) Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. *Appl Environ. Microbiol.* **43**: 1379-1379.

Mehta, T., Coppi, M.V., Childers, S.E. and Lovley, D.R. (2005) Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in Geobacter sulfurreducens. *Appl. Environ. Microb.* **71**: 8634-8641.

Meyerdierks, A., Kube, M., Lombardot, T., Knittel, K., Bauer, M., Glöckner, F.O. et al. (2005) Insights into the genomes of archaea mediating the anaerobic oxidation of methane. *Environ. Microbiol.* **7**: 1937-1951.

Montagna, P.A., Bauer, J.E., Prieto, M.C., Hardin, D. and Spies, R.B. (1986) Benthic metabolism in a natural coastal petroleum seep. *Mar. Ecol. Prog. Ser.* **34**: 31-40.

Morton, T., Runquist, J., Ragsdale, S., Shanmugasundaram, T., Wood, H. and Ljungdahl, L. (1991) The primary structure of the subunits of carbon monoxide dehydrogenase/acetyl-CoA synthase from Clostridium thermoaceticum. *J. Biol. Chem.* **266**: 23824-23828.

Nauhaus, K., Boetius, A., Krüger, M. and Widdel, F. (2002) In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediments from a marine gas hydrate area. *Environ. Microbiol.* **4**: 296-305.

Nauhaus, K., Albrecht, M., Elvert, M., Boetius, A. and Widdel, F. (2007) In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environ. Microbiol.* **9**: 187-196.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K. et al. (2006) Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* **443**: 854-858.

Orcutt, B., Boetius, A., Elvert, M., Samarkin, V. and Joye, S.B. (2005) Molecular biogeochemistry of sulfate reduction, methanogenesis and the anaerobic oxidation of methane at Gulf of Mexico cold seeps. *Geochim. Cosmochim. Ac.* **69**: 4267-4281.

Orcutt, B.N., Boetius, A., Lugo, S.K., MacDonald, I.R., Samarkin, V.A. and Joye, S.B. (2004) Life at the edge of methane ice: microbial cycling of carbon and sulfur in Gulf of Mexico gas hydrates. *Chem. Geol.* **205**: 239-251.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D. and DeLong, E.F. (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc. Natl. Acad. Sci.* **99**(11), 7663-7668. .

Preuß, A., Schauder, R. and Fuchs, G. (1989) Carbon isotope fractionation by autotrophic bacteria with three different CO 2 fixation pathways. *Z. Naturforsch.* **44**: 397–402.

Ragsdale, S.W. (2007) Nickel and the carbon cycle. *J. Inorg. Biochem.* **101**: 1657-1666.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T. and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098-1101.

Sprott, G., Ekiel, I. and Patel, G. (1993) Metabolic pathways in Methanococcus jannaschii and other methanogenic bacteria. *Appl. Environ. Microb.* **59**: 1092.

Thauer, R. (1998) Biochemistry of methanogenesis: A tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. *Microbiology* **144**: 2377-2406.

Field Experience

Mar. 2006 RV SONNE **Cruise**, **SO191-2** IfM-GEOMAR (COMET/MUMM)
Investigation of new methane driven habitats on the New Zealand continental margin using biogeochemical and microbiological methods

Apr. 2006 RV METEOR/ROV QUEST **Cruise M67/2b**, RCOM Bremen
Quantification of microbial activity in different habitats of the Campeche Asphalt Volcano sulfate reduction measurements by "In situ incubation (Insinc)"

Nov. 2005 RV Heincke **Cruise HE 242,** BGR Hannover. (DFG project RE 2424/1-1 'Nordsee') Mapping of potential seep structures in the North Sea.

Sept. 2005 RV Alkor **Cruise AL267**: (MUMM test cruise)
Microbial turnover at North Sea seep sites Tommeliten and Gullfaks, Organic geochemistry, biogeochemistry and microbial ecology, sea floor mapping

May 2004 RV Heincke **Cruise HE208**
Investigation of microbial communities at different seep sitesof the North Sea with emphasis on Gullfaks seep (METROL)

Presentation List

Oral presentation: G.Wegener, H. Niemann, M. Elvert, K.-U. Hinrichs, & A. Boetius (2008) Assimilation of methane and inorganic carbon by microbial communities mediating the anaerobic oxidation of methane. AMO-meeting, Aselage, Germany.

Oral presentation: G. Wegener, H. Niemann, M. Elvert, K.-U. Hinrichs, & A. Boetius (2007): The combination of isotope labeling and biomarker analyses to trace carbon flows in anaerobic methanotrophy. Geologische Vereiningung

Oral presentation: G. Wegener, M. Bowles, J. Felden, F. Wenzhöfer, F. Schubotz, K.-U. Hinrichs, M. Zabel, G. Bohrmann and A.Boetius. (2007) Microbial activity associated with asphalt volcanism at the Campeche Knolls, Gulf of Mexico (Results from research cruise Meteor M67/2b), Goldschmidt Conference, Cologne, Germany

Oral presentation: G. Wegener, H. Niemann, M. Elvert & A. Boetius: (2007) Which microorganisms benefit from methane oxidation in seep sediments: Tracing carbon sources by isotope labeling experiments. EGU, Vienna, Austria

Poster presentation: G. Wegener, M. Shovitri, H. Niemann, K. Knittel, M. Hovland, A. Boetius (2007) Active methane seepage in the North Sea: Gullfaks and Tommeliten, EGU, Vienna, Austria

Poster presentation: Gunter Wegener, M. Shovitri, H. Niemann, K. Knittel, M. Hovland, A. Boetius (2007) Active methane seepage in the North Sea: Gullfaks and Tommeliten, EGU, Vienna, Austria

Oral presentation: G. Wegener (2007) Methane based ecosystems in the deep sea. Auckland University of Technology. Host: Dr. Lindsey Zemke-White

Poster presentation: G. Wegener, M. Shovitri, H. Niemann, M. Hovland, G. Wendt, A. Boetius (2005) Gullfaks seep area: Anaerobic oxidation of methane in marine sediments. EGU, Vienna, Austria

Oral Presentation: G. Wegener (2005): Life without oxygen. Max Planck PhD Earth Science Seminar. Jena, Germany

Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit erkläre ich, dass ich die vorliegende Arbeit

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- keine anderen, als die von mir im Text angegebenen Quellen und Hilfsmittel benutzt habe,

- die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, 19. März 2008

Gunter Wegener