Microbial Aspects of Anaerobic Methane Oxidation with Sulfate as Electron Acceptor

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Table of contents

Abstract

Anaerobic oxidation of methane (AOM) is an important methane sink in the ocean but the microbes responsible for AOM are as yet resilient to cultivation. It was shown that AOM was coupled to sulfate reduction (SR) and this gave rise to current research which aims to develop a biotechnological process in which methane is used an electron donor for SR.

This thesis describes the microbial analysis of an enrichment capable of high rate AOM (286 $\mu mol.g_{dry\ weight}^{-1}.day^{-1}$) coupled to SR using a novel submerged membrane bioreactor system. Initially AOM rates were extremely low (0.004 mmol L^{-1} d^{-1}), but AOM and SR increased exponential over the course of 884 days to 0.60 mmol L^{-1} d^{-1}. The responsible organisms doubled every 3.8 months.

By constructing a clone library with subsequent sequencing and fluorescent in situ hybridization (FISH), we showed that the responsible methanotrophs belong to the ANME-2a subgroup of anaerobic methanotrophic archaea, and that sulfate reduction is most likely performed by sulfate reducing bacteria commonly found in association with other ANME related archaea in marine sediments. Another relevant portion of the bacterial sequences can be clustered within the order of *Flavobacteriales* but their role remains to be elucidated. FISH analyses showed that the ANME-2a cells occur as single cells without close contact to the bacterial syntrophic partner. Incubation with ^{13}C labeled methane showed substantial incorporation of ^{13}C label in the bacterial C_{16} fatty acids (bacterial; 20, 44 and 49%) and in archaeal lipids, archaeol and hydroxyl-archaeol (21 and 20%, respectively). This confirms that both archaea and bacteria are responsible for the anaerobic methane oxidation in a bioreactor enrichment inoculated with Eckernförde bay sediment. To unravel the pathway of this syntrophic conversion, the effect of possible intermediates on AOM and SR was assessed.

To investigate which kind of waste and process streams can be treated by the methanotrophic sulfate-reducing enrichment, the effect of environmental conditions and different substrates was assessed. The optimum pH, salinity and temperature for SR with methane by the enrichment were 7.5, 30‰ and 20°C, respectively. The biomass had a good affinity for sulfate (K_m < 1.0 mM), a low affinity for methane (K_m > 75 KPa) and AOM was completely inhibited at 2.4 (±0.1) mM sulfide. The enrichment utilized sulfate, thiosulfate, sulfite and elemental sulfur as alternative electron acceptors for methane oxidation and formate, acetate and hydrogen as

alternative electron donors for sulfate reduction. As a co-substrate for methane oxidation only methanol stimulated the conversion of ^{13}C labeled CH_4 to $^{13}CO_2$ in batch incubations of Eckernförde bay sediment, other possible co-substrates had a negative effect on the AOM rate.

The research described in this thesis shows the possibility of enriching slow growing methane oxidizing communities but also shows the difficulties in applying this process for a biotechnological purpose because of the extreme slow doubling times and the lack of understanding of the metabolic routes used by these organisms.

Chapter 1

Introduction

Introduction

This introduction will give an overview of the importance of oxidation products and substrates in anaerobic environments. To understand the importance of the production and consumption of methane in the global carbon and sulfur cycle some major sources and sinks will be described. After that the microbial processes of anaerobic methane oxidation and the characterization of the responsible organisms will be discussed and finally the application of methane as electron donor for sulfate reduction will be discussed.

1.1 Methane properties

Methane is the smallest molecule of all organic compounds and it is the most reduced form of carbon (oxidation state -4). CH_4 is the main component of natural gas (70-95%) and biogas (50-70%). The energy yield per carbon during oxidation is for CH_4 higher than for other hydrocarbons or coal. Therefore, less CO_2 is produced when natural gas or biogas is used as fuel or as energy source for microorganisms.

Methane (CH_4) is a tetrahedral shaped molecule and is, at standard conditions, a colorless and odorless gas. CH_4 gas is only flammable when the concentration in the air is between 5 and 15%. Methane is a non-polar molecule and has therefore a relatively low solubility in water (1.44 mM in distilled water at 20°C and 0.101 MPa CH_4; Yamamoto *et al.*, 1976). Its solubility depends on salinity, temperature and hydrostatic pressure. CH_4 is a non-toxic gas which can dilute or displace the oxygen containing atmosphere. About 2.7 million years ago, CH_4 was a major component in the earth's atmosphere (Chang *et al.*, 1983). Since then the atmosphere became more oxidized. In 1998, the average atmospheric CH_4 concentration was 1.7 ppm (UN Environment Program, 2001; Nakaya *et al.*, 2000). Compared with other alkanes, CH_4 has an unusually high C-H bond strength, making it more resistant to radicals than other alkanes. The dissociation energy of the C-H bond in CH_4 is +439 kJ/mol (Thauer and Shima, 2008). CH_4 is the least reactive alkane in reactions involving hydride abstraction by an electrophile, because the C-H bond is not polarized (Crabtree, 1995). This all also makes CH_4 a difficult substrate for microorganisms.

Methane is the main component in the atmosphere of Jupiter, Saturn, Uranus and Neptune (Beyer and Walter, 1991). The methane concentration of the Earth's atmosphere has increased by 145% since 1800 (ARM, 2001). This increase coincides

with the onset of industrialization and roughly parallels world population growth, pointing to anthropogenic sources as the cause. Methane has the ability to trap and re-emit infrared radiation. It therefore belongs to the greenhouse gases and with other greenhouse gases methane is jointly responsible for global warming. Methane is 21 times more effective at trapping heat in the atmosphere than carbon dioxide. Today, its contribution to global warming is about 20%.

1.2 Sources of methane

Methane in the form of fossil natural gas is one of the main global energy sources. Natural gas is a combustible mixture of hydrocarbon gases. While natural gas consists primarily of methane (up to 87% by volume), it also contains ethane, propane, butane and pentane. The largest reservoirs of methane are located in natural gas, gas hydrates and petroleum deposits (Gornitz *et al.*, 1994; Kvenvolden, 1995) and consist of ancient deposits of organic matter that has been decomposing for millions of years. Next to these ancient deposits also more recent deposits and sources can be found that contribute to the global methane emissions such as wetlands, rice fields and ruminants like cattle being the most important. Smaller methane emissions take place in landfills, biomass burning, in marine and freshwater sediments, during gas and oil production and in the guts of termites.

Table 1.1: Global methane emission sources and sinks (after Houweling, 1999).

Sources	Methane emission (Tg of CH_4 per year)	Percentage (%)[a]
Natural sources		
Wetlands	92–237	15–40
Termites	20	3
Ocean	10–15	2–3
Methane hydrates	5–10	1–2
Subtotal	127–282	21–47
Anthropogenic sources		
Ruminants	80–115	13–19
Energy generation[b]	75–110	13–18
Rice agriculture	25–100	7–17
Landfills	35–73	6–12
Biomass burning	23–55	4–9
Waste treatment	14–25	2–4
Subtotal	267–478	45–80
Total sources	500–600	
Source: Liu *et al.*, 2008		
[a] Estimates of the relative contribution of methane emission from a source to the total global emissions of 600 Tg of CH_4 per year.		
[b] Methane deposits released by coal mining, petroleum drilling, and petrochemical production.		

The majority of recent methane production is from thermogenic transformation of organic material and by methanogenesis as the final step in fermentation of organic matter by methanogenic archaea in anoxic habitats (Reeburgh, 1996). There are also abiotic sources of methane e.g. at mid oceanic ridges where serpentinization takes place. The following sections will give an overview over global methane sources and sinks.

1.2.1 Wetlands

The sum of all wetlands such as bogs, tundra, swamps, and ponds represents the largest natural methane emitting environments on Earth (Table 1.1).

However, estimations of emission rates are difficult in these complex and diverse environments. Swamps are characterized by an imbalance of organic matter production from plants and organic matter degradation. As a result, organic matter accumulates leading to the formation of peat. Due to the high water content, anoxic conditions develop and methane is formed during the degradation of organic matter by methanogenic communities (Moore *et al.*, 1990). Hereby, methane concentrations often exceed saturation. The formed gas bubbles rise to the surface causing the typical bubbling surface of swamps (Figure 1.1). Through diffusion of methane into the upper oxic zone of the swamp, a part of the methane is oxidized to carbon dioxide by aerobic methanotrophic bacteria before it reaches the atmosphere. Methane emission from swamps was recognized already many centuries ago, better known as "swamp gas" or "marsh gas". Its mysterious spontaneous ignition was named "ghost light" and today we know that methane fires are started by traces of self-igniting hydrogen phosphide (Meredith, 2002). *Sphagnum*-dominated acidic peat bogs represent one of the most extensive wetland types in North America and Eurasia. They occupy about 3% of the Earth's terrestrial surface (Kivinen *et al.*, 1981), comprising up to 80% of the area in some regions of West Siberia. The environmental and ecological significance of peat soils is immense because of the well-recognized role of the northern wetlands in the global carbon budget and emission of methane. *Sphagnum* peat bogs support a symbiotic interaction between the mosses and anaerobic methanotrophic bacteria. this interaction results in net methane oxidation with biomass as end product (Dedysh *et al.*, 2006).
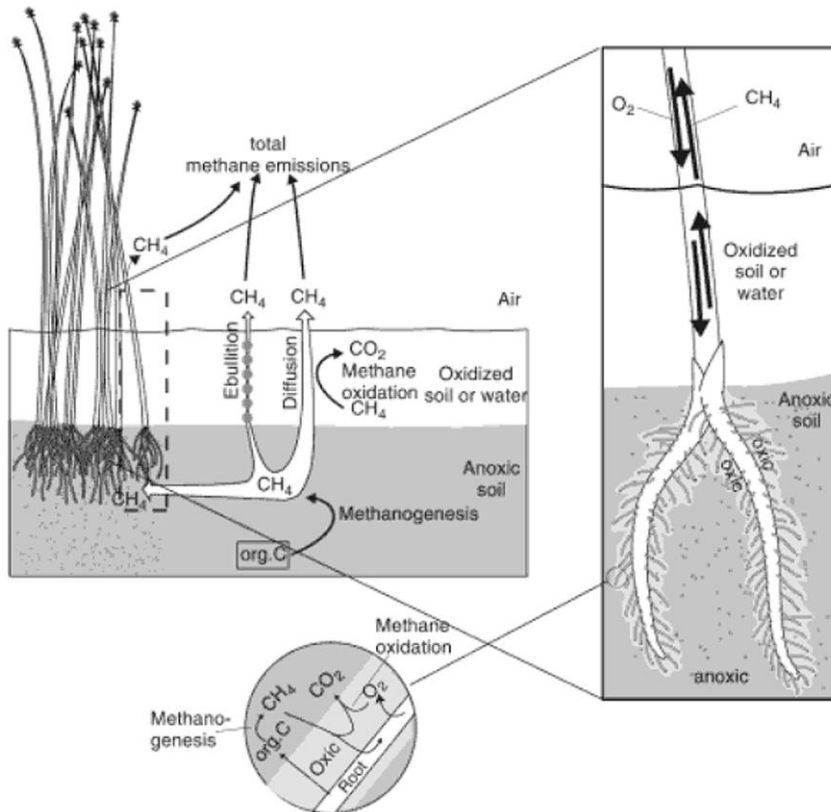
Figure 1.1 Methane cycling in a wetland (Mitsch et al., 2007)

1.2.2 Rice fields

From a global view, rice paddies are the most important man-made habitats of methane emission to the atmosphere (Furukawa and Inubushi, 2002). The magnitude of CH_4 emission is primarily a function of emission factors and assumed rice cropland area. In turn, emission factors depend on cultivation method (wet versus dry cultivation), water management practices, type of rice variety planted, and cropping patterns. (Wassmann *et al.*, 1997).

Because the paddies are flooded with water to provide optimum conditions for rice plants to grow, anoxic conditions develop, leading to strong microbial

methanogenesis due to degradation of organic matter. The rice plants play a major role in the emission of methane from the paddies as about 90% of the methane leaves the soil via the airy tissue around the roots, which allows exchange of gases between the shoot and the root (*aerenchyma*). Furthermore, the plants are suggested to stimulate methanogenesis due to the excretion of exudates, i.e. organic substances, from the roots (Seiler, 1984). However, the *aerenchyma* not only enables enhanced methane emission from the paddies, but also leads to an increase in oxygen penetration to the sediment. The oxygen is utilized by heterotrophic bacteria feeding on organic material and methanotrophic bacteria in the aerobic oxidation of methane (Krüger *et al.*, 2001; Krüger *et al.*, 2002). This explains the relatively high methane consumption rates during concurrent methane production leading to a lowered net methane emission from the paddies (Table 1.1). As rice paddies are anthropogenic, many efforts are attempted today to reduce the methane emission from this environment (Furukawa and Inubushi, 2002). Bodelier *et al.,* (1999) found that, in rice-paddy soils, ammonium (which is formed naturally but is also a major constituent of nitrogen fertilizers) stimulates methane oxidation and methanotroph growth. This phenomenon may dominate the overall response of methane cycling to fertilization in rice-paddy ecosystems.

1.2.3 Ruminants

After wetlands and rice paddies, the intestines of ruminants, especially of cattle, are the next largest source of atmospheric methane (Table 1.1). Different to other environments, the methane produced by ruminants is not partly oxidized by microbes and hence it is completely emitted into the atmosphere. The volumetric rates of methanogenesis in the intestines of a cattle are about 100-1000 times higher compared to aquatic systems (Moss *et al.*, 2000). The reason for such a high methane production is the digestive system of a ruminant. Without microbes, the ruminant is unable to utilize a major part of the polymeric substances from plants, especially cellulose, as it is lacking the essential hydrolytic enzymes (Moss *et al.*, 2000). Therefore, its intestine is inhabited by a diverse community of symbiotic microorganisms which mediate (1) the enzymatic decomposition of polymeric substances like cellulose, hemi-cellulose, pectin and starch, (2) the fermentative transformation of the hydrolysis products into low-molecular weight fatty acids that can be resorbed by the host to gain energy and to synthesize cells, (3) the synthesis

of microbial protein to meet the protein requirements of the host, and (4) the formation of vitamins. Although H_2 is one of the major end products of fermentation by protozoa, fungi and pure monocultures of some bacteria, it does not accumulate in the rumen because it is immediately used by other bacteria which are present in the mixed microbial ecosystem. The collaboration between fermenting species and H_2-utilising bacteria (e.g. methanogens) is called "interspecies hydrogen transfer" (Stams, *et al.*, 2006). Attachment of methanogens to the external pellicle of protozoa may facilitate interspecies hydrogen transfer in the rumen. (Krumholz *et al.*, 1983; Stumm *et al.*, 1982). About 800 L hydrogen is produced in a cattle intestine per day (Wolin, 1979) and is transformed to 200 L methane by the methanogens. For the host, methanogenesis means a loss of 10-15% of the total energy of the food. However, hydrogen consumption leads to an increase of the fermentative community and therefore to an increase in microbial protein usable for the host. Similar to rice paddies, most of the methane emission by cattle is caused by humans due to animal husbandry.

Efforts to reduce the methane emission by livestock includes manipulating the feed by promoting a shift in fermentation toward propionate production, but adverse effects on ruminant production cannot be avoided (Moss *et al.*, 2000). Increasing animal productivity seems to be the most effective means of reducing methane release in the short term but overall production should remain constant. The increase of productivity involves the increased use of feed containing higher quality/lower fiber sources of carbohydrate. However, the reason that ruminants are so important to mankind is that much of the world's biomass is rich in fiber and can be converted into high quality protein sources (i.e. meat and milk) for human consumption only by ruminants (Moss *et al.*, 2000).

1.2.4 Biomass burning

Besides natural fires, biomass burning caused by human activities is certainly one of the most ancient anthropogenic pollutions of the atmosphere, which started with the discovery of fire. Nevertheless its importance in the chemical composition of the atmosphere has long been ignored. During the 1980's many research campaigns were conducted, especially in the tropics, to study in detail trace gas and particle emissions from vegetation fires in various ecosystems. The burning of organic material is an oxidation process which primarily produces water vapor and carbon

dioxide, under ideal conditions of complete combustion. In natural fires, the oxygen supply is never sufficient, therefore incomplete combustion occurs leading to the formation of reduced compounds such as methane. (Delmas, 1994; Bertschi *et al.*, 2003). Huge amounts can be produced during large scale burning of woodlands, savanna and agricultural waste. In savanna regions, burning is often performed to promote regeneration of the vegetation. The emission of methane from biomass burning was ignored in global budgets up to the late 1970's but is now considered as a significant source. (Van der Werf et al., 2006)

The only route to reduce emissions from this source is to reduce the amount of burning itself. Some biomass burning is required if environments such as the savanna are to be retained, but it is the large scale destruction of forest areas for cash crop agriculture and urban spread which are the most important causes. Biomass burning under controlled condition is currently being developed as an alternative and more durable method compared to traditional fossil fuel energy production methods. By making use of a renewable resource, like pine wood chips, and avoiding incomplete combustion, these biomass power stations can significantly reduce the net greenhouse gas impact compared to equivalent coal, oil and gas fired power stations.

1.2.5 Landfills

Municipal solid waste landfills contribute for a large part to the anthropogenic sources of methane. The emission of methane from landfills due to organic matter degradation already represents an important contribution to the global methane budget (Augenstein, 1992) and in the United States landfills account for the second largest source of human-related methane emissions (United States Environmental Protection Agency). Landfill gas (LFG) is created when solid waste decomposes in a landfill by microbial activity. This gas consists of around 40-60 percent methane (CH_4), and the remainder is mainly carbon dioxide (CO_2). Landfill gas also contains varying amounts of nitrogen, oxygen, water vapor, sulfur and a hundreds of other contaminants, most of which are known as "non-methane organic compounds" or NMOCs. The aerobic oxidation of methane prior to its release into the atmosphere by methanotrophs is small (about 10%, Mancinelli and McKay, 1985). Today many efforts are made to collect the methane that is produced and to utilize it but the

main part of the LFG is flared to avoid gas migration and explosions. Also the emission of toxic components can be lowered due to the burning of the LFG.

1.2.6 Gas and coal production

Methane found in coal deposits is called Coal Bed Methane (CBM). CBM is adsorbed into the solid matrix of the coal and is called 'sweet gas' because of its lack of hydrogen sulfide. CBM is distinct from a typical sandstone or other conventional gas reservoirs, as the methane is stored in adsorbed form. The methane is in a near-liquid state, lining the inside of pores within the coal (called the matrix). The open fractures in the coal (called the cleats) can also contain free gas or can be saturated with water. During production of oil, gas or coal, large amounts of methane can be released into the atmosphere. In coal mines, the released methane is called firedamp. In an explosive mixture with air it can cause devastating pit explosions. Today, industrial production units are built to minimize loss of gas into the atmosphere.

1.2.7 Termites

Methane production by termites plays an important role in the global methane emission. Termites produce about 20 Tg methane per year, and account for approximately 3% of the global methane emissions (Table 1.1). Termites feed on wood and do not have the enzymes to hydrolyze cellulose-rich food. Because of this they are dependent on microbes that inhabit their intestines (Rasmussen and Khalil, 1983). Termites can produce numerous gases like methane, carbon dioxide and hydrogen (Zimmerman, *et al*. 1982). The termite microbial community is complex and includes also methanogens. Laboratory studies on methane emission from termites revealed higher emission rates compared to *in situ* measurements at termite hills. This can be explained by an intensive methane oxidation in the hills and the surrounding soil (Seiler *et al.*, 1984; Macdonald *et al.*, 1998). Also the amount of methane generated varies among different species. Ultimately, emissions from termites depend largely on the population of these insects, which can also vary significantly among different regions of the world.

1.2.8 Marine and freshwater sediments

The highest marine CH_4 production rates can be found near the continental margins, because the primary production in the overlying surface waters and thus also the organic matter deposition are largest in those relative shallow waters. In most of the deeper continental margin zones, primary production of organic matter is comparatively low and only 1-5% of the surface primary production reaches the bathyal and abyssal seabed due to degradation processes in the water column (Gage and Tyler, 1996). Seawater contains approximately 28 mM sulfate, therefore organic matter oxidation in marine sediments is for a large part coupled to sulfate reduction (SR). However, when the organic matter input is large enough, sulfate will be depleted in the top part of the sediment and organic matter degradation will result in CH_4 production. A large difference between marine and freshwater environments in the presence of sulfate. Sulfate is an electron acceptor used for the degradation of organic matter by sulfate-reducing bacteria (Jørgensen, 1982; Baumgartner *et al.*, 2006). As long as sulfate is present in the sediment, major methanogenesis is inhibited most probably caused by substrate competition between sulfate-reducing bacteria and methanogens (Zehnder, 1988). This is the reason that methane emission rates from marine environments are lower compared to freshwater systems (Mitsch *et al.*, 2007). Bulk methanogenesis in marine sediments is usually banished into deeper parts of the sediment. This shift might be centimeters (Martens *et al.*, 1986) to meters (Fossing *et al.*, 2000) depending on methane and sulfate fluxes. Methane diffusing upwards the sediment-water interface has consequently a longer passage in marine compared to freshwater sediments, in which methanogenesis begins right below the penetration depth of oxygen (Ferry *et al.*, 2008). In freshwater sediments, methane is mainly consumed in the very thin oxic sediment surface layer by aerobic methanotrophic bacteria. Also anaerobic methane oxidation coupled to denitrification of nitrate has been found (Raghoebarsing *et al.*, 2006). Methane passing these barriers is emitted to the hydrosphere and finally, if not consumed in the water column, to the atmosphere. In the oceans, there are two pathways of methane consumption: aerobic and anaerobic oxidation of methane (AOM). These two processes together cause the relatively low methane emission by oceans, the methane that is not anaerobically oxidized in the anoxic part of the sediment will pass though the oxic top-layer (if present) of the sediment and into the water column where aerobic oxidation of methane is very important.

There are also some less diffuse sites where CH_4 is traveling up by convection along cracks and faults. These are called cold seeps or CH_4 vents, in which pore water or fluid with dissolved CH_4 seeps up from deeper sediment layers, or in which gaseous CH_4 vents up. This results in small ecological habitats with large CH_4 inputs. These seeps can occur in many forms, e.g. as mud volcano's and brine pools. In addition to cold seeps and vents there are hydrothermal vents where mainly CH_4 is vented. These are different from the "black smokers", in which mainly hydrogen sulfide is vented.

CH_4 seeps and vents occur above fossil fuel fields or gas hydrates. The CH_4 from these vents and seeps can be produced biological, but can also be produced geochemically or thermogenic from organic matter (Levin, 2005).

1.2.9 Hydrates

Gas hydrates, also called gas clathrate, are ice-like structures in which a gas, mostly CH_4, is incorporated. The earth's methane hydrates contain more energy than all other known oil, natural gas and coal reservoirs combined (Potential Gas Committee, 1981; Kvenvolden, 2000). Extremely large deposits of methane clathrate have been found under sediments on the ocean floors and under permafrost (Makogon, 1981; Egorov *et al.*, 1999; Suess *et al.*, 1999; Borowski *et al.*, 2000; Kvenvolden, 2000). These hydrates are stable at low temperatures (<15°C), high pressures (>50 bar), in the presence of dissolved CH_4 and occur in stable ocean floor sediments below 300 meters down to about 500 meters (the Gas Hydrate Stability Zone or GHSZ). Above and below that zone hydrates do not form effectively as geothermal temperatures are too high for them to stabilize. However, the hydrates will dissociate when they come in contact with warm fluids or when dissolved CH_4 is depleted (Boetius and Suess, 2004). When methane hydrates decompose, 164 m^3 methane is released from 1 m^3 gas hydrate due to gas expansion. The remaining water has a volume of 0.8 m^3. Thus, gas hydrates contain more gas than an equivalent volume of free gas. There is quite some interest by oil and petroleum companies to commercially exploit these important sources of methane, but only a fraction of the total methane in hydrate will be economically accessible as an energy resource (Kerr *et al.*, 2004). Beside utilization of methane from gas hydrates, many speculations have been made about possible influences and reactions of gas hydrate reservoirs on global climate. It was

hypothesized that a warming of deep ocean currents during global warming could cause a destabilization of gas hydrates in the deep ocean despite the stabilizing effect of the rising sea-level (Bice and Marotzke, 2002), but recent calculations by Archer (2007) suggest that there is no risk of rapid destabilization of methane clathrate because of the buffering effect on the temperature signal due to the immense depth of most of these layers.

1.3 Methane sinks

1.3.1 Soil consumption

Only little is known of the regulation of methane production and consumption in soil. Its magnitude might vary strongly depending on water content, oxygen ventilation, organic matter content and temperature. Considering net emission from soils into the atmosphere, soil represent a sink for methane (King, 1992; King, 1996). Indeed, many investigations on soils from tropical savanna, agriculture, grasslands, and forests confirmed substantial rates of atmospheric methane consumption by methanotrophic bacteria (King, 1992 and references therein). Recent studies show that the consumption underlies seasonal changes in temperate climates (Henckel *et al.*, 2000) and is sensitive to disturbances of the soil structure (Roslev *et al.*, 1997). In many studies, aerobic methanotrophic bacteria in soil seem to be different from known groups of methanotrophs (Holmes *et al.*, 1999; Henckel et *al.*, 2000; Ragajewski *et al.*, 2002) and the pattern observed for metabolism of atmospheric methane in soils was not consistent with the physiology of known methanotrophic bacteria (Roslev *et al.*, 1997).

1.3.2 Chemical destruction

The atmosphere represents the largest sink for methane (Wuebbles and Hayhoe, 2002). In a chemical reaction, methane reacts with hydroxyl (OH) radicals, forming water and carbon dioxide. The breakup of methane is much stronger in the troposphere compared to the stratosphere (about 12:1). The consumption of hydroxyl radicals indirectly magnifies the effects of other pollutants due to the reduced oxidizing power in the atmosphere as a whole.

1.3.3 Microbial oxidation

An important sink for methane is oxidation by microorganisms. In both aerobic as anaerobic environments, methane is actively converted into CO_2 by methanotrophs. The aerobic oxidation of methane is described in Chapter 1.4.2 and the anaerobic oxidation of methane (AOM) is extensively described in Chapter 1.7.2 and further.

1.4 Microbial aspects of methane formation and consumption

1.4.1 Microbial methanogenesis in the ocean

The largest net methane formation by micro-organisms occurs in the oceans. Methanogens are not able to directly consume polymeric organic substances and methanogens are therefore always found in facultative or obligate syntrophic associations with microbial communities of the anaerobic degradation pathways (Ferry *et al.*, 2008). Only a small group of archaea is able to form methane and they include the phylogenetic groups: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Madigan *et al.*, 2000). There are over 50 described species of methanogens and the morphology of methanogens is very divers, ranging from rod-like to spiral-shaped cells and coccoid to sarcina-like aggregates (Liu *et al.*, 2008; Thauer *et al.*, 2008). The substrates of methanogenesis can be H_2/CO_2, acetate, formate, methanol, methylamines and CO (Thauer *et al.*, 2008), but H_2/CO_2 or acetate are the best known. Hydrogen and acetate are competitive substrates in marine sediments as they are also used by sulfate-reducing bacteria (Zehnder, 1988). Methanogens that use these substrates are usually outcompeted by sulfate reducers. Other substrates like methylamines, methanol are non-competitive and with these substrates methanogenesis proceeds despite the presence of sulfate (Oremland *et al.*, 1982).

Figure *1.2* shows four pathways for methanogenesis in *Methanosarcina barkeri* with different substrates including the novel pathway (D) that bypasses the Mtr enzyme reaction (Welander *et al.*, 2005).
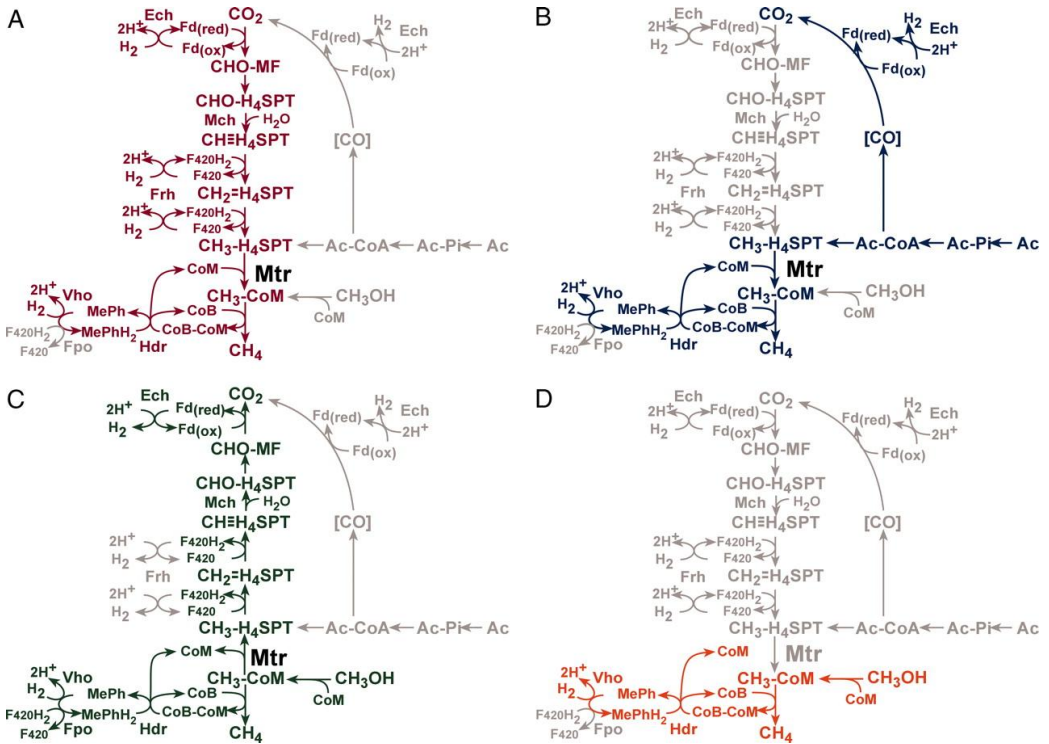
Figure 1.2: Four overlapping methanogenic pathways found in Methanosarcina barkeri. Many methanogens reduce CO_2 to methane by using electrons derived from the oxidation of H_2 (hydrogenotrophic pathway, shown in red in A). Alternatively, acetate can be split into a methyl group and an enzyme-bound carbonyl moiety. The latter is oxidized to CO_2 to provide the electrons required for reduction of the methyl group to methane (aceticlastic pathway, shown in blue in B). C-1 compounds such as methanol or methylamines can also be disproportionated to CO_2 and methane. In this pathway, one molecule of the C-1 compound is oxidized to provide electrons for reduction of three additional molecules to methane (methylotrophic pathway, shown in green in C). Finally, C-1 compounds can be reduced by using electrons derived from hydrogen oxidation (methyl reduction pathway, shown in orange in D). Steps not required by each pathway are shaded gray. The step catalyzed by the Mtr protein is indicated: note that this enzyme is predicted to be required for all pathways except the methyl-reduction pathway. CHO-MF, formyl-methanofuran; CHO-H_4SPT, formyl-tetrahydrosarcinapterin; CH=H_4SPT, methenyl-tetrahydrosarcinapterin; CH≡H_4SPT,

methylene-tetrahydrosarcinapterin; CH$_3$-H$_4$SPT, methyl-tetrahydrosarcinapterin; CH$_3$-CoM, methyl-coenzyme M; CoM, coenzyme M; CoB, coenzyme B; CoM-CoB, mixed disulfide of CoM and CoB; Mph/MphH$_2$, oxidized and reduced methanophenazine; F$_{420}$/F$_{420}$H$_2$, oxidized and reduced Factor 420; Fd(ox)/Fd(red), oxidized and reduced ferredoxin; Ac, acetate; Ac-Pi, acetyl-phosphate; Ac-CoA, acetyl-CoA; Ech, ferredoxin-dependent hydrogenase; Frh, F$_{420}$-dependent hydrogenase; Vho, methanophenazine-dependent hydrogenase; Fpo, F$_{420}$ dehydrogenase. (Image from Welander et al., 2005)

Methyl-coenzyme M (CH$_3$-CoM) is the central intermediate in this oxidative reaction and is unique for methanogens. It is formed from coenzyme M, the smallest coenzyme known to date, and the substrate (e.g. CO$_2$, acetate). Methyl-coenzyme-M is subsequently reduced with coenzyme B to methane with the concurrent formation of heterodisulfide of co-enzyme M and co-enzyme B (Thauer, 1998 and references therein). The key enzyme of this reaction is Methyl-coenzyme M reductase. This last step in methane formation is, as far as known, not coupled with energy conservation. The energy required for growth must be generated in the reductive part, i.e. the exergonic reduction of the heterodisulfide.

1.4.2 Aerobic oxidation of methane

The ability to oxidize methane with oxygen is restricted to a diverse group of specialized *Alpha-* and *Gammaproteobacteria* (Madigan *et al.*, 2000). Their existence is known from the beginning of the 20th century. The first isolated methanotrophic organism was named *Bacillus methanicus* (Söhngen, 1906), although the isolate turned out not to be a pure culture. Methane-oxidizing bacteria, or methanotrophs, are crucial players in the global cycle of the greenhouse gas methane. They are strict aerobes that use methane as their only source of carbon and energy (Anthony, 1982; Amaral and Knowles, 1995) except for the serine pathway methanotrophs (Jahnke *et al.*, 1999) The bacteria oxidize methane to formaldehyde, which is then either assimilated into cell biomass or further oxidized to carbon dioxide.

There are three types of aerobic methanotrophs, which differ in the intracellular membrane arrangement, pathways of carbon assimilation, and phospholipid fatty acid (PLFA) composition (Chistoserdova, *et al.*, 2005). Type I methanotrophs are *Gammaproteobacteria* that have stacked membranes with methane monooxygenase (pMMO), the enzyme for primary methane oxidation, and that use the ribulose

monophosphate (RuMP) cycle, which converts formaldehyde into multicarbon compounds, for building cell biomass (Lidstrom, 2001). Type II methanotrophs belong to the genera *Methylocystis* and *Methylosinus* and form a distinct clade within the *Alphaproteobacteria*, they have rings of membranes that contain pMMO at the periphery of the cells, and use the serine cycle, an alternative pathway for converting formaldehyde into biomass; these bacteria also often contain a soluble sMMO in addition to pMMO. The third type, type X methanotrophs, belong to the genus *Methylococcus* (*Gammaproteobacteria*) and combine features characteristic of the other two types: they have stacked membranes and the RuMP cycle, but they also have elements of the serine cycle and sMMO (Lidstrom, 2001). The well known type I and type II methanotrophs typically inhabit the aerobic interfaces of methanogenic environments and are found at both freshwater conditions (muds, swamps, rivers, rice paddies, ponds, soils from meadows, deciduous woods, streams and sewage sludge) but also at marine conditions (marine sediments, marine water column) (Hanson *et al.*, 1996). These methanotrophs reduce the release of methane into the atmosphere (King, 1992; Reeburgh, 1996; Horz *et al.*, 2001; McDonald *et al.*, 2008). Because of the usually high methane supply in these environments, methane is oxidized with low apparent half-saturation constants (Km(app) >1 µM CH_4) but Km values as low as 7-14 ppmv (10-20 nM dissolved CH_4) have been reported in the literature (Bender *et al.* 1992; Dunfield, *et al.*, 1999; Knief *et al.*, 2005). Methanotrophs are able to metabolize methane even at low oxygen concentrations down to 6.3×10^{-3} mM (Hanson and Hanson, 1996). This enables them to inhabit oxic-anoxic transition zones.

Aerobic CH_4 oxidation proceeds according to equation 1. The oxidation proceeds via a pathway with cyclic electron flow, in which CH_4 is first converted to methanol by a (NADH)-dependent monooxygenase. The methanol is further oxidized via formaldehyde and formate to carbon dioxide by NADH-independent methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase. The electrons released in these steps are passed to the electron transport chain for adenosine triphosphate (ATP) synthesis (Hanson *et al.*, 1996).

(1) $CH_4 + 2\ O_2 \rightarrow CO_2 + 2H_2O$ $\Delta G° = -773\ kJ.mol_{CH4}^{-1}$
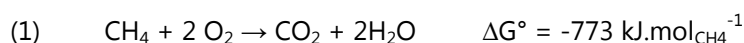
Under oxygen limiting conditions, methanotrophs can produce methanol (Xin *et al.*, 2004; Lee *et al.*, 2004) or acetate (Costa *et al*, 2000) from CH_4. Denitrifiers are able to utilize these products. In this way, denitrification with CH_4 as electron donor is possible at oxygen limiting conditions (Costa *et al.*, 2000; Waki *et al.*, 2004). A similar process for sulfate reduction has thus far not been described, although some sulfate reducers can tolerate the presence of oxygen (Muyzer and Stams, 2008).

1.4.3 Sulfate reduction the main oxidative pathway in anoxic marine habitats

Sulfate reduction, and in particular heterotrophic sulfate reduction, is a dominant anaerobic carbon oxidation pathway in marine sediments, (Jørgensen, 1982; Brandes *et al.*, 1995; Hartnett, *et al.*, 2003). Although other electron acceptors such as oxygen, nitrate, iron and manganese yield higher energy outputs compared to sulfate, their combined concentration at the sediment-water interface is more than 50 times lower compared to the total sulfate concentration (D´Hondt, *et al.*, 2002). The general reaction of heterotrophic sulfate reduction is (Jørgensen, 1982):

(2) $2\ CH_2O + SO_4^{2-} \rightarrow 2\ HCO_3^- + H_2S$
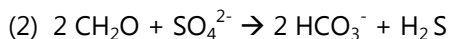
SR only occurs when electron acceptors with a higher redox potential (e.g. oxygen and nitrate) are absent. These sulfate reducing conditions are found in sediments and stratified waters due to the limited penetration depth of oxygen. Sulfide produced in the anoxic compartment will be partly transported to the aerobic compartment where sulfide is oxidized back to sulfate, and vice versa (Bottrell *et al.*, 2006; Holmer *et al.*, 2001). SR and sulfide oxidation form the main routes of the biological sulfur cycle (Figure 1.3).
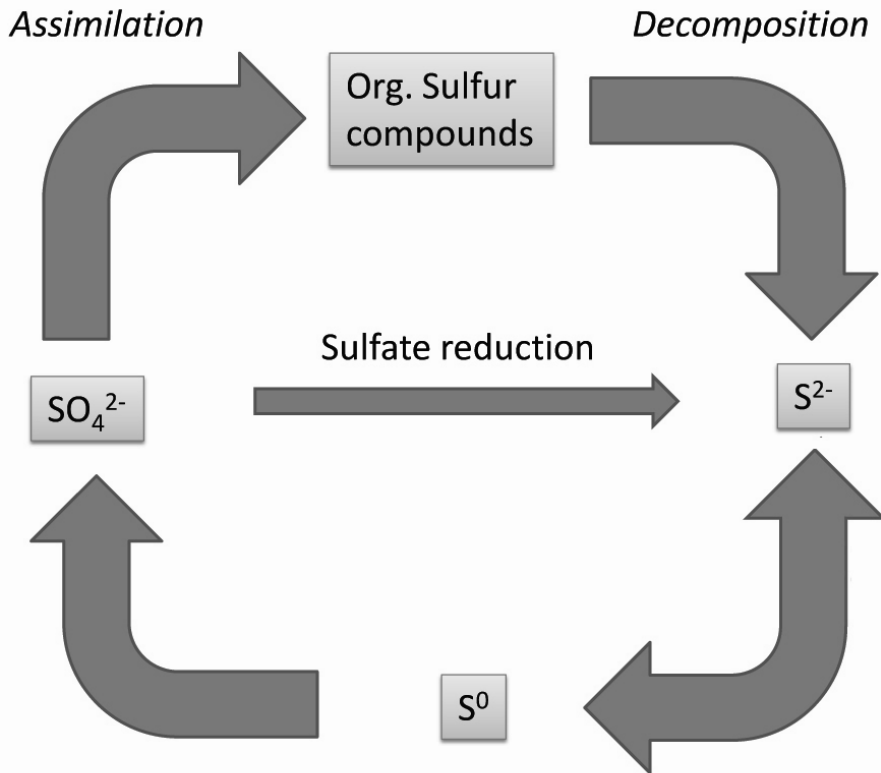
Figure 1.3: The sulfur cycle

SRB are a diverse group of prokaryotes (Castro *et al.*, 2000), the known SRB can be grouped into seven phylogenetic lineages, five within the bacteria and two within the archaea (Madigan *et al.*, 2000; Muyzer and Stams, 2008). Typically SRB occur in anoxic marine and freshwater sediments or waters (Postgate, 1984). Eight mol reduction equivalents are needed for the reduction of one mol sulfate to one mol sulfide. These reduction equivalents are obtained by the oxidation of an electron donor to carbon dioxide and water. They use a large variety of electron donors like hydrogen, acetate, lactate, pyruvate, butyrate, formate, methanol, ethanol, carbon monoxide, methanethiol and sugars. (Widdel *et al.*, 2007; Muyzer and Stams, 2008) Some groups, e.g. *Desulfosarcina, Desulfonema, Desulfococcus*, are able to live chemoautolithotrophic with hydrogen as the electron donor, sulfate as electron

acceptor and CO_2 as the solely carbon source. Among the sulfate reducers there are obligate anaerobic forms, however, some also tolerate oxygen in small amounts or even use it as electron acceptor (Madigan *et al.*, 2000). Sulfate reducing bacteria can often outcompete methanogens because they can use larger substrates and have a higher affinity for hydrogen. But aceticlastic methanogens can compete with acetate-degrading sulfate reducers (van Bodegom and Stams, 1999; Stams *et al.*, 2005). Thus far, no SRB was found to be able to utilize CH_4 as electron donor or carbon source.

1.7.1 Anaerobic oxidation of methane in anoxic marine habitats

AOM is a microbial process in anoxic marine sediments whereby methane is oxidized with sulfate as the terminal electron acceptor according to equation 3 (Barnes and Goldberg, 1976):

(3) $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$
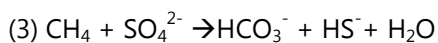
AOM is thought to be mediated by a syntrophic consortium of methanotrophic archaea and sulfate-reducing bacteria (Zehnder and Brock, 1980; Hoehler *et al.*, 1994; Boetius *et al.*, 2000; Orphan *et al.*, 2001; Stams *et al.*, 2009), but there are also indications that methanotrophic archaea are able to perform the AOM reaction without a direct coupling with a bacterial partner (Chapters 2 and 3). The methanotrophic archaea involved in AOM are commonly referred to as ANME archaea. In contrast to aerobic CH_4 oxidation, anaerobic oxidation of methane (AOM) by microbes is a less understood process and for many years AOM was thought to be impossible (Thauer and Shima, 2008). The first investigation of AOM dates back to the year 1974, when Martens and Berner speculated about the cause for conspicuous methane and sulfate profiles in organic rich sediments (Figure 1.4). The scientists observed that methane was not accumulating before sulfate was exhausted. From the decrease of methane concentrations in the sulfate-reducing zone, they concluded that methane must be consumed with sulfate.
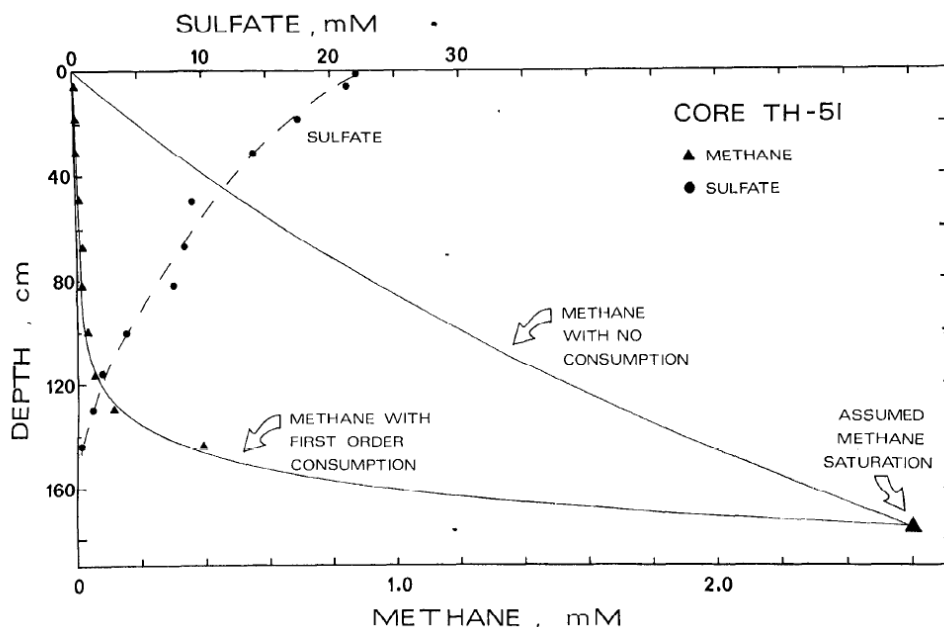
Figure 1.4 Methane and sulfate concentrations versus depth for core TII-51. Dashed line is an exponential fit to scientific data. (Figure from Martens and Berner (1974)

Zehnder and Brock (1979 and 1980) found methane oxidation by methanogenic communities and proposed a coupled two-step mechanism of AOM. They postulated that methane is first activated by methanogenic archaea, working in reverse, leading to the formation of intermediates, e.g. acetate or methanol. In a second step, the intermediates are oxidized to CO_2 coupled to sulfate reduction by other non-methanogenic members of the microbial community. The methane oxidation co-occurring with methanogenesis is called trace methane oxidation (TMO) and only a small portion of the methane formed is oxidized back to CO_2 (Zehnder and Brock, 1980). No net methane oxidation by isolated methanogens has been reported. With the discovery of net methane oxidation by marine microorganisms, the knowledge of AOM increased substantially involving biogeochemical, microbiological, and molecular methods adding one peace after the other to the big puzzle. Radiotracer measurements enabled the first direct quantification of AOM and sulfate reduction rates in anoxic marine sediments (Reeburgh, 1976; Iversen and Blackburn, 1981; Devol, 1983). By this technique, traces

of $^{14}CH_4$ and $^{35}SO_4$ are added to the sediment and their conversion into $^{14}CO_2$ and H_2S are determined. Including the total methane and sulfate concentration of the sediment, turnover rates can be calculated. Iversen and Blackburn (1981) were the first to measure a 1:1 ratio of AOM and sulfate reduction, demonstrating the close coupling between these processes. In 1994, Hoehler *et al.* confirmed by thermodynamic modeling that a consortium of methanogenic archaea and sulfate-reducing bacteria could gain energy from AOM. The hypothetical pathway involves hydrogen and CO_2 production from methane by methanogens. The hydrogen is consumed by sulfate-reducing bacteria, thereby maintaining hydrogen partial pressure low enough for favorable free energy yields. Further evidence was gained by inhibition experiments (Hoehler *et al.*, 1994; Hansen *et al.*, 1998). Chemical substances were added to anoxic methanotrophic sediments inhibit the activity of either methanogens or sulfate reducers. For methanogens, 2-bromoethanesulfonic acid (BES) was used (Hoehler *et al.*, 1994). This inhibitor is an analogue of methylcoenzyme M (Gunsalus *et al.*, 1978), an enzyme cofactor present only in methanogens (see section 1.4.1). The enzymatic pathway of sulfate reduction was inhibited by the addition of molybdate (Hansen *et al.*, 1998). In both experiments, AOM was strongly reduced. When sulfate was removed from the sediment, AOM was completely inhibited (Hoehler *et al.*, 1994). Again, a close coupling between AOM and sulfate reduction was demonstrated.

1.8 Molecular analyses on AOM communities

1.8.1 Biomarkers

The research on AOM continued using lipid biomarker assays. Biomarkers are specific biologically produced molecules that allow identifications of organisms on the level of kingdoms or sometimes orders (Peters and Moldowan, 1993).
The lipids in the phospholipid bilayer of the cell membrane can be used for the differentiation between archaeal and bacterial cells. Typical archaeal biomarkers are characterized by isoprenoid chains and ether linkages, whereas bacterial cells are characterized by fatty acids and ester linkages (Jahnke *et al.*, 2008; Thiel *et al.*, 2001).
The carbon isotopic composition is a indicator for the diagnostic information on the carbon source and/or metabolic carbon fixation pathways utilized by its producer.

During methanogenesis the uptake of the heavier ^{13}C-substrate is lower, due to the lover reactivity of the ^{13}C-substrate. This kinetic isotope fractionation results in an enrichment of ^{12}C in the produced methane (Whiticar, 1999). The isotope ratio of ^{12}C and ^{13}C is expressed as the ^{13}C-value, which is the ratio relative to a known standard (Vienna Peedee belemnite; VPDB). The methane-derived carbon that is incorporated into methanotrophic biomass causes a second step of carbon fractionation. This reveals a very light signal in biomass molecules like lipids. Biomass of organisms involved in AOM contain methanogen-specific lipids, named crocetane, archaeol and hydroxyarchaeol. (Elvert and Suess, 1999; Hinrichs *et al.*, 1999; Pancost *et al.*, 2000; Thiel *et al.*, 2001; Stadnitskaia, *et al.*, 2008; Meulepas *et al.*, 2009). The carbon isotopic signature of archaeol and hydroxyarchaeol from the methane hydrate associated sediment was extremely light, and showed ^{13}C values of -100 and -110‰, respectively. Archaeol and hydroxyarchaeol could not be detected in sediments surrounding the methane hydrate area (Hinrichs *et al.*, 1999). Because the lipid biomarkers commonly found in archaea are so strongly depleted in ^{13}C that methane must be the carbon source, rather than the metabolic product, for the organisms that have produced them. During incubations of AOM sediment with ^{13}C-labled CH_4, ^{13}C was incorporated both in archaeal lipids associated with ANME and bacterial lipids of associated SRB, this incorporation in bacterial lipids might proceed via a carbon compound produced from CH_4 by ANME rather than by the direct uptake of CH_4 by SRB (Blumenberg *et al.*, 2005). The ^{13}C-values of the bacterial lipids were somewhat heavier ranging from -50 to -100 ‰ (Hinrichs *et al.*, 2000; Hinrichs and Boetius, 2002) and Blumenberg showed that ^{13}C-labelled methane is mainly taken up by bacteria rather than archaea which is a different outcome then we have with our Eckernförde bay enrichments in membrane bioreactors (Chapter 3). Lipid analysis of the enriched biomass showed that bacterial lipids were dominating over those of archaea but ^{13}C-label from methane was substantially incorporated in both archaeal and bacterial lipids during batch incubation with bioreactor sludge. The difference can be explained by the much higher AOM rates observed here and the much more active archaea in the AOM consortium studied. Interestingly, the degree of labeling of the bacterial lipids observed is much larger than that found by Blumenberg *et al.* for the same lipids and after the same period of incubation (e.g. 44% versus 0.2% for the C16:1 fatty acid), suggesting that the SRB were also much more active (Chapter 2).

1.8.2 Fluorescence in situ hybridisation

After rate measurements, inhibition experiments and lipid biomarkers is was clear that AOM was coupled to the reduction of sulfate and both archaea and SRB were involved. The next step was to visualize the responsible microorganisms and to find their physical association. Fluorescence *in situ* hybridization (FISH), revealed for the first time a consortium of archaeal cells surrounded by a shell of sulfate reducing bacteria both involved in AOM (Boetius *et al.*, 2000). FISH allows the identification and quantification of individual microbial cells in environmental samples (Amann *et al.*, 1990). Figure 1.5 shows a FISH image from a consortium from a Black sea sample, The fluorescent probes were aiming at ANME-2 archaea (in red) and SRB affiliated with AOM consortia (green), the image was made by C.G. Jagersma with a confocal laser scanning microscope at the Max Planck Institute Bremen where A. Boetius and co-workers made the first images of the AOM consortia (Boetius *et al.*, 2000).

The probes used in FISH (nucleic acid strands) carry different fluorescent dyes enabling a visual identification of the targeted cells by fluorescence microscopy. The probes bind to ribosomes in intact fixed cells and are unique to different phylogenetic groups or even species. Typical aggregates grow to a size of about 6-10 μm before they break apart into sub aggregates and sometimes multiple consortia form a flock with other non ANME/SRB related organisms (Figure 1.6)
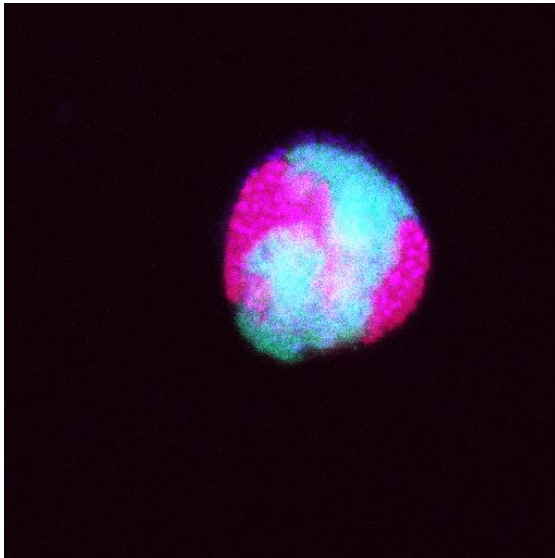
Figure 1.5: Aggregate of methane-oxidizing archaea (stained red) and sulfate-reducing bacteria (stained green) found in AOM sediments from the Black sea (image by C.G. Jagersma)
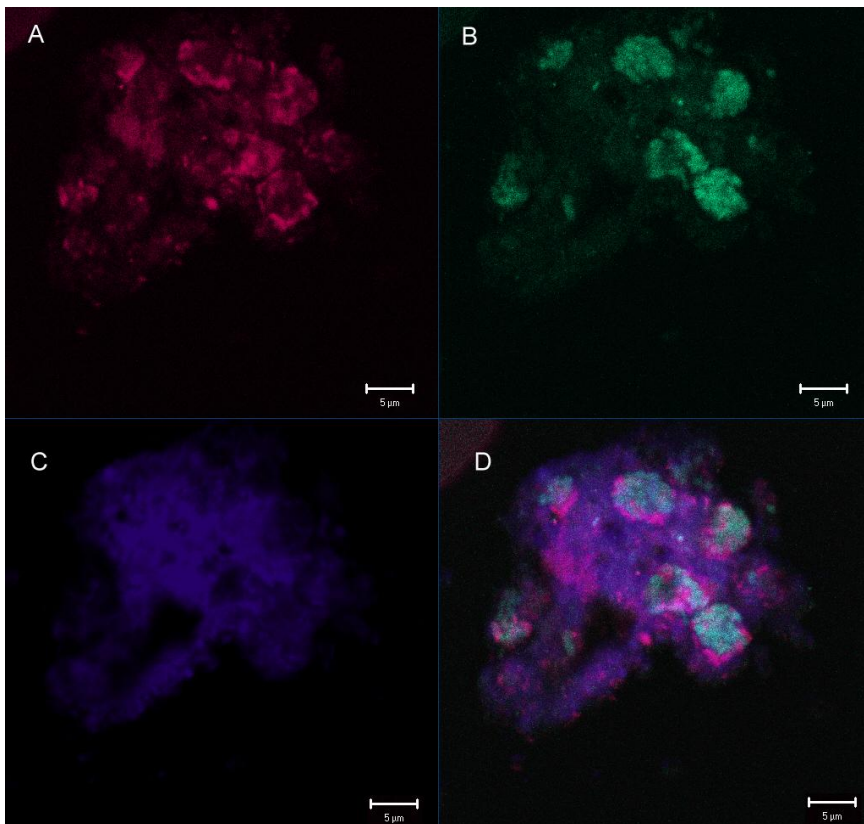
Figure 1.6: Confocal-Laser scanning microscope image of multiple consortia in a floc from Black sea sediment. ANME Archaea (stained red, A), SRB from the DSS subgroup (stained green, B), Universal probe DAPI (stained blue, C). Individual images of each probe signal are used to visualize the overlay (D), (image by C.G. Jagersma).

1.8.3 Fluorescence *in situ* hybridization-secondary ion mass spectrometry

Further methods were used to obtain direct evidence for the methanotrophy of the AOM consortium. The microanalytical method FISH-SIMS (fluorescence *in situ* hybridization-secondary ion mass spectrometry) can be used to describe the physiological traits and anabolic activity of individual methanotrophic consortia, specifically tracking ^{15}N-labelled protein synthesis to examine the effects of organization and size on the metabolic activity of the syntrophic partners (Orphan *et al.*, 2009a). Another possibility is measuring the ^{13}C-profiles of the biomass of single aggregates (Orphan *et al.*, 2001). A recent publication by Orphan *et al.* (2009b) showed enhanced ^{15}N assimilation in ANME-2 cells relative to the co-associated SRB revealing a decoupling in anabolic activity between the partners. Overall, the metabolic activity of both syntrophic partners within consortia was greater than activity measured in representatives of the ANME-2 and DSS observed alone, with smaller ANME-2/DSS aggregates displaying a tendency for higher ^{15}N uptake and faster growth rates with doubling times ranging from 3 to 5 months. When looking at the ^{13}C-profiles they found high depletion in ^{13}C in both the archaeal cells and in the bacterial cells with values down to -96 ‰ and of -62 ‰. The results confirmed the assimilation of isotopically light methane by the consortia of archaea and bacteria. (Orphan *et al.*, 2001).

1.8.4 Real time Quantitative PCR

Real time quantitative PCR (Q-PCR) is a highly specific and quantitative method using specific probes in a PCR reaction. The Q-PCR method is based on the amplification of a specific fragment of the 16s rRNA gene. The initial amount of template fragments can be calculated by the increase in fluorescence during a real time monitoring of the amplification of the fragments. The SybrGreen molecule which is added to the PCR mixture will emit a fluorescent signal when it integrates

with double stranded DNA. This signal can be detected continuously. Because the amplification of DNA fragments is logarithmic the increase of the fluorescent signal will also be logarithmic. The time it takes for the fluorescent signal to become logarithmic is a measure for the initial amount of DNA templates. Q-PCR can show the presence/absence of the product and estimate the size of it by using a DNA ladder with fragments of different sizes. The reliability of Q-PCR depends on chosen fluorescent compound. There are two most known approaches: TaqMan and SybrGreen. The difference between them is that the TaqMan method is a more specific method compared to SybrGreen which binds every double stranded PCR product resulting in lower specify. TaqMan uses a different approach by adding a specific oligonucleotide probe with a fluorescent dye. When the probe is assembled with a corresponding DNA fragment, the molecule will be detached and will emit a fluorescent signal. Despite the higher specificity of the TaqMan method, the SybrGreen method is widely adopted by researchers because of the low costs and the robustness. Girguis *et al.* (2003; 2005) were the first to use this method in AOM research. For this they developed novel primers targeting ANME-1, ANME-2c and associated sulfate reducing bacteria from the *Desulfococcus* and *Desulfosarcina* groups (*D*SRB) used also in Chapter 4.

1.8.5 Immunomagnetic cell capture

A technique using combined fluorescence *in situ* hybridization and immunomagnetic cell capture was used to isolate cells directly from the environment (Pernthaler *et al.*, 2008). Syntrophic anaerobic methane oxidizing ANME-2c archaea and physically associated microorganisms were obtained directly from deep-sea marine sediment. Metagenomics, PCR, and microscopy of these purified consortia revealed unexpected diversity of associated bacteria, including *Betaproteobacteria* and a second sulfate-reducing *Deltaproteobacterial* partner. The detection of nitrogenase genes within the metagenome and subsequent demonstration of $^{15}N_2$ incorporation in the biomass of these methane-oxidizing consortia suggest a possible role in new nitrogen inputs by these syntrophic assemblages (Pernthaler *et al.*, 2008).

1.8.6 MAR-FISH

A combination of fluorescent *in situ* hybridization and microautoradiography (MAR-FISH) can link phylogenetic information (expressed by the fluorescent signal from a probe) to the metabolic activity (expressed by the radioactive signal of radiolabelled substrates). This method can be used for prokaryotes under conditions that approach *in situ* conditions by direct visualization of microorganisms with active substrate uptake systems within a complex community (Lee *et al.*, 1999; Ouverney *et al.*, 1999). MAR-FISH has been used for diverse ecosystems and enrichments for example activated sludge (Daims *et al.*, 2001; Nielsen *et al.*, 2000; Nielsen *et al.*, 2002; Nielsen *et al.*, 2003), an anoxically operated, methanol-fed sequencing batch reactor (Ginige *et al.*, 2004), marine samples (Cottrell *et al.*, 2000; Ouverney *et al.*, 2000; Riemann *et al.*, 2002), freshwater sediments (Gray *et al.*, 2000), sewer biofilms (Ito *et al.*, 2002), and autotrophic nitrifying biofilms (Kindaichi *et al.*, 2004). These studies have demonstrated that the MAR-FISH technique has significant potential for providing a direct link between rRNA-based phylogenetic identification and *in situ* substrate uptake patterns (metabolic capability) without a requirement for cultivation and could be a very promising method to link the metabolic activity of ANME archaea and associated SRB with the uptake of specific substrates. This method could shed a light on the suggested syntrophy between these two groups of microorganisms when it can be proven which member of the consortium is using the methane and which one is taking up the sulfate. Also the intermediate could be found with this method when a possible candidate intermediate is used that contains a ^{14}C radiolabel. MAR-FISH has not been applied to AOM sediments or enrichments because the MAR signal has a high spread making it impossible to distinguish between the members of the dense consortium of ANME and SRB. Because the membrane bioreactor enrichment described in Chapters 2 and 3 show the presence of ANME-2a and DSS bacteria in single cell distribution this enrichment might be a more suitable material for MAR-FISH then the sediments with dense consortia. In collaboration with Jeppe Lund Nielsen (Aalborg University, Denmark) we have started some MAR-FISH experiments with samples from the bioreactor enrichment and preliminary results show that both the ANME archaea (shown by specific FISH probe for ANME-2a) and the DSS bacteria (shown by specific DSS FISH probe) take up the ^{14}C radiolabel when incubated with $^{14}CH_4$. It is not clear if the archaea or the bacteria take up the methane directly. It could also be that an

intermediate which contains the ^{14}C radiolabel is taken up by one of the organisms which in turn shows that the intermediate is an organic compound and that the electrons are not directly transferred.

1.8.7 Flow Cytometry

Flow cytometry is a well-established method for counting and examining microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. Multiple parameters (e.g., forward and 90° light scatter and fluorescence emission at wavelengths of interest) can be determined individually for a large number of cells in a short time (up to several thousand cells per second). Flow cytometry has been applied to ecological studies, especially to measure the distribution and abundance of marine picoplankton (Amann *et al.*, 1990).

Flow cytometry can be performed by using the morphological and physiological characteristics of the cells (e.g., size and pigment content of photosynthetic organisms) (Olson *et al.*, 1988). But these criteria generally are not sufficient for identification at the genus or species level. Staining with DNA-specific fluorochromes offers information about numbers of bacterial cells but not about their identity. With the combination of 16S rRNA-targeted oligonucleotide probes and flow cytometry it is possible to quantify specific microorganisms using their phylogenetic identity (Amann *et al.*, 1990).

A very promising application of flow cytometry is Fluorescence-activated cell sorting (FACS). FACS is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into multiple containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell (Herzenberg and Herzenberg, 1978). The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream

breaks into droplets. A charge is placed on the ring based on the immediately-prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off (Herzenberg and Herzenberg, 1978).

It is only possible to use cells that are not closely attached to each other or other particles. The current methane-oxidizing cultures mainly consists of dense consortia of ANME and SRB. The cells in these consortia cannot readily be separated, not even with short pulses of sonfinication, making them unsuitable for use in FACS. The enrichment obtained in the membrane bioreactor (Chapters 2 and 3) contains single ANME-2a cells and these cells have been successfully separated from other cells (SRB and residual archaea) at the Max Planck institute Bremen by using FACS (T. Holler, Personal communication). This pure culture can now be used for whole genome sequencing and for developing highly sensitive primers and probes for FISH, Q-PCR, DGGE and PCR amplification.

1.8.8 Drawbacks

The drawback of the FISH technique and FACS is that the fixation of cells prior to the incubation with the fluorescent probes causes lethality and the cells will not be able to grow after the treatment. To demonstrate the use of certain metabolic pathways by the microorganisms, the cells should be able to survive an enrichment technique.
One way to enrich these slow growing organisms is to optimize the growth conditions (temperature, pressure, substrate concentration, salinity, product removal rate, pH, etc.). Nauhaus *et al.* (2002) demonstrated methane-dependent sulfate reduction in Hydrate Ridge sediment as well as a 1:1 ratio of AOM and sulfate reduction rates as predicted by the stoichiometry of the two processes. A special incubation tube which allowed maintenance of dissolved methane concentrations above ambient-pressure saturation without any gas phase, enabled to measure methane-dependent sulfate reduction at high hydrostatic pressure and thereby at
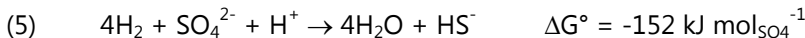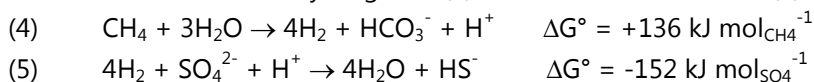
higher methane concentrations than can be reached under atmospheric pressure. The AOM-consortium revealed an increase in sulfate reduction rates with increasing methane concentration showing the dependency of AOM on the availability of dissolved methane (Nauhaus *et al.*, 2002).

It is still not clear if AOM is an enzymatic reversal of methanogenesis. Hints for this reversal can be found in genomic studies looking at the genes coding for certain pathways (Hallam *et al.*, 2004). The presence of methyl coenzyme M reductase A (mcrA), a coenzyme specific for the process of methanogenesis, could be linked to archaea involved in AOM (Hallam *et al.*, 2003; Krüger *et al.*, 2003, Nunoura *et al.*, 2006). Furthermore, a modified form of mcrA was found in the cell extract from organisms from active methane-oxidizing sediment. The modified mcrA indicates a specialization of this enzyme possibly in catalyzing the first step in AOM. Moreover, it is still unknown, which kind of intermediate is exchanged between the archaea and sulfate-reducing bacteria. All substrates from which methanogens produce methane are preliminary suspected to be the product of AOM, i.e. the intermediate of the syntrophic consortium. This aspect will be described in Chapters 4 and 5.
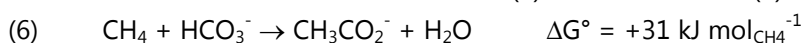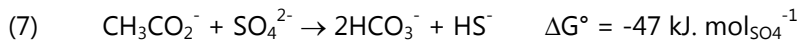
1.9 Intermediates in AOM

Further laboratory experiments and Gibbs free energy calculations resulted in the inclusion or exclusion of possible intermediates like hydrogen/CO_2, acetate, methanol, formate, carbon-monoxide, methyl sulfides or methylamine (Hoehler *et al.*, 1994; Valentine and Reeburgh, 2000; Sørensen *et al.*, 2001; Nauhaus *et al.*, 2002; Moran *et al.*, 2008; Larowe *et al.*, 2008). Nevertheless, methods for the direct identification of the intermediate exchanged during AOM are lacking. The hypothetical gross reactions with hydrogen/CO_2 or acetate as intermediate are as follows (Valentine and Reeburgh 2000; Hinrichs and Boetius 2002):

The reactions with hydrogen: (4) archaeal cell and (5) bacterial cell:
(4) $CH_4 + 3H_2O \rightarrow 4H_2 + HCO_3^- + H^+$ $\Delta G° = +136 \text{ kJ mol}_{CH4}^{-1}$
(5) $4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^-$ $\Delta G° = -152 \text{ kJ mol}_{SO4}^{-1}$

The reactions with acetate as intermediate (6) archaeal cell and (7) bacterial cell:
(6) $CH_4 + HCO_3^- \rightarrow CH_3CO_2^- + H_2O$ $\Delta G° = +31 \text{ kJ mol}_{CH4}^{-1}$

(7) $CH_3CO_2^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$ $\Delta G° = -47$ kJ. mol_{SO4}^{-1}

A syntrophic relationship is defined to be a process by which two or more microorganisms cooperate to degrade a substance that cannot be degraded by one organism alone (Madigan *et al.*, 2000; McInerney *et al.*, 2008). For the syntrophic AOM consortium a very small cell distance (<70 nm) was calculated for a thermodynamic favorable exchange of the assumed intermediates (Sørensen *et al.*, 2001).

An alternative theory for the shuttling of electrons between ANME archaea and SRB is the transfer of reduction equivalents between the sytrophic partners via extracellular redox shuttles (Widdel and Rabus 2001; Wegener *et al.*, 2008), or via membrane bound redox shuttles or so called "nanowires" (Reguera *et al.*, 2005; Stams *et al.*, 2006; Thauer and Shima, 2008; Wegener *et al.*, 2008). The primer requires the shuttle to be transported back to the ANME after donating the electrons to the SRB, giving rise to an additional loss in Gibbs free energy change, available for the microorganisms, due to the concentration gradients between the syntrophic partners. (This problem could partly be overcome when a shuttle is chosen with a much better redox potential.) The latter would require the ANME archaea and SRB to make direct physical contact, which is not always the case (Michaelis *et al.*, 2002; Knittel *et al.*, 2005; Orphan *et al.*, 2002; Treude *et al.*, 2005a; this thesis (Chapters 2 and 3)). It therefore remains unclear if and how reducing equivalents are transferred from the ANME to a sulfate-reducing partner.

Inhibitor experiments confirmed the involvement of methanotrophs and sulfate reducers but the chemicals used inhibited only processes and not specific organisms. Recent findings suggest that it is possible that AOM is mediated by one organism that comprises the enzymatic apparatus to mediate both methane oxidation and sulfate reduction. Chapter 2 and 3 describe the enrichment of Eckernförde bay sediment and FISH analysis show the occurrence of ANME-2a cells in single cells without a direct coupling with SRB or other ANME cells. Ettwig *et al.* show the isolation of a bacteria capable of both anaerobic methanotrophy coupled to denitrification showing the possibility for one organism to harbor pathways which thought to occur only between syntrophic partners. (Ettwig *et al.*, 2008)

Since the discovery of the microbes involved in AOM, much effort has been put in the identification and phylogenetic classification of AOM organisms from different habitats. Their phylogenetic classification is investigated using the relationships of

16S rDNA archaeal clones. Today there are three major groups identified: ANME-1, ANME-2 and ANME-3 (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Orphan *et al.*, 2001; Lösekann *et al.*, 2007). All of them belong to the *Euryarchaeota*, the group that also comprises all methanogens. ANME-2 and ANME-3 belong to the *Methanosarcinales*. ANME-1 is distinct from, but related to, methanogenic archaea of the orders *Methanomicrobiales* and *Methanosarcinales* (Knittel *et al.*, 2003).

1.10 Occurrence of AOM in marine habitats

In general, AOM can be expected wherever methane and sulfate coexist in anoxic environments. This includes all kinds of anoxic marine sediments but also anoxic marine waters. The methane source can be either recent or ancient, it can be microbial, thermogenic or abiotic, methane can occur dissolved, gaseous or enclosed in gas hydrates and can be transported by diffusive or advective flux. One main factor that determines the magnitude of AOM is the methane supply because methane turnover rates increase with methane concentration (Nauhaus *et al.*, 2002). Hinrichs and Boetius (2002) gave a first overview of AOM rates in marine sediments of different water depths as well as methane seeps. Table 1.2 gives an overview of the most studied sites and their AOM rates. The surveys of AOM field measurements and modeling suggests a direct link between methane supply and methane consumption in the habitat.

Table 1.2: Overview of AOM sites and rates reported thus far in the literature (Meulepas, 2009b)

Location	Depth (m)	CH$_4$ source	AOM (μmol g$_{dw}^{-1}$ day^{-1})		Reference
			radio-tracers	*in vitro*	
Eckernförde Bay, Baltic Sea	28	Organic matter decomposition	0.03-0.06	0.1-0.3	Treude *et al.*, 2005a
Kattegat, Baltic See	0.5	Organic matter decomposition	0.05-0.2	0.05-1	Küger *et al.*, 2005
Spiekeroog, North Sea	0-5	Organic matter decomposition	n.d.	0.01-0.2	Küger *et al.*, 2005
Aarhus Bay, Denmark	16	Organic matter decomposition	n.d.	n.d.	Thomsen *et al.*, 2001
Black Sea	250	Fossil-methane seep	0.2-7.5 8-21	0.5-3.5	Küger *et al.*, 2005 Treude *et al.*, 2007
Haakon Mosby Mud Volcano, Atlantic Ocean	1250	Fossil methane	n.d.	0.1-1	Damm and Budéus, 2003
Golf of Cadiz, Atlantic Ocean		Mud Volcano	n.d.	n.d.	Niemann *et al.*, 2006; Stadnitskaia *et al.*, 2006
Namibian margin, Atlantic Ocean	25	Organic matter decomposition	n.d.	n.d.	Niewöhner *et al.*, 1998
Gulf of Mexico	650	Gas hydrates	n.d.	1-13	Joye *et al.*, 2004 Krüger, 2005
Hydrate Ridge, Pacific Ocean	700	Gas hydrates	0.3-6	2-8	Boetius *et al.*, 2000; Treude *et al.*, 2003; Krüger *et al.*, 2005
Monterey Bay, Pacific Ocean		Cold seep	n.d.	0.03	Girguis *et al.*, 2003; 2005.
Eel River Basin, Pacific Ocean	516-556	Gas hydrates	n.d.	n.d.	Orphan *et al.*, 2002
Chilean margin, Pacific Ocean	800-4600	Organic matter decomposition	0.001-0.07	n.d.	Treude *et al.*, 2005b
Pearl River estuary, Pacific Ocean	3-4	Organic matter decomposition	n.d.	n.d.	Wu Zijun *et al.*, 2006
n.d. not determined					

Although the data reveal a large scatter, it is clear that the highest rates can be found at gas hydrate areas and methane seeps. Gage and Tyler (1996) found that AOM rates of non-seeps are on average slightly higher in shelf sediments (290 ± 332 mmol m^{-2} a^{-1}, n = 10) compared to continental margins sediments (117 ± 157 mmol m^{-2} a^{-1}, n = 12). This could be correlated with the general decrease of organic matter supply with water depth, resulting in weaker degradation processes including methanogenesis. In diffusive systems AOM rates are relatively low molecular diffusion is the only transport mechanism for methane. This is different from methane seeps, were additional advective processes like rising fluid and bubbling gases accelerate the overall methane flux (Judd *et al.*, 2002). The source of methane in diffusive systems is often methanogenesis from organic matter decomposition. The methane slowly diffuses upwards into the sulfate zone, were it is consumed by AOM (Iversen and Blackburn, 1981; Fossing *et al.*, 2000; Krüger *et al.*, 2005; Treude *et al.*, 2005b). The sulfate-methane transition zone is sometimes located very deep - several meters to decameters - in the sediment, but the AOM community may still be limited to a narrow zone of a few centimeters depending on the concentrations of the substrates. Diffusive systems can be found in every kind of marine environments from coastal sediments (Iversen and Jørgensen, 1985; Thomson *et al.*, 2001) to continental margins (Niewöhner *et al.*, 1998; Fossing *et al.*, 2000; Jørgensen *et al.*, 2001; Treude *et al.*, 2005b). With water depth, i.e. hydrostatic pressure, methane solubility is increasing enabling more methane to be available in the pore water of the sediment.

1.11 Occurrence of AOM in non-marine habitats

There is some indication that AOM coupled to sulfate reduction is taking place in non-marine environments. There is a report regarding Lake Plussee (Germany) showing the co-occurrence of aerobic and anaerobic methane oxidation in the water column (Eller *et al.*, 2005), and there are more reports that suggest AOM in non-marine sediments (Briee *et al.*, 2007; Grossman *et al.*, 2002) but none of these give a conclusive answer to the question if AOM coupled to sulfate is an important sink for methane in freshwater environments.
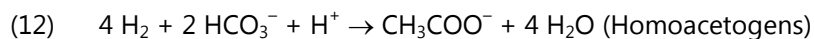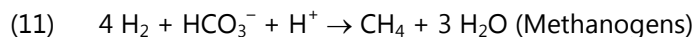
AOM coupled to nitrate reduction has recently been described (Raghoebarsing *et al.*, 2006) and it was shown that a bacteria was responsible for the anaerobic methanotrophy coupled to denitrification (Ettwig *et al.*, 2008).

1.12 Application of AOM for sulfate reduction

Research on anaerobic methane oxidation in marine environments have up to now always been focused on *in situ* conditions and unraveling the processes in marine sediments. The possible application of the AOM process coupled to sulfate reduction has not received much attention. Since CH_4 is readily available and relatively cheap the direct use of methane for sulfate removal processes could be a excellent option. Industrial activities have caused an increase in the emission of sulfuric compounds to surface waters and atmosphere causing multiple environmental problems: the acidification of surface waters, the release of heavy metals from sediments, increased salinity of freshwaters and the production of toxic sulfide in anaerobic soils. Conventional treatment techniques to remove heavy metals from wastewater result in the production of solid waste. Therefore, an alternative treatment technique, in which sulfate is biologically reduced to sulfide, was developed. Sulfide precipitates with metals to form metal sulfides (MeS) which can be reused in the process. The electron donor for SR forms the major operation costs. Cheap electron donors such as organic waste streams are not easily degradable and often contain some inert material. Therefore pre or post treatment is required. Fully degradable pure bulk chemicals are therefore a better option. Ethanol (C_2H_5OH) and CH_3OH are interesting electron donors on smaller and middle scale, but on large scale the best electron donor is H_2. The best known and least expensive method of producing commercial bulk H_2 is the steam reforming of natural gas, sometimes referred to as steam CH_4 reforming (SMR). At high temperature (700 – 1000 °C) and high pressure (3-25 bar) in the presence of a metal-based catalyst (nickel), steam reacts with CH_4 to yield CO and H_2 according to equation 8. Additional H_2 can be recovered by a lower-temperature gas-shift reaction, in which the produced CO is utilized, according to equation 9.

(8) $CH_4 + H_2O \rightarrow CO + 3\ H_2$
(9) $CO + H_2O \rightarrow CO_2 + H_2$
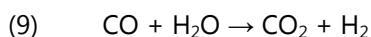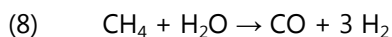
At the Nyrstar zinc factory in Budel (the Netherlands), H_2 produced by SMR is used as electron donor for biological SR. However, the efficiency of the SR process decreases when CH_4 and CH_3COO^- formation from H_2/CO_2 occurs, according to equations 10-12:

(10) $4 H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4 H_2O$ (SRB)

(11) $4 H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3 H_2O$ (Methanogens)

(12) $4 H_2 + 2 HCO_3^- + H^+ \rightarrow CH_3COO^- + 4 H_2O$ (Homoacetogens)

When H_2 is limiting and SO_4^{2-} is in excess, SRB compete with methanogens and homoacetogens for the available H_2. Growth kinetics, quantified by the maximum specific growth rate, substrate affinity and substrate threshold are often used to explain the outcome of bacterial competition. Reported values for these parameters reveal an order of competitivity of heterotrophic SRB > methanogens > homoacetogens at low H_2 concentration (van Houten, 1996). Also CO_2 limitation, can reduce CH_4 production (van Houten et al., 1994).

However, the SMR process has a low efficiency and requires high temperatures and high pressures. Therefore the possibility of coupling AOM to SR in industrial processes is attractive. Using CH_4 directly as electron donor for biological SR, will greatly reduce the costs of the wastewater treatment. This is due to the fact that CH_4 is 4 times cheaper than H_2 and 8 times cheaper than C_2H_5OH in addition natural gas distribution networks are commonly available. Furthermore biological SR directly with CH_4, will reduce CO_2 emission to the atmosphere. Additional advantages when CH_4 is used are: per amount of SO_4^{2-} reduced only ¼ of the H_2 volume is needed, there will be no competition for substrate with methanogens and/or acetogens and the low yield of the CH_4 oxidizing archaea prevents energy loss due to growth, makes their application in an efficient biological SR system suitable.

Application of the process of sulfate removal with methane as substrate in an industrial process for heavy metal removal requires a stable enrichment capable of high rate removal of sulfate. The process should be possible at ambient temperature and pressure, with a high sulfide and heavy metal tolerance. This thesis describes the efforts to enrich a community capable of high rate AOM and SR in a bioreactor (Chapters 2 and 3) and the search for optimal growth conditions and the pathways

used in the alleged syntrophic relation between the ANME archaea and the SRB (Chapters 4 and 5).

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Chapter 2

Microbial diversity and community structure of a highly active anaerobic methane oxidizing sulfate-reducing enrichment

2.1 Abstract

Anaerobic oxidation of methane (AOM) is an important methane sink in the ocean but the microbes responsible for AOM are as yet resilient to cultivation. Here we describe the microbial analysis of an enrichment obtained in a novel submerged-membrane bioreactor system and capable of high rate AOM (286 μmol.g$_{dry\ weight}^{-1}$.day^{-1}) coupled to sulfate reduction (SR). By constructing a clone library with subsequent sequencing and fluorescent in situ hybridization (FISH), we showed that the responsible methanotrophs belong to the ANME-2a subgroup of anaerobic methanotrophic archaea, and that sulfate reduction is most likely performed by sulfate reducing bacteria commonly found in association with other ANME related archaea in marine sediments. Another relevant portion of the bacterial sequences can be clustered within the order of *Flavobacteriales* but their role remains to be elucidated. FISH analyses showed that the ANME-2a cells occur as single cells without close contact to the bacterial syntrophic partner. Incubation with ^{13}C labeled methane showed substantial incorporation of ^{13}C label in the bacterial C$_{16}$ fatty acids (bacterial; 20, 44 and 49%) and in archaeal lipids, archaeol and hydroxyl-archaeol (21 and 20%, respectively). The gathered data confirms that both archaea and bacteria are responsible for the anaerobic methane oxidation in a bioreactor enrichment inoculated with Eckernförde bay sediment.

2.2 Introduction

Large amounts of methane are formed by biotic and abiotic processes in marine sediments. The major part of methane that is formed in marine sediments is oxidized anaerobically before it can reach the earth's atmosphere (Crutzen, 1994; Reeburgh, 1996; Hinrichs *et al*, 2002). Reeburgh (1976) was the first to suggest that the anaerobic oxidation of methane (AOM) is coupled to sulfate reduction (SR)). Microorganisms which couple AOM to SR have indeed been reported in methane seeps and gas hydrate sediments (e.g. Hinrichs *et al.*,1999; Boetius *et al.*, 2000; Pancost *et al.*, 2000; Lanoil *et al.*, 2001; Knittel *et al.*, 2005; Treude *et al.*, 2007) and in non-seep sediments (Bian *et al.*, 2001; Treude *et al.*, 2005; Parkes *et al.*, 2007).
The leading explanation suggests that AOM is mediated by a syntrophic community of methanotrophic archaea, performing reversed methanogenesis, and sulfate-

reducing bacteria (SRB) that use compounds excreted by the archaea as electron donor for sulfate reduction (Orphan *et al.*, 2001; Blumenberg *et al.*, 2005). The methanotrophic archaea are represented by three different phylogenetic clusters (ANME-1, -2 and -3). Archaea in the ANME-2 and -3 clusters are closely affiliated with methanogenic archaea of the order of *Methanosarcinales* (Hinrichs *et al.*, 1999; Orphan *et al.*, 2001; Niemann *et al.*, 2006). ANME-1 archaea are distinct from, but related to, the methanogenic orders *Methanomicrobiales* and *Methanosarcinales* (Hinrichs *et al.*, 1999). The known ANME clusters are associated with specific SRB belonging to the *Desulfosarcina/Desulfococcus* (DSS) group (Boetius *et al.*, 2000; Michaelis *et al.*, 2002; Knittel *et al.*, 2003) and the *Desulfobulbus* group (Niemann *et al.*, 2006) of the *Deltaproteobacteria*. Despite several investigations, the exact mechanism of metabolic interaction between the syntrophic partners is still unclear (Hoehler *et al.*, 1994; Nauhaus *et al.*, 2002; Moran *et al.*, 2008; Stams and Plugge, 2009). Obtaining pure cultures of the microorganisms for physiological studies could solve this problem but the extremely low growth rates with reported doubling times varying from 2 to 7 months (Girguis *et al.*, 2005; Nauhaus *et al.*, 2007; Krüger *et al.*, 2008) and product inhibition by sulfide toxicity make isolation of these microorganisms difficult.

To overcome some of these problems several designs of bioreactors and incubation systems have been developed, but they did not prevent product inhibition and the outflow of suspended cells. Girguis *et al.* (2005) developed a flow-through reactor to reproduce the *in situ* conditions of methane seep sediments. In these reactors, the number of ANME archaea increased, and the rate of AOM increased but did not exceed 140 nmol/g dry sediment per day. Nauhaus (2002) found that methane-driven sulfate reduction rate increased five times in ANME-2 dominated sediments by increasing the methane partial pressure from atmospheric pressure to 1.1 MPa. In a later study they developed a fed-batch system that was operated at a methane partial pressure of 1.4 MPa, corresponding to 21 mM dissolved CH_4 (12°C) and an AOM rate of 230 $\mu mol.g_{dry\ weight}^{-1}.day^{-1}$ was reached (Nauhaus *et al.*, 2007).

In this study, we analyzed the microbial community that was enriched in a continuous submerged-membrane bioreactor inoculated with Eckernförde Bay sediment. The reactor design and and its performance are described in Chapter 3. The enrichment obtained after 809 days was characterized by fluorescent *in situ* hybridization (FISH), using specific probes for AOM archaea and SRB, and by constructing a clone library with 16S rRNA genes from the archaeal and eubacterial

community. The reactor biomass was incubated with ^{13}C labeled methane and the label incorporation into archaeal and bacterial lipids was measured.

2.3 Materials and methods

2.3.1 Reactor and sampling

Sediment samples were taken in Eckernförde Bay (Baltic Sea) at a water depth of 28 m (position 54°31'N, 10°01'E), during a one day cruise of the German research vessel *Littorina* in June 2005. This sampling site has been described by Treude *et al.* (2005)(see also Chapter 4.2.1). To enrich for anaerobic methanotrophs, a novel submerged-membrane bioreactor was developed (Chapter 3). The liquid volume, pH and temperature in the bioreactor were maintained at 1 L, 7.2 and 15ºC, respectively. The reactor was continuously supplied with 0.13 L day^{-1} marine medium (description can be found in chapter 3.3.2) and 4.8 L day^{-1} pure methane gas, which was supplied via a gas sparger at the bottom of the bioreactor. To provide additional mixing and to suspend the sediment/biomass, the reactor suspension was recirculated from top to bottom at a rate of 0.3 L min^{-1}. The effluent was extracted via 4 polysulfone membranes (Triqua BV, Wageningen, the Netherlands). The mean pore size of 0.2 µm guaranteed complete cell retention. Sampling and reactor maintenance were done under anaerobic conditions and the reactor was kept anaerobic for the duration of the run. The effluent was checked regularly for the outflow of solids and cells and for the presence of oxygen. During a total reactor run of 884 days, liquid samples from mixed reactor sludge were taken regularly to perform DGGE and FISH and to construct a clone library. Batch incubations with diluted reactor sludge taken at day 570 of the reactor run were used for ^{13}C labeling experiments for lipid analyses and AOM activity tests.

2.3.2 DNA extraction and construction of a clone library

DNA was extracted from the bioreactor sludge using the FastDNA SPIN for Soil Kit (MP Biomedicals, Ohio, USA). To construct an archaeal and a bacterial 16S rRNA gene library, almost full-length 16S rRNA gene fragments were amplified using primers ARCH-4f and Uni1492r (Sousa *et al.*, 2007). 16S rRNA-gene PCR was performed in a G-storm cycler (G-storm, Essex, UK) starting with 2 min at 94°C,

followed by 35 cycles of 94°C for 30 s, 52°C for 40 sec and 72°C for 1.5 min. The final PCR extension step was at 72°C for 5 min. PCR products were ligated into pGEM-T (Promega) and transformed into *E. coli* XL1-blue cells (Stratagene) as specified by the manufacturer. For screening of the clone library by DGGE (Schabereiter-Gurtner *et al.*, 2003), 10 µl of the overnight culture of the clones were mixed with 90 µl of TE, and lysed by heating 10 min at 95°C. 400 bp 16S rRNA gene fragments were amplified from 1 µl of the lysed clones using the primer pair ARCH-109T-f (Großkopf *et al.*, 1998) plus Uni515r-GC clamp (Lane, 1991). The DNA clean and concentrator-5 kit (Zymo research) was used for the purification of almost full-length 16S rRNA gene fragments.

2.3.3 Phylogenetic analyses

A phylogenetic analysis of the sequences was performed by using the standard operating procedure for phylogenetic inference (SOPPI) developed by Peplies *et al.* (2008). Purified PCR products from the plasmid clones were used as the templates for sequence analysis and sequenced commercially by BaseClear (Leiden, The Netherlands). The complete sequences were obtained by primers from previously published work: BACT-27f, Uni-515r, Uni-519f, BACT-1100r (Lane, 1991) and Uni-1492r (Sousa *et al.*, 2007) for eubacterial sequences and ARCH-4f, Uni-515r (Lane, 1991), ARCH-340f (Øvreås *et al.*, 1997), ARCH-915r (Stahl *et al.*, 1991) and Uni-1492r for Archaeal sequences. The overlapping set of sequences were assembled into one contiguous sequence by using the DNASTAR Lasergene 6 package (Madison, WI, USA) and verified by BlastN (Altschul *et al.*, 1997). The possible chimerical sequences were checked using the Pintail program (Ashelford *et al.*, 2005) and Vector sequences were removed by using the VecScreen system (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Sequences have been analysed using the ARB software package (version December 2007)(Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 96 database (Pruesse *et al.*, 2007). After importing, all sequences were automatically aligned according to the SILVA SSU reference alignment. Manual refinement of the alignment was carried out taking into account the secondary structure information of the rRNA.

Tree reconstruction was performed with up to 1000 sequences using the neighbor joining (ARB), MP (DNAPars v1.8, Felsenstein, 2005) and ML (RAxML v7.04, (Stamatakis, 2006) methods. Tree topology was further tested by the application of 30%, 40% and 50% positional conservatory filters. The final tree was calculated with 500 sequences based on 1280 valid columns (50% conservatory filtering) with RAxML (model: GTRGAMMA). Partial sequences were added to the tree using the ARB parsimony tool. Multifurcations were manually introduced in the case where tree topology could not be unambiguously resolved based on the different treeing methods and the underlying dataset. For better clarity, only selected subsets of the sequences used for treeing are shown in the figure. Only sequences from the bioreactor clones with 2 or more identical migration patterns in DGGE have been used to construct the phylogenetic tree (Figure 2.2, 2.3, and 2.4). Table 2.2 shows the phylogenetic affiliation of the clones. All sequences described in the paper have been deposited in the databases of Genbank, under accession numbers FJ555674-FJ555687 (archaeal sequences) and FJ615406-FJ615417 (eubacterial sequences).

2.3.4 ^{13}C-CH$_4$ incubation

120-ml serum bottles were closed with butyl rubber stoppers and caps, and the gas phases were replaced 8 times with nitrogen gas and made vacuum thereafter. Subsequently, 20 ml reactor suspension was sampled (570 days after reactor inoculation) and immediately transferred into the bottles, using a syringe and a hypodermic needle (internal diameter of 0.2 mm). To ensure homogeneous sampling of the reactor suspension, the gas sparging rate in the reactors was temporally increased to 1 L min^{-1}. Subsequently, the headspaces of the bottles were made vacuum again and filled with 0.15 MPa pure ^{13}C-CH$_4$ gas (Campro, Veenendaal, the Netherlands). The bottles were incubated in an orbital shaker (rotating at 100 rpm) at 15°C. To remove the accumulation sulfide, the suspension and headspace were monthly flushed with N$_2$, during which HCl was added anaerobically to keep the pH between 7.2 and 7.5. Subsequently, the headspace was replaced by new ^{13}C-CH$_4$. After three months, the biomass was sampled for lipid analyses.

2.3.5 Lipid analyses

The ^{13}C-methane incubated biomass was extracted using the procedure of Bligh and Dyer (1959), with minor modifications. The freeze dried biomass was extracted 3 times in ultrasonic bath for 10 min with methanol (MeOH)/dichloromethane (DCM)/phosphate buffer in a volume ratio 2/1/0.8. The phosphate buffer was composed of 8.7 g of K_2HPO_4 dissolved in 1.0 L of bi-distilled H_2O and pH adjusted to 7 with 1 M HCl. The supernatant was collected, and DCM and phosphate buffer were added to the supernatant in a final volume ratio of 1/1/0.9. The mixture was centrifuged for 1 min at 3000 rpm. The methanol/phosphate buffer phase was removed and the DCM phase was collected in a round-bottom flask. The methanol/phosphate buffer was re-extracted twice with DCM. The combined DCM phases were reduced under rotary vacuum and dried under N_2. The extract was subsequently hydrolysed by refluxing with 2 ml 2 N HCl/MeOH (1:1 v/v) for 3 h after which the pH was adjusted to 5 with 1 N KOH/MeOH 1:1 v/v). Subsequently, 2 ml double distilled H_2O and 2 ml DCM were added and the MeOH/H_2O layer was washed twice with 2 ml DCM. The DCM layers were combined and dried. The hydrolyzed extract was methylated by adding 0.5 ml of BF_3-MeOH to the dried extract and incubation for 10 min at 60°C. Then, 0.5 ml of bi-distilled water was added and the water layer was washed three times with DCM. The DCM layer containing the total lipid extract (TLE) was collected and dried with N_2. The TLE was dissolved in ethyl acetate, and transferred on a small silica gel 60 column, and eluted with ethyl acetate (3x column volumes). Subsequently, the TLE was silylated by dissolving in 25 µl pyridine and 25 µl BSTFA and incubated for 20 min in 60°C. Samples were then diluted with ethyl acetate to a final concentration of 1mg/ml. The methylated and silylated hydrolysed extract was analysed by gas chromatography (GC), GC/mass spectrometry (MS) and isotope ratio monitoring GC/MS. GC analyses was performed using an Agilent 6890 instrument equipped with a flame ionization detector (FID) and an on-column injector. A fused silica capillary column (25 m x 0.32 mm) coated with CP-Sil 5 (film thickness 0.12 µm) was used with helium as carrier gas. The oven was programmed at a starting (injection) temperature of 70 °C, which rose to 130 °C at 20°C/min and then to 320 °C at 4 °C/min, at which it was maintained for 20 min. GC/MS analysis was done using a Thermofinnigan TRACE gas chromatograph under the same GC conditions as described above. The gas chromatograph was coupled with a Thermofinnigan DSQ quadrupole mass spectrometer with an ionization energy of 70 eV using GC conditions as described above. Samples were analysed in full scan mode with a mass range of m/z 50-800 at

three scans per second. Stable carbon isotopes were measured using an Agilent 6890 GC coupled via a combustion interface to a ThermoFisher Delta V irm-MS. The stable carbon isotopic compositions were measured against external calibrated reference gas. Derivatised compounds were corrected for added methyl and trimethylsilylgroups.

2.3.6 Fluorescent in situ hybridization (FISH)

Samples were fixed overnight at 4°C with 3% formaldehyde, centrifuged and washed twice with PBS and finally stored in PBS/EtOH (1:1) at -20 °C. Stored samples were diluted and treated by 1 s pulsed sonication for 20 s (Branson sonifier B-12, probe from Heinemann, Germany) at an amplitude of 40% of the maximum power of 70W. Dilution series of samples were prepared in order to determine the optimal cell concentration for counting with the different probes. 10 µl of the fixed sample was spotted on the well of a gelatin coated slide (8 mm well, 10 well Multitest slide, MP Biomedicals) and dried for 10 min at 46 °C. The cells were dehydrated for 2 to 3 min in a graded ethanol series with the ethanol concentration increasing from 50 to 80% and finally in 96% ethanol in H_2O. 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) was added to each well, and 1 µl of each probe (50 ng/µl) was added to the wells and this was followed by incubation at 46°C for 2-3 h. After hybridization the slides were washed in 50 ml of pre-warmed (48°C) washing buffer with SDS for 10 min. For total counts 4',6-diamidino-2-phenylindole (DAPI) was added to the washing buffer at a final concentration of 100 ng/ml. After the slides were rinsed in water, they were immediately air dried, mounted in Vectashield (Vector Labs, Burlingame, USA) and covered with a cover slide (42x60mm, Menzel-Glaser, Germany) Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMR epifluorescence microscope, were taken with a Leica DFC 340FX camera. The oligonucleotide probes with CY3- and carboxyfluorescein- (FLUOS-) labels were obtained from Eurogentec (Belgium)

2.4 Results

After 884 days of reaction operation, the AOM rate in the bioreactor had increased exponentially from 0.4 to 286 $\mu mol.g_{dry\ weight}^{-1}.day^{-1}$ (Chapter 3). Microscopic observations revealed that the biomass in the reactor, after 884 days of operation,

was mainly present as loose flocks with an average size of 0.1 mm. Besides the flocks, also single cells were detected (Figure 2.1).
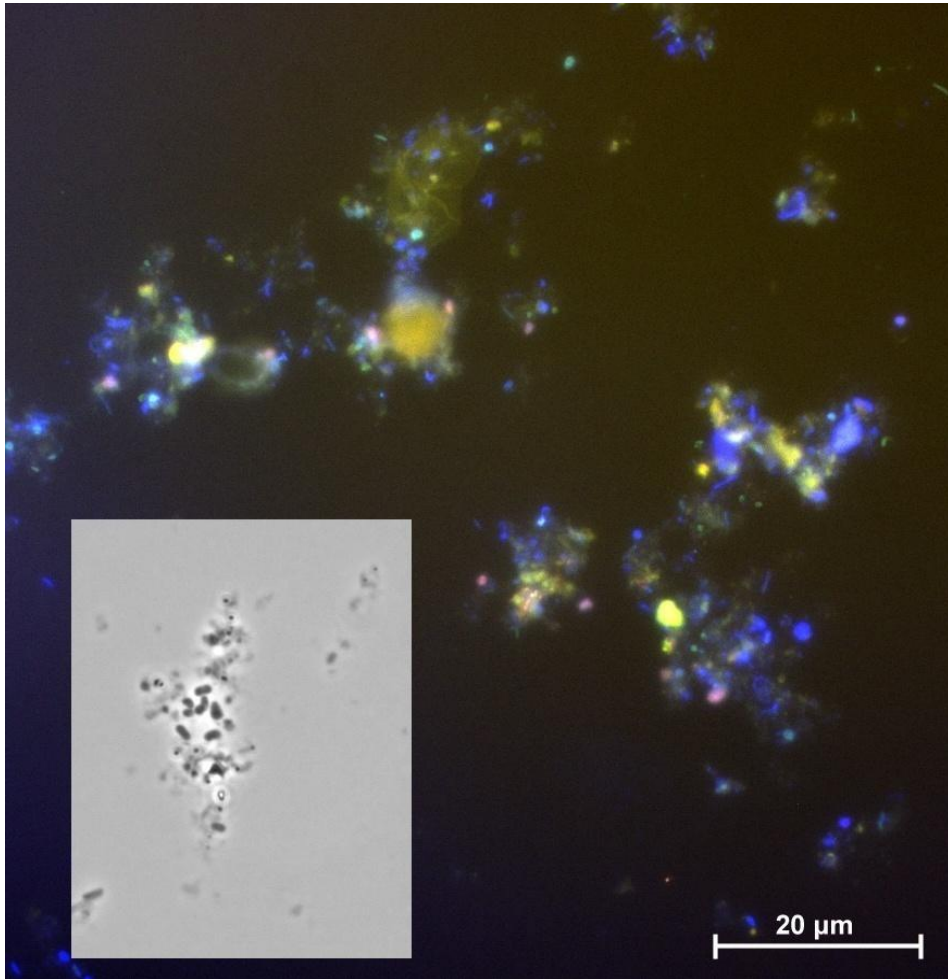

Figure 2.1: FISH image with a probe for the ANME-2a subgroup (ANME-IIa-647 (Knittel et al., 2005), red) ,probe for the DSS subgroup of the sulfate reducing bacteria (DSS658 (Manz et al., 1998), green), and nonspecific stain for DNA (DAPI, blue). Insert is a bright field microscopy image of a typical loose aggregate found in the bioreactor enrichment.

Analysis of the 16S rRNA gene sequences obtained with the clone library showed that the archaeal community is dominated by ANME-2a archaea (90%, N=172 clones, Table 2.2, Figure 2.2). The second most dominant group clustered in the *Thermoplasmatales* group of the *Euryarchaeota* (8%, N=172, Table 2.2, Figure 2.3).

Table 2.2: Phylogenetic affiliation of 16S rRNA gene sequences obtained with clone library analysis.

| 16S rRNA phylotypes, number	16S rRNA phylotypes, %	Closest relative
172 total		*Archaea*
155	90	ANME-2a
11	6	*Thermoplasmatales*
3	2	Uncultured *Methanococcoides*
1	1	Uncultured *Methanosarcinales*
1	1	Uncultured *Methanomicrobiales*
1	1	Uncultured *Methanolobus*
68 total		*Bacteria*
32	47	*Deltaproteobacteria*
23	34	*Bacteroidetes*
6	9	*Planctomycetes*
2	3	*Alphaproteobacteria*
2	3	Uncultured *Chloroflexi*
1	1	Uncultured *Spirochaetes*
1	1	*Gammaproteobacteria*
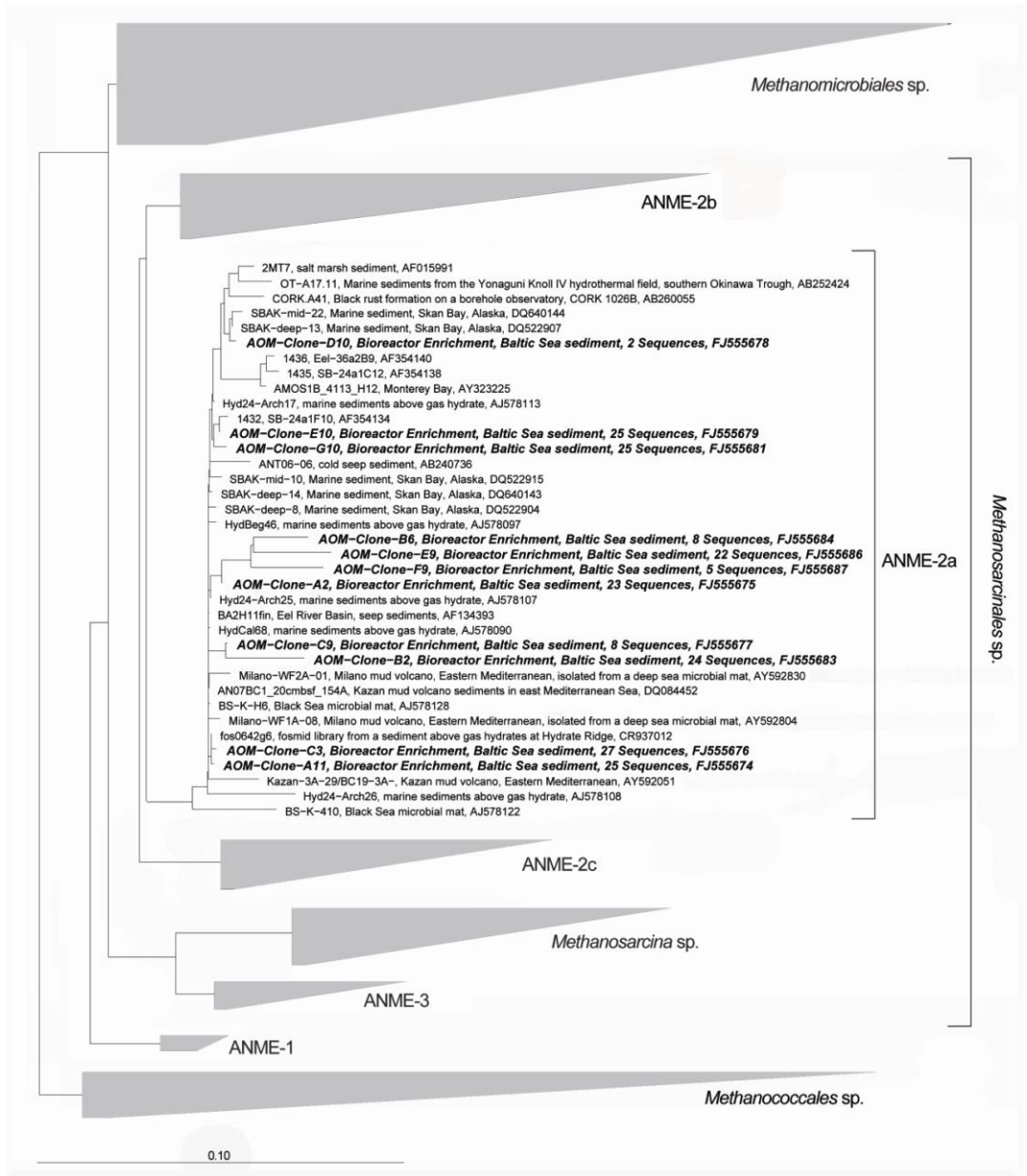
Figure 2.2: Phylogenetic tree showing the affiliation of archaeal 16S rRNA gene sequences (N=172 clones) retrieved from the submerged membrane bioreactor library (printed in boldfaced type) including representative sequences of the ANME-2a subgroup.
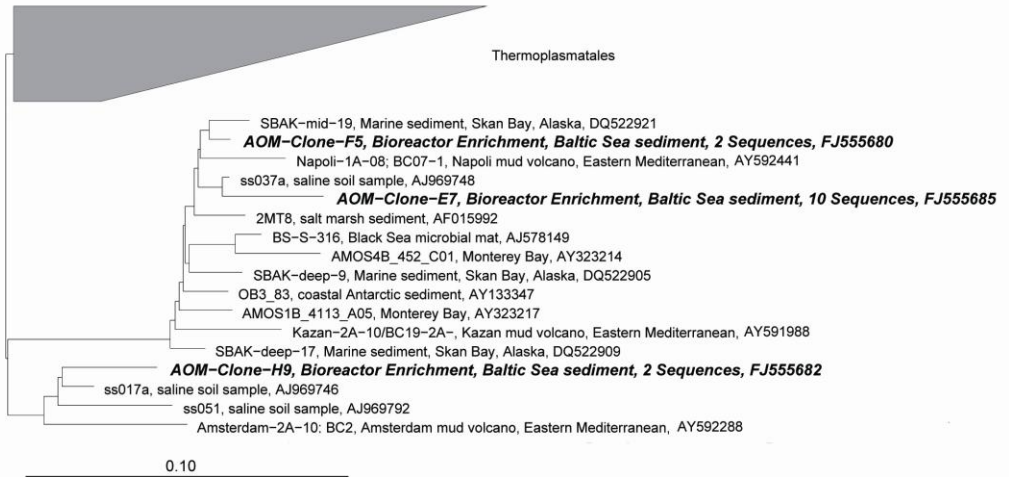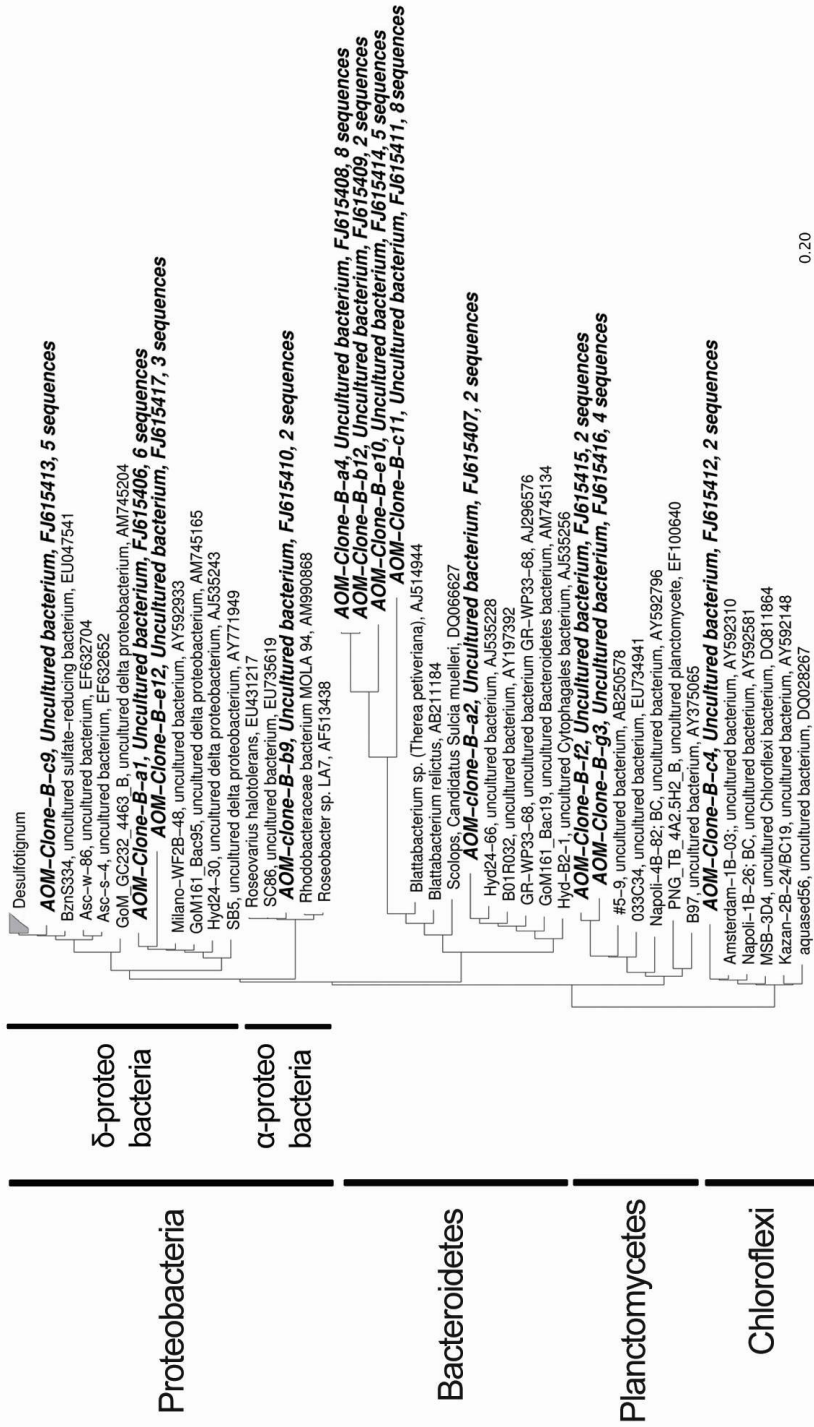
Figure 2.3: Phylogenetic tree showing the affiliation of archaeal 16S rRNA gene sequences (N=172 clones) retrieved from the submerged membrane bioreactor library (printed in boldfaced type) including representative sequences of the Thermoplasmatales.

The closest relatives within this group are clones from other marine sediments where AOM occurs like Skan Bay (Alaska), Mediterranean mud volcanoes and the Black Sea (Kendall *et al.*, 2007; Knittel *et al.*, 2005; Heijs *et al.*, 2005, 2007). The bacterial sequences showed a dominance of organisms belonging to the *Deltaproteobacteria (50%, N=68)* and the *Flavobacteriales (34%, N=68,* Figure 2.4).

Desulfotignum
AOM-Clone-B-c9, Uncultured bacterium, FJ615413, 5 sequences
BznS334, uncultured sulfate–reducing bacterium, EU047541
Asc–w–86, uncultured bacterium, EF632704
Asc–s–4, uncultured bacterium, EF632652
GoM_GC232_4463_B, uncultured delta proteobacterium, AM745204
AOM-Clone-B-a1, Uncultured bacterium, FJ615406, 6 sequences
AOM-Clone-B-e12, Uncultured bacterium, FJ615417, 3 sequences
Milano–WF2B–48, uncultured bacterium, AY592933
GoM161_Bac95, uncultured delta proteobacterium, AM745165
Hyd24–30, uncultured delta proteobacterium, AJ535243
SB5, uncultured delta proteobacterium, AY771949
Roseovarius halotolerans, EU431217
SC86, uncultured bacterium, EU735619
AOM-clone-B-b9, Uncultured bacterium, FJ615410, 2 sequences
Rhodobacteraceae bacterium MOLA 94, AM990868
Roseobacter sp. LA7, AF513438
AOM-Clone-B-a4, Uncultured bacterium, FJ615408, 8 sequences
AOM-Clone-B-b12, Uncultured bacterium, FJ615409, 2 sequences
AOM-Clone-B-e10, Uncultured bacterium, FJ615414, 5 sequences
AOM-Clone-B-c11, Uncultured bacterium, FJ615411, 8 sequences
Blattabacterium sp. (Therea petiveriana), AJ514944
Blattabacterium relictus, AB211184
Scolops, Candidatus Sulcia muelleri, DQ066627
AOM-clone-B-a2, Uncultured bacterium, FJ615407, 2 sequences
Hyd24–66, uncultured bacterium, AJ535228
B01R032, uncultured bacterium, AY197392
GR–WP33–68, uncultured bacterium GR–WP33–68, AJ296576
GoM161_Bac19, uncultured Bacteroidetes bacterium, AM745134
Hyd-B2–1, uncultured Cytophagales bacterium, AJ535256
AOM-Clone-B-f2, Uncultured bacterium, FJ615415, 2 sequences
AOM-Clone-B-g3, Uncultured bacterium, FJ615416, 4 sequences
#5–9, uncultured bacterium, AB250578
033C34, uncultured bacterium, EU734941
Napoli–4B–82; BC, uncultured bacterium, AY592796
PNG_TB_4A2.5H2_B, uncultured planctomycete, EF100640
B97, uncultured bacterium, AY375065
AOM-Clone-B-c4, Uncultured bacterium, FJ615412, 2 sequences
Amsterdam–1B–03;, uncultured bacterium, AY592310
Napoli–1B–26; BC, uncultured bacterium, AY592581
MSB–3D4, uncultured Chloroflexi bacterium, DQ811864
Kazan–2B–24/BC19, uncultured bacterium, AY592148
aquased56, uncultured bacterium, DQ028267

0.20

Proteobacteria

δ-proteo bacteria

α-proteo bacteria

Bacteroidetes

Planctomycetes

Chloroflexi

Figure 2.4: Phylogenetic tree showing the affiliation of eubacterial 16S rRNA gene sequences (N=68 clones) retrieved from the submerged membrane bioreactor library (Printed in boldfaced type) to selected reference sequences.

FISH imaging showed an increase of cells hybridizing with both the ANME-2 and ANME-2a probes, and combining these probes with universal archaeal probes, showed that the dominant archaeal species belong to the ANME-2a subgroup of anaerobic methanotrophs (Figure 2.1). With probes specific for ANME-2 archaea and DSS, some consortia consisting of ANME-2/DSS cells can be detected in the bioreactor sludge, but quantitative analyses did not show an increase of consortia compared to the original Eckernförde Bay sediment (results not shown). The ANME-2a cells present in the bioreactor sludge are not directly associated with a bacterial partner. Quantitative analyses using FISH showed an equal number of ANME-2a cells compared with cells that hybridize with probes specific for DSS. The ANME-2a cells have an average count of 150/1000 DAPI signals (± 40) measured in triplicate from separately hybridized slides. FISH analyses using universal probes for archaea and eubacteria show an abundance of bacterial to archaeal cells in a 10:1 ratio.

An aliquot of the reactor biomass taken on day 570 of the run, was incubated for three months under a pure 0.15 MPa ^{13}C-CH$_4$ headspace and the distribution and ^{13}C-content of the lipids were analyzed. The lipid extract was dominated by C$_{14}$-C$_{18}$ fatty acids with no, one or two double bonds, lipids which are ubiquitously present in bacteria (Table 2.1). In addition, small amounts of C$_{14}$ and C$_{16}$ glycerol monoethers and a C$_{16}$ glycerol diether are present, compounds which have been found in some SRB (Rütters *et al.*, 2001) and in sediments where AOM occurs (Hinrichs *et al.*, 2000; Pancost *et al.*, 2001).

Finally, archaeol and *sn2*-hydroxyarchaeol, lipids typical for archaea, including those involved in AOM (Blumenberg *et al.*, 2004), were also present but in much lower abundances than the bacterial lipids. Carbon isotopic analysis revealed large amounts of incorporation of ^{13}C label in both bacterial and archaeal lipids (Table 2.1). These results confirm that both bacteria and archaea of the reactor have incorporated label derived from methane into their biomass. The degree of labeling in the bacterial C$_{16}$ fatty acids (bacterial; 20, 44 and 49%) was similar or even higher

than in the archaeal lipids, archaeol and hydroxyl-archaeol (21.3 and 20.1%, respectively).

Table 2.1. Relative abundance and degree of ^{13}C labelling of bacterial and archaeal lipids in the lipid extract of the bioreactor enrichment after 3 months of batch incubation.

Compound	Relative abundance (%)	Stable carbon isotopic composition [% ^{13}C]
C14:0 FA	3.4	36.1
iso C15:0 FA	1.8	6.0
anteiso C15:0 FA	1.8	8.1
C15:0 FA	0.7	20.5
C16:1 FA	8.6	49.0
C16:1 FA	5.2	44.0
C16:0 FA	24.2	20.3
C18:2 FA	1.2	14.6
C18:1 FA	1.3	7.4
C18:1 FA	7.4	10.9
C18:0 FA	27.0	2.9
C20:0 FA	1.8	2.8
C14 monoether (1-tetradecanoyl-O-glycerol	1.2	16.2
C22:1 FA	4.5	1.5
C16 monoether (1-hexadecanoyl-O-glycerol	2.1	10.3
C16 monoglyceride	1.3	1.0
C24:0 FA	1.5	1.2
C18 monoglyceride	1.1	1.7
C26:0 FA	0.4	1.8
C28:0 FA	0.2	1.7
C16 diether	1.0	3.1
archaeol	1.3	21.3
SN2-hydroxyarchaeol	0.9	20.1

FA = fatty acid.

2.5 Discussion

The dominance of ANME-2a sequences in the archaeal clone library and the increase in single ANME-2a cells in the FISH analysis, suggest that archaea from the ANME-2a subgroup of the anaerobic methanotrophs are responsible for the exponential increase in AOM rate in the bioreactor. No other ANME sequences were detected by denaturing gradient gel electrophoresis (DGGE) (Chapter 3) or the clone library analysis. The known SRB capable of growth under mesophilic conditions and the DSS-affiliated sequences belong to the *Deltaproteobacteria*. This is consistent with the presence of *Deltaproteobacteria* in the clone library. The sequences in the clone library confirm the presence of sulfate-reducing bacteria related to *Desulfotignum* sp. and uncultured environmental clones from anaerobic methanotrophic sediments (Musat *et al.*, 2008; Heijs *et al.*, 2005).

The other dominant group of sequences found in the bacterial clone library belongs to the phylum *Bacteroidetes* and form a cluster within the order of *Flavobacteriales*. The novel cluster is phylogenetically distantly related to *Blattabacteria*, isolated from cockroach hindgut (Wren *et al.*, 1987). The functioning of these intracellular endosymbionts of insects is not yet fully understood, but it is reported to be linked to the conversion of inorganic sulfate to organic sulfur compounds (Wren *et al.*, 1987; Henry *et al.*, 1960) or the nitrogen-metabolism (Cruden *et al.*, 1987). Recent findings also indicate a much larger role of bacteria not related to known SRB in AOM like *Betaproteobacteria*, most similar to members of the *Burkholderiaceae*, and *Alphaproteobacteria*, related to *Sphingomonas* (Pernthaler *et al.*, 2008). Other clones from the bioreactor enrichment can be linked to known marine micro-organisms and because of their low abundance after more than 800 days of continuous incubation, they are most probably residual micro-organisms from the original Eckernförde bay sediment or organisms that use cross-feeding for their metabolism.

The presence of single cells which hybridize with the ANME-IIa-647 or the DSS658 FISH probe without a directly associated bacterial or archaeal partner does not correspond with the idea that AOM is a syntrophic process that requires a close physical interaction of the micro-organisms involved (Boetius *et al.*, 2000; Schink, 2002). In some sediments highly structured ANME-2/*Desulfosarcina* consortia are not the sole entities responsible for AOM, but also monospecific consortia and single cells can be active (Orphan *et al.*, 2002).

Lipid analysis of the enrichment biomass showed that bacterial lipids were dominating over those of archaea, in agreement with the FISH results which showed a dominance of bacteria over archaea. ^{13}C-label from methane was substantially incorporated in both archaeal and bacterial lipids during batch incubation with bioreactor sludge. Our results are different from those of Blumenberg *et al.* (2005), who showed that ^{13}C-labelled methane is mainly taken up by bacteria rather than archaea. The difference can be explained by the much higher AOM rates observed here and the much more active archaea in the AOM consortium studied. Interestingly, the degree of labeling of the bacterial lipids observed in our study is much larger than that of Blumenberg *et al.* for the same lipids and after the same period of incubation (e.g. 44% versus 0.2% for the C16:1 fatty acid), suggesting that the SRB were also much more active. The reason why the ^{13}C-label is taken up by bacteria in this and previous studies (Blumenberg *et al.*, 2005) is yet unclear. Possibly they have taken up [13]CO_2 or organic compounds produced by ANME-2a. However, the direct uptake of methane by bacteria cannot be excluded. Raghoebarsing *et al.* (2006) found low uptake rates of ^{13}C-labelled methane in archaeal lipids in batch reactors in which AOM was performed by a consortium of archaea and denitrifying bacteria and methane oxidation coupled to denitrification was later found to be a bacterial process not involving archaea (Ettwig *et al.*, 2008)

Acknowledgements

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Chapter 3

Enrichment of Anaerobic Methanotrophs in Sulfate-Reducing Membrane Bioreactors

3.1 Abstract

Anaerobic oxidation of methane (AOM) in marine sediments is coupled to sulfate reduction (SR). AOM is mediated by distinct groups of archaea, called anaerobic methanotrophs (ANME). ANME co-exist with sulfate-reducing bacteria, which are also involved in AOM coupled SR. The microorganisms involved in AOM coupled to SR can be are extremely difficult to grow *in vitro*. Here, a novel well-mixed submerged-membrane bioreactor system is used to grow and enrich the microorganisms mediating AOM coupled to SR. Four reactors were inoculated with sediment sampled in the Eckernförde Bay (Baltic Sea) and operated at a methane and sulfate loading rate of 4.8 L L^{-1} day^{-1} and 0.29 g L^{-1} day^{-1}. Two bioreactors were controlled at 15ºC and two at 30ºC. At 15ºC, the volumetric AOM and SR rates doubled approximately every 3.8 months. After 884 days, an enrichment culture was obtained with an AOM and SR rate of 1.0 mmol g_{VSS}^{-1} day^{-1} (286 µmol $g_{dry\ weight}^{-1}$ day^{-1}). No increase in AOM and SR was observed in the two bioreactors operated at 30ºC. The microbial population of one of the 15ºC reactors was analyzed. ANME-2a became the dominant archaea. This study showed that sulfate reduction with methane as electron donor is possible in well-mixed bioreactors and that the submerged-membrane bioreactor system is an excellent system to enrich slow-growing microorganisms, like methanotrophic archaea.

3.2 Introduction

3.2.1 Anaerobic methanotrophs

The anaerobic oxidation of methane (AOM) by microbes was first discovered during geochemical studies, which showed that AOM in marine sediments is coupled to sulfate reduction (SR), according to equation 13 (Barnes and Goldberg, 1976; Martens and Berner, 1974; Reeburgh, 1976; 1980; Iversen en Jørgensen, 1985). The AOM rates in marine sediments are low, between 0.001 and 21 µmol $g_{dry\ weight}^{-1}$ day^{-1} (Krüger *et al.*, 2005; Treude *et al.*, 2007).

(13) $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ $\Delta G° = -16.6\ kJ.mol^{-1}$
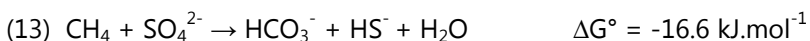
AOM in marine sediments is mediated by uncultured archaea, termed anaerobic methanotrophs (ANME). ANME are phylogenetically distantly related to cultivated methanogenic members from the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrichs *et al.*, 1999; Orphan *et al*, 2002; Knittel *et al.*, 2005; Niemann *et al.*, 2006). Three groups of ANME have been distinguished so far, of which ANME-1 and ANME-2 are the most abundant and geographically widespread groups (Hinrichs *et al.*, 1999; Orphan *et al.*, 2002; Knittel *et al.*, 2005; Niemann *et al.*, 2006). Thus far, no gene analogs for enzymes involved in dissimilatory SR have been found in archaea belonging to the ANME groups (Thauer and Shima, 2008). It has been suggested that the archeaon produces an electron carrier compound from CH_4 that is utilized by the sulfate-reducing partner (Zehnder and Brock, 1980; Alperin and Reeburgh, 1985; Hoehler *et al.*, 1994 and DeLong, 2000). This is supported by the fact that consortia of ANME and associated sulfate-reducing bacteria (SRB) are found in specific methane oxidizing marine sediments (Boetius *et al.* 2000, Hinrichs *et al.*, 2000). However, ANME may also occur not or only loosely associated to SRB, e.g. ANME-1 in Black Sea sediment (Treude *et al.*, 2007) and ANME-2 in Eckernförde Bay sediment (Treude *et al.*, 2005). Thus far, the exact metabolic pathway via which AOM coupled to SR proceeds is not known.

3.2.2 Sulfate reduction in biotechnology

Nauhaus *et al.* (2002; 2007) demonstrated *in vitro* AOM coupled to SR and growth at a rate of 0.003 day^{-1}. Therefore, AOM coupled to SR might also be possible in bioreactors, which would open a wide range of process applications. Biological sulfate reduction in bioreactors is applied for the removal and recovery of metal and sulfur compounds from waste or process streams produced in the mining and metallurgical industry (Weijma *et al.*, 2002). The produced sulfide and the dissolved metals form insoluble metal sulfides, which are separated from the water and reused in the metallurgical industry. The excess sulfide can be biologically oxidized to elemental sulfur, which is a reusable product as well. This process allows complete sulfur and metal recovery from a waste stream, however, the costs of the electron donor limit the application of this process. Natural gas (70-90% CH_4) is 2 to 4 times cheaper per amount of reducing capacity than conventional electron donors, like hydrogen and ethanol (Mueller-Langer *et al.*, 2007; www.ethanolmarket.com). To assess the potential of CH_4 as electron donor for biological sulfate reduction in

wastewater treatment, insight in the growth and conversion rates that can be achieved in bioreactors is required. In addition, the obtainment of an active methane-oxidizing sulfate-reducing enrichment is desired for studying the physiological aspects of AOM coupled to SR.

3.2.3 Current research

In the present study, a well-mixed ambient-pressure submerged-membrane bioreactor (MBR) was used to enrich anaerobic methanotrophs. In this bioreactor system, the washout of cells and growth limitation, due to product depletion (SO_4^{2-} and CH_4) or product inhibition (H_2S toxicity), can be prevented. The MBRs were inoculated with sediment from the Eckernförde Bay (Baltic Sea) and operated at 15 or 30°C. One MBR at 30°C was additionally inoculated with methanogenic granular sludge. This was done to assess if microorganisms from anaerobic granular sludge could play a role in SR with CH_4 as electron donor, directly or indirectly by providing unknown compounds that may support growth (e.g. growth factors). Volumetric AOM and SR rates were followed in time and used to estimate the growth rate. The microorganisms responsible for the conversion were identified by constructing a clone library of the enrichment and by monitoring the changes in microbial composition by Denaturing Gradient Gel Electrophoresis (DGGE).

3.3 Material and methods

3.3.1 Origin and storage of the inoculates

Sediment samples were taken in Eckernförde Bay (Baltic Sea) at station B (water depth 28 m, position 54°31) during a cruise of the German research vessel *Littorina* in June 2005. This sampling site has been described by Treude *et al.* (2005). Eckernförde Bay sediment is a non-seep sediment, AOM is fueled by CH_4 produced by organic matter degradation. Sediment samples were taken with a small multicore sampler based on the construction described by Barnett *et al.* (1984). The cores had a length of 50 cm and reached 30-40 cm into the sediment bed (see also chapter 4.2.1). Immediately after sampling, the content of the cores was collected in a large bottle, which was made anaerobic by replacing the headspace by anaerobic artificial seawater. Back in the laboratory, the sediment was homogenized and transferred

into 1L bottles in an anoxic glove chamber. The 1L bottles were closed with butyl rubber stoppers and the headspace was replaced by CH_4 (0.15 MPa). The bottles were stored at 4°C in the dark for 4 months until the experiments were started.

Methanogenic granular sludge samples were obtained from two full-scale methanogenic mesophilic UASB reactors, one UASB reactor treating paper mill wastewater (Industriewater Eerbeek, Eerbeek, the Netherlands, June 2005) and one treating distillery wastewater (Nedalco, Bergen op Zoom, the Netherlands, July 2005), described in detail by Roest *et al.* (2005) and Gonzalez *et al.* (2001), respectively, and stored anaerobically at 4°C in the dark.

3.3.2 Medium

The basal medium consisted of: NaCl (19.8 g L^{-1}), KCl (0.45 g L^{-1}) $MgCl_2.6H_20$ (4.25 g L^{-1}), NH_4Cl (0.25 g L^{-1}), $CaCl_2.2H_2O$ (1.19 g L^{-1}), $MgSO_4.7H_2O$ (5.10 g L^{-1}), KH_2PO_4 (0.34 g L^{-1}), $K_2HPO_4.3H_2O$ (1.25 g L^{-1}), a trace element solution (1 ml L^{-1}), a vitamin solution (1 ml L^{-1}), a 0.5 g L^{-1} resazurin solution (1 ml L^{-1}), a 0.1 M Na_2S solution (1 ml L^{-1}) and demineralized water. The trace elements and vitamin solutions were made according to Widdel and Bak (1992). Prior to the addition of the vitamins and sulfide solutions, the medium was boiled, cooled down under a nitrogen (N_2) atmosphere and transferred into a 10 L bottle with a CH_4 headspace (kept at an overpressure of 10 - 20 kPa). The bottle was kept at 4°C and connected to the influent pumps of the reactors.

3.3.3 Experimental set-up of membrane bioreactors

To enrich for anaerobic methanotrophs, 4 submerged-membrane bioreactors were built (Figure 3.1).
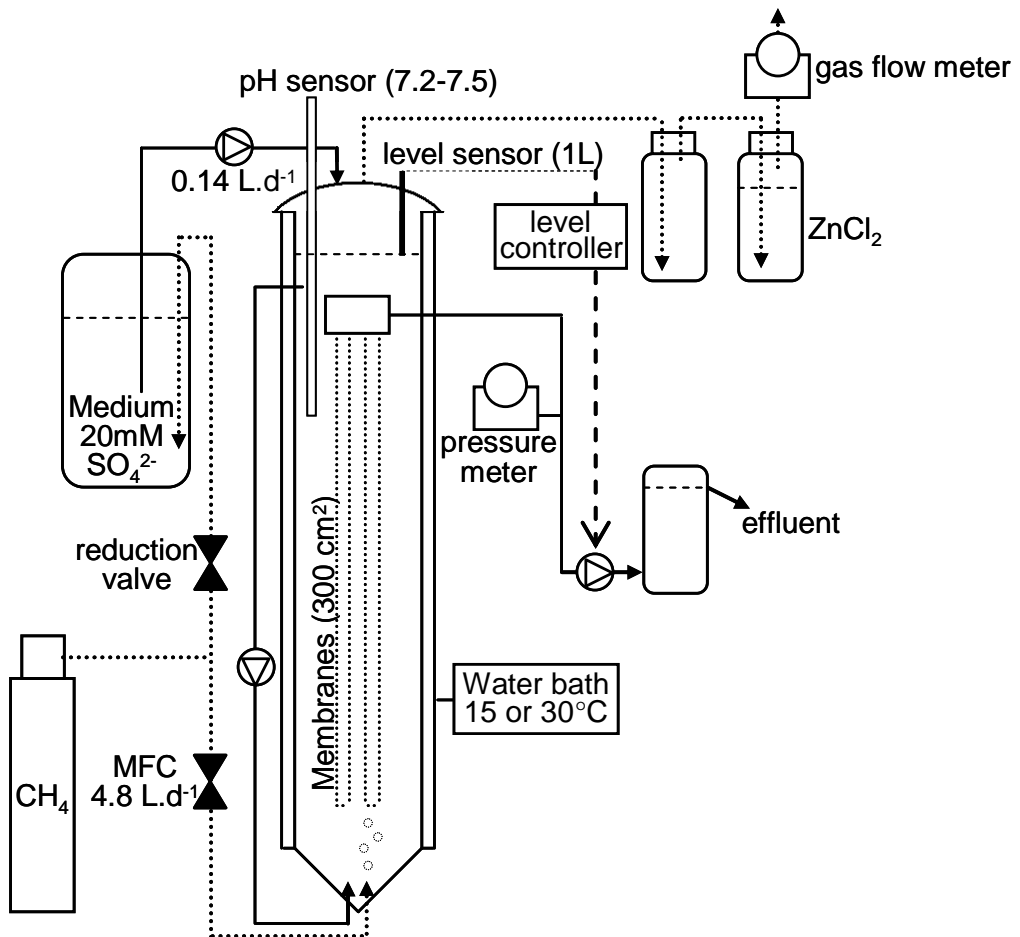
Figure 3.1 Schematic overview of a submerged-membrane bioreactor used for the enrichment experiments.

The reactor system consisted of a cylindrical glass vessel (height: 520 mm, internal diameter: 70 mm), the vessel was equipped with sampling ports for the headspace and the reactor suspension (mixture of liquid and suspended solids in the bioreactor). The glass reactor was covered with opaque plastic to prevent phototrophic conversions. STEPDOS® diaphragm metering pumps (KNF Flodos, Sursee, Switzerland) continuously supplied the reactors with medium, the hydraulic retention time (HRT) was 7 days, which resulted in a sulfate loading of 0.29 g L^{-1} day^{-1}. Each reactor was equipped with 4 polysulfone membranes (Triqua BV,

Wageningen, the Netherlands), with a total effective surface of 0.028 m^2, via which the effluent was extracted by means of a peristaltic pump (Watson Marlow 505S, Cornwall, UK). The mean pore size of 0.2 µm guaranteed complete cell retention. The transmembrane pressure was monitored using a pressure sensor (Sensortechnics, Puchheim, Germany). Due to weekly manual back flushing, the transmembrane pressure remained below 20 kPa. The effluent pump was controlled by a level switch (Electronics ATV, Wageningen, the Netherlands), which kept the liquid volume at 1.0 L. Each reactor was equipped with a water-jacket, through which water, cooled or heated in a thermostatic water bath (Julabo, Seelbach, Germany) was recirculated to maintain a constant temperature of 15 (±1)°C or 30 (±1)°C in the reactor, measured with a PT-100 electrode. The pH was monitored with a sulfide resistant Hamilton flushtrode pH-electrode (Reno, USA) connected to a pH monitor (Electronics ATV, Wageningen, the Netherlands). The pH was maintained at 7.2 (±0.2) by means of the phosphate buffer in the medium and manual addition of diluted hydrochloric acid (1M).

CH$_4$ gas (Praxair, Danbury, USA), with a purity of 99.9995%, was supplied via a gas sparger at the bottom of the reactor. Not only to supply methane to the microorganism, but also to promote reactor mixing, to strip off the sulfide and to prevent fouling of the membrane surface (Chang et al., 2002). The influent CH$_4$ flow was measured and controlled at a gas loading rate of 4.8 L L^{-1} day^{-1} by a thermal mass flow meter type 5850E and control unit type 5878 (Brooks, Veenendaal, The Netherlands). The gas with the hydrogen sulfide (H$_2$S) and carbon dioxide (CO$_2$) stripped from the liquid, left the reactor via two gas cleaning bottles and a gas flow meter (Ritter, Bochum, Germany). The first bottle (1L) collected reactor liquid that was eventually transported with the gas out of the reactor. The second bottle (1L) was filled with a 0.5 M zinc chloride solution to selectively retain the H$_2$S, and was placed on a magnetic stirrer. The zinc chloride solution was replaced when the sulfide concentration (including the precipitated zinc sulfide) reached 10 to 15 mM. The overpressure in the headspace of the MBRs was 25 mbar. To provide additional mixing and to suspend the sediment/biomass, the reactor suspension was recirculated from top to bottom at a rate of 0.3 L min^{-1}.

3.3.4 Operation of the membrane bioreactors

Initially, 3 reactors were started, two at 30°C and one at 15°C, all three were inoculated with 10 $g_{dry\ weight}$ Eckernförde Bay sediment. One of the reactors at 30°C (R2) was additionally inoculated with 1.0 $g_{dry\ weight}$ Eerbeek sludge and 1.0 $g_{dry\ weight}$ Nedalco sludge. During the first 330 days, 0.5 mM acetate was added to the medium, the volumetric acetate loading rate was 70 µmol L^{-1} day^{-1}. From day 330 onwards, CH_4 was the sole electron donor and carbon source. A fourth reactor was started 18 months later, it was inoculated with 20 $g_{dry\ weight}$ Eckernförde Bay sediment and operated at 15°C with CH_4 as sole electron donor and carbon source from the start onwards. Table 3.1 shows the differences, in inoculation and operation of the four bioreactors.

Table 3.1 Inoculation and operational conditions of the MBRs used in this study.

	Inocula	Inoculation date	Duration run (days)	Temp (°C)	Addition 70 µmol L^{-1} day^{-1} acetate
R1	10 $g_{dry\ weight}$ Eckernförde Bay sediment	15-8-05	520	30	till day 330
R2	10 $g_{dry\ weight}$ Eckernförde Bay sediment & 2 $g_{dry\ weight}$ Methanogenic biomass	15-8-05	520	30	till day 330
R3	10 $g_{dry\ weight}$ Eckernförde Bay sediment	15-8-05	884	15	till day 330
R4	20 $g_{dry\ weight}$ Eckernförde Bay sediment	18-1-07	355	15	None

The influent pumps, mass flow meters, pH-electrodes and gas flow meters were checked every two months and recalibrated when needed. The sulfate and sulfide concentrations of the influent and effluent (supernatant of the membranes), and the sulfide concentration in the wash bottle, were analyzed approximately every three weeks. Samples of the reactor suspension were taken for activity assays, DNA isolation and quantification, and volatile suspended solids (VSS) and total suspended

solids (TSS) analysis. In theory, a membrane bioreactor allows an indefinite solid retention time. However, due to frequent sampling the solid retention time was approximately one year.

3.3.5 Activity assays

CH_4 oxidation rates were estimated from the ^{13}C-labeled CO_2 ($^{13}CO_2$) production rate during batch incubations of reactor suspension with ^{13}C-labeled CH_4 ($^{13}CH_4$). After determination of the exact weight and volume of the 35-mL serum bottles, they were closed with butyl rubber stoppers and caps, and the gas phase was replaced 8 times with nitrogen gas and made vacuum thereafter. Subsequently, 20 ml reactor suspension was transferred into the bottles, using a syringe and a hypodermic needle (internal diameter of 0.2 mm). To ensure homogeneous sampling of the reactor suspension, the gas sparging rate in the reactors was temporally increased to 1 L min^{-1}. After day 420, the reactor suspension of R3 was diluted with fresh medium, the dilution factor was the last obtained rate divided by 50 µmol L^{-1} day^{-1}; the exact amounts of added reactor suspension and medium were determined by weighing. Subsequently, the headspaces of the bottles were made vacuum again and filled with pure $^{13}CH_4$ gas (Campro, Veenendaal, the Netherlands). The bottles were incubated in an orbital shaker (rotating at 100 rpm) at the operation temperature of the source reactor. Weekly, 100 µl headspace samples were taken for gas analysis ($^{12}CH_4$, $^{13}CH_4$, $^{12}CO_2$ and $^{13}CO_2$). In addition, the headspace pressure, liquid and gas volume and pH were measured.

To investigate the stoichiometry in batch, activity assays were done with reactor suspension diluted with medium with a reduced sulfate concentration. The headspace contained or $^{13}CH_4$ or N_2. Liquid samples were taken and used for sulfide and sulfate analyses, after filtering over a 0.2 mm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany).

3.3.6 Chemical analyses

Sulfide was measured photometrically using a standard kit (LCK 653) and a photo spectrometer (Xion 500) both from Hach Lange (Dusseldorf, Germany). This method accounted for all dissolved sulfide species (H_2S, HS^- and S^{2-}) and, if no filtration or centrifugation was applied, precipitated sulfide (e.g. the ZnS in the wash bottle).

Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA) as described previously (Sipma *et al.*, 2004). Acetate was analyzed on a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) according to Weijma *et al.* (2000).

The headspace composition was measured on a gas chromatograph-mass spectrometer (GC-MS) from Interscience (Breda, the Netherlands). The system was composed of a Trace GC equipped with a GS-GasPro column (30 m by 0.32 mm; J & W Scientific, Folsom, USA), and a Ion-Trap MS. Helium was the carrier gas at a flow rate of 1.7 ml min^{-1}. The column temperature was 30°C. The fractions of CH_4 and CO_2 in the headspace were derived from the peak areas in the gas chromatograph. The fractions of ^{13}C-labeled CH_4 ($^{13}CH_4$) and ^{13}C-labeled CO_2 ($^{13}CO_2$) were derived from the mass spectrum as done by Shigematsu *et al.* (2004), the method was checked using standards with known mixtures of $^{12}CO_2$, $^{13}CO_2$, $^{13}CH_4$ and $^{12}CH_4$.

The pressure in the bottles and tubes was determined using a portable membrane pressure unit, WAL 0-0.4 MPa absolute (WalMess- und Regelsysteme, Oldenburg, Germany). The pH was checked by means of pH paper (Macherey-Nagel, Düren, Germany). The VSS and TSS content of the reactor suspension and the dry weight content of the inocula were analyzed according to standard methods (American Public Health Association, 1995).

3.3.7 DNA isolation and quantification

DNA was extracted from the reactor suspension using the FastDNA SPIN for Soil Kit (MP Biomedicals, Ohio, USA). The 16S rRNA gene was amplified from genomic DNA by PCR using the archaea-specific forward primer 4F (5'-TCCGGTTGATCCTGCCRG-3') and the universal prokaryotic reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3'). 16S rRNA gene PCR was performed in a G-storm cycler (G-storm, Essex, UK) starting with 2 minutes at 94°C, followed by 35 cycles of 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1.5 min. The final PCR extension step was at 72°C for 5 min. PCR products were ligated into pGEM-T (Promega Benelux BV, Leiden, The Netherlands) and transformed into *E. coli* XL1-blue cells (Stratagene, La Jolla, USA) as specified by the manufacturer. For screening of the gene library by denaturing gradient gel electrophoresis (DGGE), 10 µl of the overnight cultures of the clones were mixed with 90 µl of TE and lysed for 10 minutes at 95°C. 400 bp 16S rDNA gene fragments were amplified from 1 µl of the lysed clones using the primer pair A109T-F (ACT GCT

CAG TAA CAC GT, original Grosskopf *et al.* (1998) but with a third nucleotide changed into T) plus 515R (ATC GTA TTA CCG CGG CTG CTG GCA , Lane, 1991) with a GC clamp (Muyzer *et al.*, 1993). The DNA clean and concentrator-5 kit (Zymo research, Orange, USA) was used for the purification and the DNA fragments were partially sequenced commercially (400-740 bp) by BaseClear (Leiden, The Netherlands).

3.3.8 Denaturing Gradient Gel Electrophoresis

DGGE analysis was directly performed on extracted DNA from the submerged membrane bioreactor. 400 basepair fragments of 16S rRNA genes were amplified by PCR using the universal archaeal primer pairs A109T-F plus 515R- GC clamp (as described above). DGGE was performed by following a published protocol (Muyzer *et al.*, 1998); the temperature was 60°C, the denaturant (urea and formamide) gradient was 30 to 60%, the electrophoresis time was 16 h, and the voltage was 85 V. Gels were stained with silver according to Sanguinetty *et al.* (1994) with minor modifications. Selected DGGE bands were excised. The DNA was extracted in 25 µl of TE buffer and incubated overnight at 37°C. 1 µl of DNA was reamplified with the same primers and sequenced commercially by BaseClear (Leiden, The Netherlands).

3.3.9 Phylogenetic analyses

Partial sequences were processed using the DNASTAR Lasergene 6 package (Madison, WI, USA) and verified by BLASTN (Altschul *et al.*, 1997), possible chimerical sequences were checked using the Pintail program (Ashelford *et al.*, 2005). The phylogenetic affiliation of the novel clones was deduced by means of BLASTN analyses (http://blast.ncbi.nlm.nih.gov). Identical migration patterns in DGGE were used to cluster the clones (Schabereiter-Gurtner *et al.*, 2003). Corrected sequences from representative clones were deposited in GenBank (www.ncbi.nlm.nih.gov; accession numbers FJ210915 and FJ210925).

3.3.10 Calculation of volumetric activities

The volumetric sulfate removal rate (r_{SR}) and the volumetric sulfide production rate (r_{SP}) in the bioreactors were calculated according to the equations:

$$r_{SR} = \frac{\left(\left[SO_4^{2-}{}_{\text{inf}}\right] - \left[SO_4^{2-}{}_{\text{eff}}\right]\right)}{HRT}$$

$$r_{SP} = \frac{\left(\left[sulfide_{\text{eff}}\right] - \left[sulfide_{\text{inf}}\right]\right)}{HRT} + \frac{\Delta\left[sulfide_{\text{gaswash}}\right] \times V_{\text{gaswash}}}{\Delta t}$$

The absolute amount of gaseous $^{13}CO_2$, dissolved $^{13}CO_2$ and ^{13}C-labeled bicarbonate ($=\sum{}^{13}CO_2$) in the activity assay bottles was plotted against time, the volumetric CH_4 oxidation rate (r_{AOM}) was obtained from the $\Delta\sum{}^{13}CO_2/\Delta t$ over the period in which the increase was linear, at least four successive measurements were used.

$$\sum{}^{13}CO_2 = {}^{13}CO_2 + H^{13}CO_3^- = f^{13}CO_2 \times P \times (V_{gas} + V_{liquid}/k \times (1 + K_z/[H^+]))$$

$$r_{AOM} = \frac{\left(\Delta\sum{}^{13}CO_2/\Delta t\right)}{V_{inoculum}}$$

3.4 Results

3.4.1 Reactor operation

The MBRs were kept anaerobic during operation and the biomass was retained. During operation, five times the feeding, mixing, heating and/or cooling were interrupted for short periods of time (maximum 48h). This was because of power failure, equipment failure or the depletion of CH_4 gas or medium. During these interruptions, the redox potential of the reactor suspension always stayed below -50 mV (at which the liquid would become pink because of the presence of rezasurin). Also when the membranes, sparging stones or electrodes were cleaned or replaced, which was done under a nitrogen flow, the redox potential stayed below that value.

No organic acids or alcohols could be detected in the supernatant of the bioreactor suspension (data not shown). If any of those compounds were produced, the concentrations did not exceed the detection limit of the GC, which is in the µg L^{-1} range. Also hydrogen and carbon monoxide could not be detected in the

headspaces. Although the MBRs were not ideally mixed, the mixing was sufficient to keep the biomass in suspension.

The phosphate buffer in R3 was, after day 590, not sufficient to cope with the increased alkaline production. By manual dosing of hydrochloric acid two times a week the pH was kept below 7.6. Before day 590 and in the other three reactors the pH remained between 7.2 and 7.5.

3.4.2 Conversion rates and stoichiometry

Figure 3.2 presents the volumetric sulfide production, sulfate removal, AOM and acetate consumption rates in the 4 MBRs in time. Three phases can be distinguished. During the first phase (phase I), the SR rates were higher than the AOM and acetate consumptions rates. Immediately after start-up, the sulfate removal and sulfide production rate were relatively high, between 0.1 and 0.6 mmol L^{-1} day^{-1}, but then over the course of a few weeks the SR rates dropped and stabilized around 0.07 mmol L^{-1} day^{-1}. During phase I, SRB are able to utilize substrates that were present in the inoculum or became available by decay of biomass. This endogenous activity dropped after the readily available endogenous organic compounds were depleted.
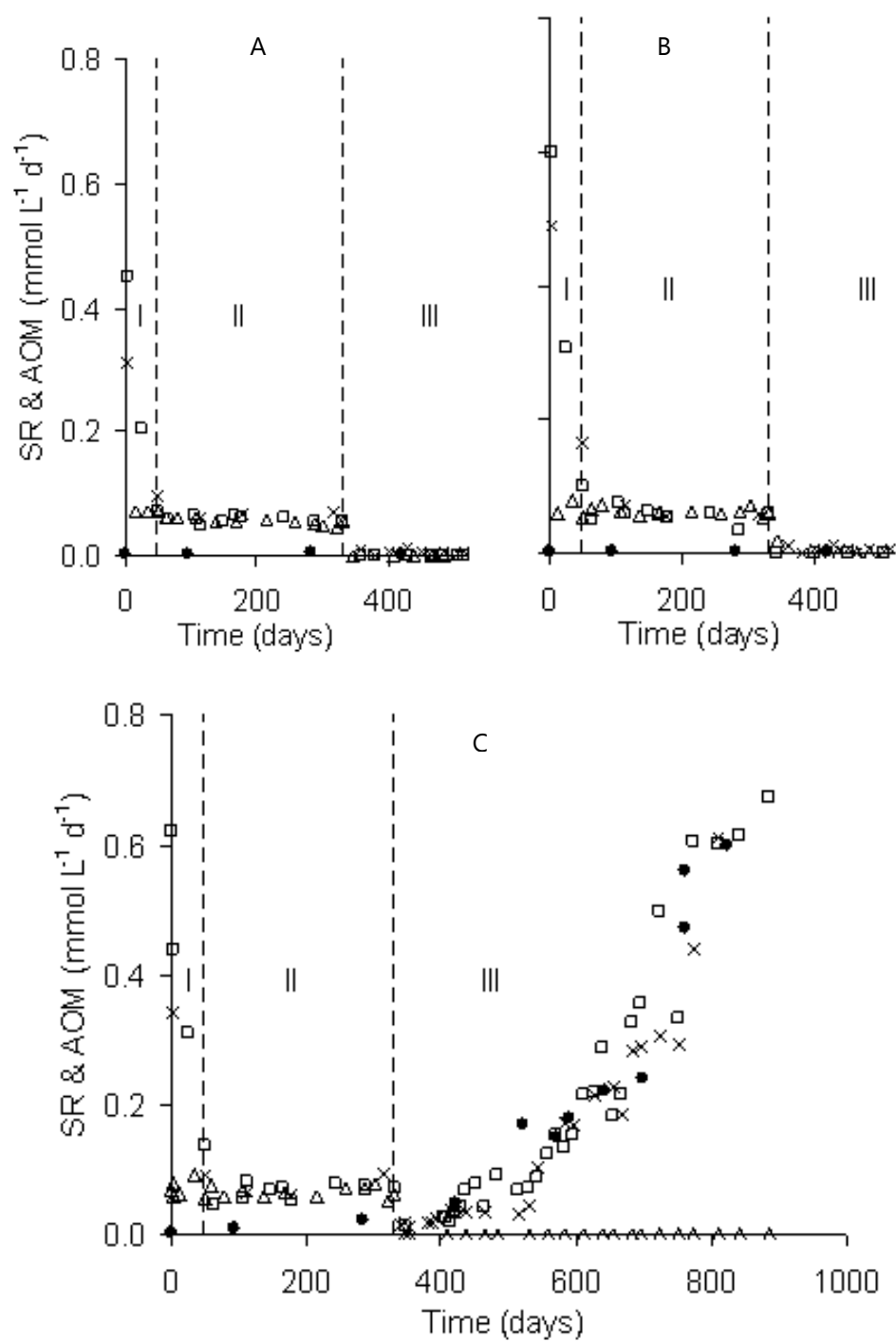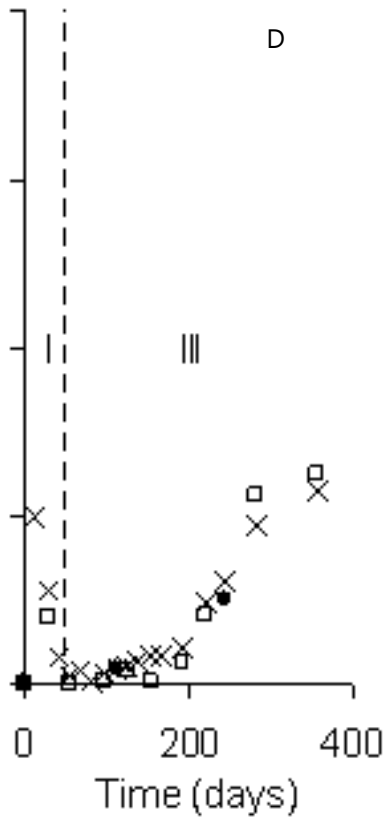
*Figure 3.2 Volumetric conversion rates over time of four reactors inoculated with Eckernförde Bay sediment, R1 (**A**) and R2 (**B**) both operated at 30°C, and R3 (**C**) and R4 (**D**), operated at 15°C. R2 was additionally inoculated with anaerobic granular sludge. Symbols indicate: sulfide production rate (X), sulfate removal rate (□), AOM rate (●) and acetate consumption (Δ). Three phases can be distinguished in R1, R2 and R3 and two in R4: during phase I endogenous organic matter from the inoculum was fueling sulfate reduction, during phase II 0.07 mmol L^{-1} day^{-1} (0.5 mM) acetate was added besides CH$_4$, during phase III CH$_4$ was the sole electron and carbon source.*

In the acetate-fed MBRs (R1, R2 and R3), a subsequent phase can be distinguished (phase II) in which acetate is completely removed and during which sulfate removal, sulfide production and acetate consumption rates are almost equal, circa 0.07 mmol L^{-1} day^{-1}. AOM rates during phase II were at least 5 times lower. The dominant

process in the reactors in this period was, therefore, sulfate reduction with acetate according to equation 14.

(14) $CH_3COOH + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$ $\Delta G° = -47\ kJ\ mol^{-1}$
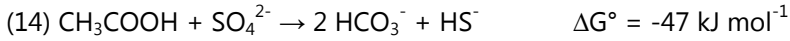
Acetate was omitted from the feed of R1, R2 and R3 from day 330 onwards, CH_4 was thus the only available electron donor and carbon source in this period (phase III). No acetate has been added to R4, therefore no phase II can be distinguished for that reactor. In the reactors operated at 15°C (R3 and R4), the sulfate removal, sulfide production and AOM rates are coupled during phase III, according to equation 13. Also in the activity assays done with reactor suspension from R3, taken during phase III, simultaneous $^{13}CH_4$ and sulfate consumption was accompanied by $^{13}CO_2$ and sulfide production, according to equation 14 (Figure 3.3a).
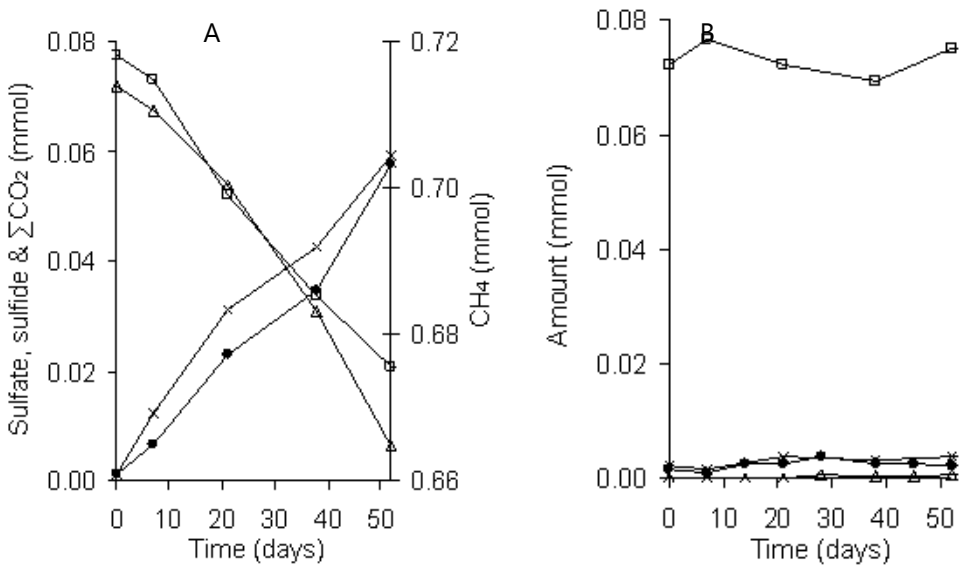
*Figure 3.3 Stoichiometric sulfide production (X), sulfate consumption (□), $\sum CO_2$ production (●) and CH_4 consumption (Δ) in 35-ml batch bottles containing 20 ml marine medium with 2.5 mM sulfate and a headspace of 0.15 MPa CH_4 (**A**) or 0.15 MPa nitrogen (**B**). The bottles were inoculated with 2.5 ml reactor suspension taken 760 days after start-up from R3 and incubated shaken at 15°C.*

In control incubations with nitrogen gas instead of $^{13}CH_4$ in the headspace, no conversion was observed (Fig. 3.3b). During the entire incubation, the AOM rates obtained from activity assays, increased from 0.004 to 0.60 mmol L^1 day^{-1} in 884 days for R3, and from 0.008 to 0.19 mmol L^{-1} day^{-1} in 280 days for R4 (Fig. 3.2c and 3.2d). In the reactors operated at 30°C (R1 and R2), the sulfate removal rate and sulfide production rate during phase III were always below 0.01 mmol L^{-1} day^{-1} (Fig. 3.2a and 3.2b). There was no increase in AOM or sulfate reduction over a period of 640 days, after which the reactors where stopped.

3.4.3 Biomass concentration and composition of R3

To assess which microorganisms are responsible for the 150 fold increase in AOM rate in R3, the biomass concentration and composition were analyzed. Just after inoculation the TSS and VSS content in R3 were 8.4 g L^{-1} and 1.1 g L^{-1}, respectively. After 884 days the TSS and VSS content in R3 had decreased to 2.1 g L^{-1} and 0.59 g L^{-1} respectively. The DNA concentration in R3 also decreased over time (Fig.3.4b), despite the exponential increase of the AOM and SR rate. The potential growth of microorganisms mediating AOM and SR did not result in a net increase in biomass. The decrease in solids can be explained by the frequent sampling of reactor suspension for chemical analyses and activity assays, in totally 2.3 L was sampled during the 884 day incubation (dilution factor ≥ 3.3), and by the decomposition of particulate organic matter present in the inoculum (e.g. inactive and dead biomass). The products of particular organic matter decomposition can be used as electron donor for SR, this resulted in the relative high SR rate during phase I (Fig. 3.2). Maximum 8 mmol sulfate can be reduced from the 0.5 g VSS that was lost during the experiment, when it is assumed that the average molecular structure of the particular organic matter is CH_2O. These 8 mmol form only 3.2% of the total amount of sulfate that was reduced during the 884 days of incubation. Therefore endogenous SR could not have contributed significantly to SR in R3 during phase III.
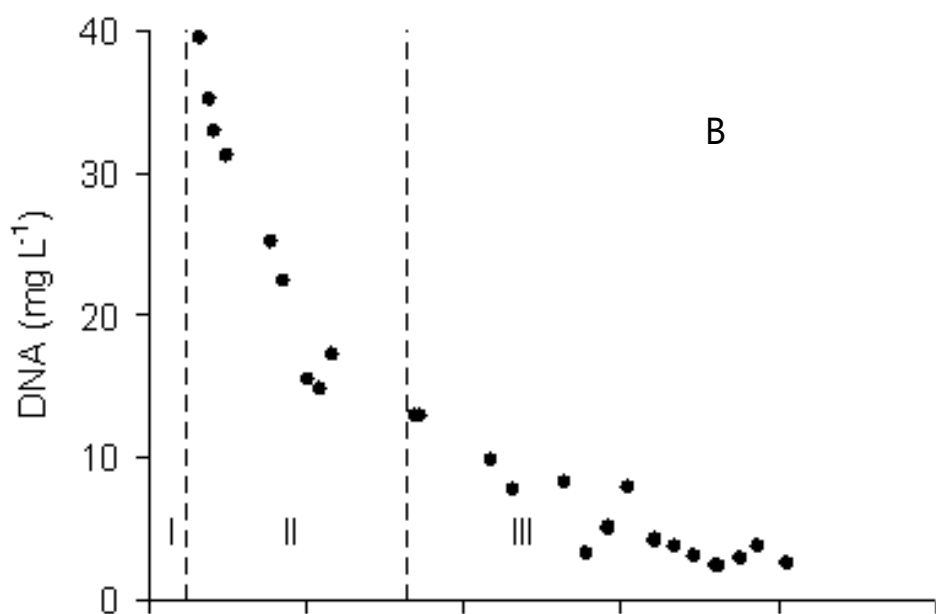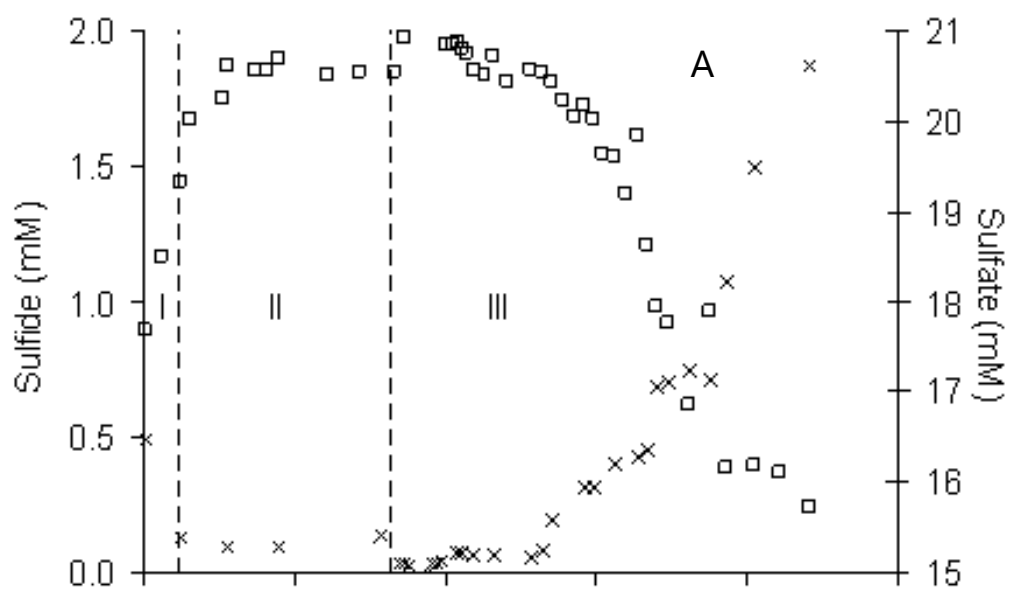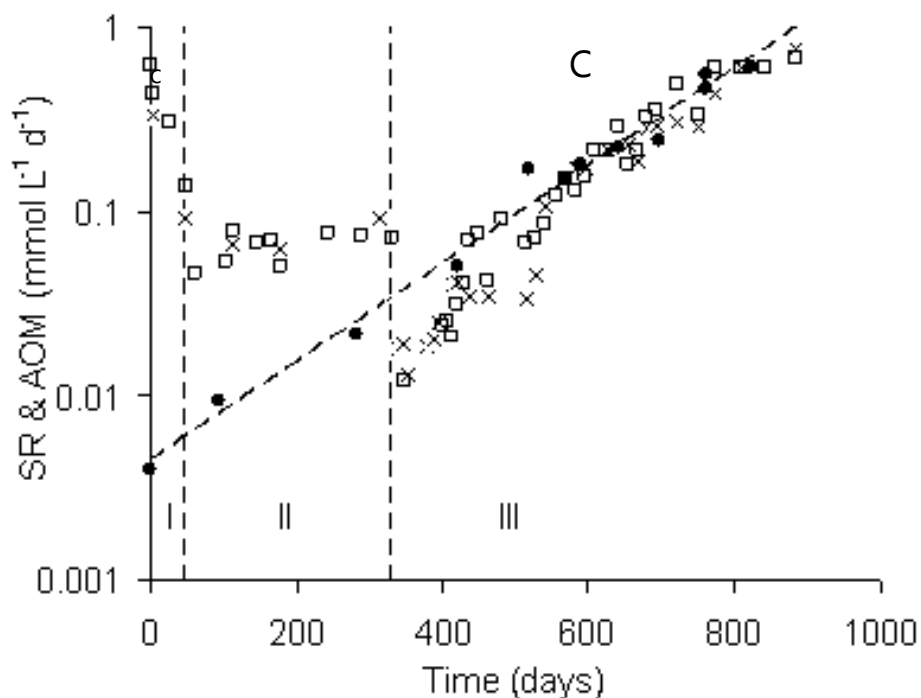
*Figure 3.4 The dissolved sulfide (x) and sulfate (□) concentrations (**A**), the DNA concentration (**B**) and the volumetric sulfide production (x), sulfate removal (□) and CH$_4$ oxidation (●) rates on logarithmic scale (**C**) over time for a membrane bioreactor inoculated with 10 g$_{dry\ weight}$ Eckernförde Bay sediment, continuously fed with CH$_4$ and sulfate and controlled at 15°C (R3).*

The biomass was mainly present as small flocks (up to 0.1 mm in diameter). When the reactor suspension is left undisturbed, the flocks agglomerated to bigger flocks and settled at velocities between 16.8 and 3.4 m h^{-1}. Table 3.2 shows an overview of the clone library of the archaea obtained from the biomass in R3, 809 days after inoculation. 91% of the obtained archaeal clones had similarities with ANME-2a sequences found by BLASTN analysis (http://blast.ncbi.nlm.nih.gov). The DGGE scan of different samples of R3 in time show the proliferation of two bands at the bottom of the DGGE gel (Fig. 3.5), of which the sequences had high similarity with ANME-2a related clones (Band A. 96% (300bp) with clone SBAK-mid-10 (DQ522915) and Band B 92% (253 bp) with clone Hyd24-Arch25. (AJ578107).

Table 3.2. Phylogenetic summary based on clone library analysis of partial archaeal sequences from the MBR inoculated with Eckernförde Bay sediment and operated at 15°C for 809 days (R3).

Representative clone	Accession no.	No. of clones	Sequence with highest similarity in Genbank (Blastn) with accession no.*	Identity (%)	Putative taxon
R3-1A3	FJ210916	27	Clone fos0642g6 (CR937012)	99%	ANME-2a
R3-1A2	FJ210915	23	Clone Hyd24-Arch25 (AJ578107)	99%	ANME-2a
R3-1A11	FJ210917	22	Clone GoM_GC232_4463_Arch65 (AM745238)	99%	ANME-2a
R3-1E5	FJ210918	8	Clone IV.4.Ar15 (AY367329)	99%	ANME-2a
R3-1D10	FJ210919	2	clone SBAK-mid-74 (DQ640234)	99%	ANME-2a
R3-1B6	FJ210920	2	Clone WHA34-14 (AB426391)	95%	*Methanococcoides*
R3-1G4	FJ210921	2	Methanomicrobiales archaeon 'SBAK-CO2-reducing Enrichment-4' (DQ280485)	99%	*Methanomicrobiales*
R3-1A6	FJ210922	1	clone MOB7-2 (DQ841237)	98%	*Methanosarcinales*
R3-1E8	FJ210923	1	Uncultured euryarchaeote EHB95 (AF374283)	97%	*Methanosarcinales*
R3-1F5	FJ210924	1	clone SBAK-mid-25 (DQ522923)	96%	Marine Benthic Group –D
R3-1H9	FJ210925	1	clone ss017b (AJ969786)	91%	*Thermoplasmatales* - related Group

Similarity to nearest neighbor in the GenBank nucleotide database as determined by BLAST results. A similarity of 100% indicates that the sequences were indistinguishable.
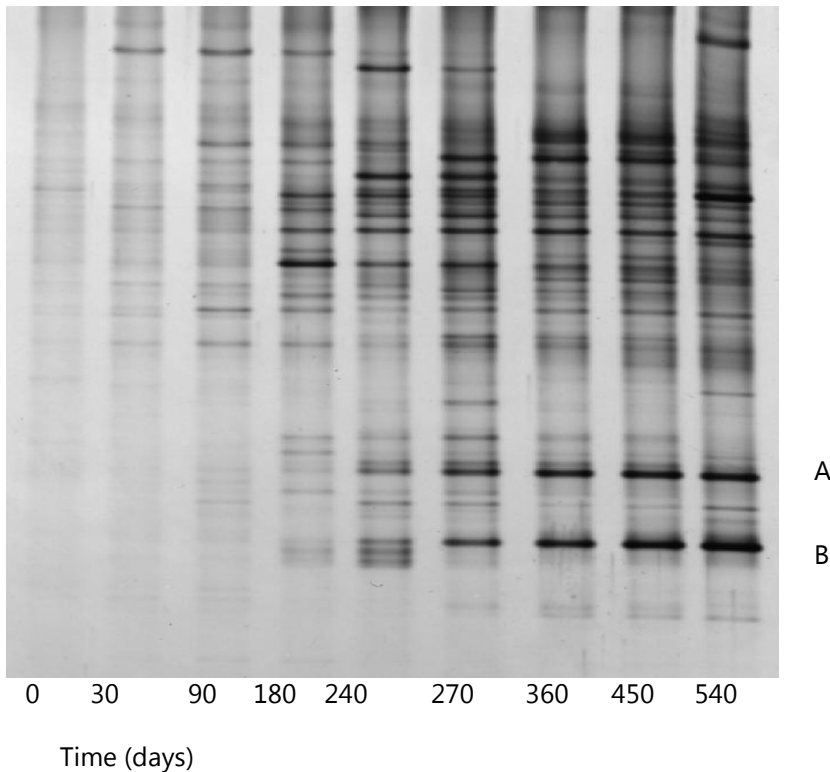
Figure 3.5: Analysis of changes in archaeal community over time, by 16S rRNA gene-targeted PCR- denaturing gradient gel electrophoresis, in a MBR inoculated with Eckernförde Bay sediment, fed with CH_4 and sulfate and operated at 15°C (R3). of a membrane bioreactor inoculated with Eckernförde Bay sediment, continuously and controlled at 15°C (R3). The sequences obtained from bands A and B can be affiliated with clones from the ANME-2a cluster.

3.5 Discussion

3.5.1 Bioreactor system

In Fig. 3.4c, the AOM, sulfate removal and sulfide production rates of R3 are shown on a logarithmic scale, during the 884 days of incubation the AOM rate increased exponential, corresponding to a doubling time of 3.8 months (R=0.99). In this way a very active enrichment was obtained (1.0 mmol g_{VSS}^{-1} day^{-1}). The used submerged-

membrane bioreactor system was therefore an excellent system for growing the microorganisms mediating AOM coupled to SR. The temperature, pH and salinity in this reactor (15°C, 7.5 and 30‰ respectively) were comparable with conditions found at the sampling site in Eckernförde Bay in early September: a temperature between 10 and 22°C and a salinity between 14 and 19‰ (Treude *et al.*, 2005a). However, in contrast to the *in situ* situation, the microorganisms in the bioreactors were continuously exposed to high shear forces, due to the liquid recirculation and gas sparging, and were suspended in the liquid phase. Another difference was that gaseous and dissolved compounds were continuously stripped out, due to the gas sparging, or washed out of the reactors system with the effluent. These issues are of importance if AOM is a syntrophic conversion, in which an intermediate compound is transported between the partners (DeLong, 2000). Syntrophic partners could get separated due to the breakup of the sediment-biomass matrix under conditions of high shear forces. In addition, intermediate compounds could be transported away before reaching the syntrophic partner. However, this study demonstrates that liquid recirculation, gas sparging and a hydraulic retention time of 7 days did not prevent the exponential development of the AOM rate. Because these features help to prevent mass transfer limitation, high volumetric conversion rates can be obtained. This research opens possibilities for a biotechnological sulfate reduction process with CH_4 as electron donor. The volumetric rate that was obtained in this study (0.6 mmol L^{-1} day^{-1}), is still too low for application. In a full-scale sulfate-reducing bioreactor fed with hydrogen as electron donor, a maximum volumetric sulfate reduction rate of 175 mmol L^{-1} day^{-1} has been reached (Weijma *et al.*, 2002). However, the volumetric methane oxidation and sulfate reduction rates can be increased further by increasing the biomass concentration in the bioreactor, MBR's can operated at suspended solid concentrations up to 31 $g_{dry\ weight}$ $^{-1}$ L^{-1} (Stephenson *et al*, 2000). Figure 3.4a shows that the sulfate concentration in the effluent of R3 decreased over time and the dissolved sulfide concentration increased. Stripping with CH_4 only partly removed the sulfide from the liquid. Till approximately day 800, the exponential increase in the AOM rate was not affected by the decreasing sulfate or increasing sulfide concentrations. After that, the increase in sulfate removal rate slowed down. Possibly this was caused by the increased dissolved sulfide concentration. A minimum sulfate concentration of 15.7 mM and a maximum sulfide concentration of 1.9 mM was reached.

3.5.2 Doubling time

Girguis *et al.* (2003; 2005), Nauhaus *et al.* (2007) and Krüger *et al.* (2008) also showed *in vitro* growth of anaerobic methanotrophs (Table 3.3).

Table 3.3: Comparison of the doubling times and maximum AOM conversion rates found in different enrichment experiments with marine sediments.

Origin inoculum	Monterey Bay	Hydrate Ridge	Gulf of Mexico	Eckernförde Bay
Incubation technique	Continuous feeding, prop flow	Fed-batch, not mixed	Batch, shaken once a week	Continuous feeding, well-mixed
CH_4 partial pressure (during incubation)	1.5 mM (≈0.1MPa)	1.4 Mpa	1.5 Mpa	0.10 Mpa
Incubation temp.	5°C	n.r.	12°C	15°C
Involved microorganisms	ANME-1, ANME-2 and SRB	Consortia of ANME-2 and SRB	ANME-1 dominated	ANME-2a, possible partner not known
Estimated doubling time (months)	1.1 (ANME-2) 1.4 (ANME-1)	7.5	2	3.8
Maximum AOM rate (μmol $g_{dry\ weight}^{-1}.d^{-1}$)	0.1	230	13.5	286
Maximum AOM rate (mmol $g_{VSS}^{-1}\ d^{-1}$)	n.r.	n.r.	n.r.	1.0
Reference	Girguis *et al.* 2005	Nauhaus *et al.* 2006	Krüger *et al.* 2008	This study

n.r. not reported.

The difference in reported doubling times can be related to the inoculates that were used. However, there were also differences in incubation conditions (e.g. CH_4 partial pressure and temperature) and techniques. One important difference is that the

relative short doubling times found by Girguis *et al.* (2005) were obtained with sediment in which the AOM rates were low, while Nauhaus *et al.* (2007) found much slower growth using the active Hydrate Ridge sediment. For this study, both initial rates and doubling times were in between reported values, but growth did not slow down when rates exceeded those of the Hydrate Ridge sediment. Further research should clarify which parameters are critical to obtain optimal growth. An important difference in the approach of this research with those of others is that the aim was not to mimic the natural conditions, but to apply conditions that allowed high conversion rates. The AOM activity of the enrichment obtained in this research is the highest reported so far (Table 3.3).

3.5.3 Responsible microorganisms

The exponential increase in activity in R3 (Fig 3.4c) indicates growth. However, VSS content and DNA concentration (Fig. 3.4b) decreased over time. This decrease indicates that the original sediment contained many organisms not involved in the exponential increase in AOM coupled to SR and that these organisms were slowly decaying. One aspect of the submerged-membrane bioreactor used in this study is that inactive and dead cells will not wash-out with the effluent. The bands in the DGGE gel (Fig. 3.5) that were not linked to ANME could be attributed to methanogens that were already present in the original Eckernförde Bay inoculum. Despite the presence of these inactive microorganisms, over 90% of the partial sequences (N=90, 400-750 bp) of the archaeal clones cluster in the ANME-2a subgroup. Especially in samples taken from R3 in the period that AOM coupled to SR was the only conversion taking place (phase III) clearly showed the dominance of ANME-2a clones in the archaeal clone library and in the sequences from DGGE bands. Indicating that ANME-2a were involved in the exponential increase in AOM coupled to SR. ANME-2a were also detected in the original Eckernförde Bay sediment by Treude et al. (2005). ANME have been shown to directly consume CH_4 (Orphan et al., 2001) and to have enzymes that can play a role in reversed methanogenesis (Hallam et al., 2004). However, ANME have not been shown to be capable of sulfate reduction nor to possess enzymes involved in SR (Thauer and Shima, 2008). Therefore, further research is required to unravel the AOM pathway in the obtained enrichment. The bacterial composition of the active biomass in the

bioreactor is not unraveled yet, but further research will focus on the quantitative and phylogenetic aspects of these sulfate-reducing bacteria.

3.5.4 Temperature

The AOM and SR rates increased in both reactors operated at 15°C (Fig. 3.2c and 3.2d). In contrast, the AOM activity and the SR during phase III, did not increase in the two reactors operated at 30°C (Fig. 3.2a and 3.2b). Nauhaus *et al.* (2005) found a very low AOM rate of ANME-2 dominated Hydrate Ridge sediment when the temperature exceeded 16°C. The enrichment obtained in this research at 15°C was also ANME-2 dominated (Chapter 2). Despite a possible inhibitory effect of temperatures exceeding 16°C, AOM still occur during the initial activity assays done at 30°C (Fig. 3.2a and 3.2b). Treude *et al.* (2005) found AOM with Eckernförde Bay sediment at 28°C. However, the conversion does not have to be coupled to net growth, as the energy required for maintenance can be equal or larger than can be conserved by the available Gibbs free energy change. The maintenance Gibbs free energy is positively related with the temperature; it doubles for every 10°C increase (Tijshuis *et al.*, 1993). This could explain why no net growth was possible at 30°C.

3.5.4 AOM activity assays

The AOM rates are estimated from the $^{13}CO_2$ production in batch bottles to which only pure $^{13}CH_4$ and reactor liquid were added. The reactor suspension contains biomass and dissolved components (that were not stripped from the liquid by flushing during sampling), containing carbon with a natural isotopic signature (approximately 1.07% ^{13}C). The oxidation of these compounds will contribute to the $^{13}CO_2$ formation. However, the $^{13}CO_2$ production was always at least 10% of the $^{12}CO_2$ production, therefore this contribution was neglected.

The AOM rate presented is the net $^{13}CH_4$ oxidation rate, thus the $^{13}CH_4$ oxidation to $\sum^{13}CO_2$ minus the backward reaction ($\sum^{13}CO_2$ reduction). This CO_2 reduction during AOM might be similar to observed methane oxidation during methanogenesis (Zehnder and Brock, 1979; Harder, 1997). Treude *et al.* (2007) showed that in Black sea sediments the CO_2 reduction rate was about 10 % of the methane oxidation rate. Krüger *et al.* (2008) reported that the AOM rates are reduced by 30-80% if the fraction $^{13}CH_4$ (of the total CH_4) exceeds 25%. This could be due to the inability of

the microbial community to use sufficient amounts of $^{13}CH_4$ as substitute for $^{12}CH_4$ to sustain viability (Krüger *et al.*, 2008) but a more likely explanation could be impurities in the methane used or the dilution of essential intermediates or trace elements. In this study, all activity assays were performed with 100% $^{13}CH_4$ or N_2 in the headspace, this was done to more directly and accurately quantify AOM. The reactors on the other hand were fed with unlabeled CH_4. An inhibitory effect of $^{13}CH_4$ will therefore result in a discrepancy between the AOM rate obtained from the activity assays and the sulfate removal and sulfide production achieved in the MBRs (during phase III). However, the AOM rates are not systematically lower than sulfate removal and sulfide production rates (Fig. 3.2c). If the inhibitory effect of $^{13}CH_4$ was due to a loss in viability of the cells, the effect can be expected to be less profound during the relative short activity assays done in this research.

At the start of phase III, the coupling between sulfate removal, sulfide production and AOM is poor. This was because the SR rate was so low that the measurements of the differences between influent and effluent sulfate concentrations and sulfide accumulation was less accurate.

The AOM rate in R3 just after start up was 0.5 µmol $g_{dry\ weight}^{-1}$ day^{-1}. Treude *et al.* (2005) found AOM activities between 0.1 and 0.3 µmol.$g_{dry\ weight}^{-1}$.day^{-1} with sediment sampled three years earlier at the same site (station B in Eckernförde Bay). The difference between the rates could be the result of growth prior to reactor inoculation, as the wet sediment was stored for 69 days at 4°C in an unshaken bottle with 100% CH_4 in the headspace.

3.5.5 Acetate as co-substrate

Acetate was initially fed to the reactors, in addition to CH_4, in order to obtain and maintain sulfate reducing conditions. Another reason to add acetate during start-up was the stimulation of sulfate reducers that use acetate as energy or carbon source, which may play a role in AOM. However, acetate did not stimulate AOM coupled to SR given the exponential increase in AOM and SR in R4 (to which no acetate was fed; Fig. 3.2d) and in R3 after acetate was omitted (Fig. 3.2c, phase III). Acetate removal rates and sulfate reduction rates were coupled during phase II; therefore it is likely that (during phase II) acetate was the m ain electron donor for sulfate reduction. However, it cannot be excluded that some acetate was converted to CH_4 and an equal amount of CH_4 was used for sulfate reduction.

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Chapter 4

The effect of possible co-substrates on the rate of anaerobic methane oxidation with sulfate

Abstract

Organisms involved in the process of anaerobic oxidation of methane (AOM) have not been isolated. In an attempt to stimulate growth of ANME archaea and associated sulfate reducing bacteria (SRB) responsible for AOM we incubated Eckernförde Bay sediment with alternative substrates for methane. The organisms involved in AOM with sulfate as electron acceptor were monitored using Quantitative-PCR and FISH using specific primers and probes for different ANME archaea and SRB. In addition, the effect of possible co-substrates on AOM was assessed. In long term experiments we have found that methanol was the only substrate that increases the relative AOM rate when incubated together with ^{13}C-labeled methane. More over it is shown that with thiosulfate and, acetate, pyruvate or butyrate, as substrates ANME-1 archaea became the dominant archaeal species. A possible conclusion is that ANME-1 archaea are not obligate methanotrophs but heterotrophic methanotrophs capable of switching their metabolism to methanogenesis when other substrates than methane are present.

4.1 Introduction

Anaerobic oxidation of methane (AOM) is a process that occurs in marine habitats where sulfate from the seawater is penetrating and methane rises up from the sediment (Hinrichs & Boetius, 2002). The mayor part of methane that comes from marine sediments is oxidized before it can reach the earth's atmosphere. AOM is therefore an important process in the global methane cycle (Crutzen, 1994; Reeburgh, 1996). The organisms responsible for anaerobic methane oxidation in marine systems have been identified as archaea and are represented in three different phylogenetic clusters (ANME-1, -2 and -3). Archaea in the ANME-2 and -3 clusters are closely affiliated with methanogenic archaea of the order of *Methanosarcinales* (Hinrichs *et al.*, 1999; Orphan *et al.*, 2001; Niemann *et al.*, 2006). ANME-1 archaea are distinct from, but related to, the methanogenic orders *Methanomicrobiales* and *Methanosarcinales* (Hinrichs *et al.*, 1999).

The known ANME clusters are associated with specific SRB belonging to the *Desulfosarcina/Desulfococcus* (DSS) group (Boetius *et al.*, 2000; Michaelis *et al.*, 2002; Knittel *et al.*, 2003) and the *Desulfobulbus* group (Niemann *et al.*, 2006) of the

126

Deltaproteobacteria. Despite several investigations, the exact mechanism of metabolic interaction between the syntrophic partners is still unclear (Hoehler *et al.*, 1994; Nauhaus *et al.*, 2002; Moran *et al.*, 2008). Methanotrophs from the ANME-2 lineage are almost always found in consortia with SRB suggesting a syntrophic interaction. Also thermodynamic calculations give an indication that a syntrophic interaction between the methanotrophic archaea and the sulfate reducing bacteria is the most common explanation but the process in which the organisms cooperate to oxidise methane coupled to the reduction of sulfate is not completely understood (Sørensen *et al.*, 2001)

The fact that ANME-1 archaea are often found in single cells without bacterial partner suggests that a different metabolic pathway is used in this methanotroph (Treude *et al.*, 2005). Research that looked at other substrates than methane use a very active culture and a short incubation period (Nauhaus *et al.*, 2002). To elucidate possible alternative metabolic pathways for archaea from the ANME clusters an active methane oxidizing sediment from Eckernförde bay (German Baltic sea) containing both ANME-1 and ANME-2 archaea (Treude *et al.*, 2005) was incubated under different conditions. Eckernförde bay sediment is very useful for long term growth experiments because the relatively low activity of this sediment prevents a fast build up of toxic end-products like hydrogen sulfide (Krüger *et al.*, 2005). Monitoring this sediment for a long period without altering the composition of the sediment and the frequent change of medium is therefore possible.

To obtain more information about the growth characteristics of the responsible micro organisms two separate long term incubations were started with different combinations of substrates, electron acceptors and temperatures. The first experiment (experiment A) was aimed at the effect of these incubation conditions on the growth of AOM associated organisms in the absence of CH_4 in the headspace. As electron acceptors, sulfate and thiosulfate were used and incubation temperatures were 20°C and 30°C. The effects on the microbial community was measured using fluorescence in situ hybridization (FISH) using group-specific fluorescently labelled rRNAtargeted oligonucleotides and Real Time Quantitative PCR (RT-Q-PCR). FISH has been shown to be very powerful for detecting and quantifying uncultured bacteria in environmental samples. The advantage of FISH compared with PCR-based approaches to study marine sediments (e.g. Cifuentes *et al.*, 2000) is the possibility to quantitatively determine morphology and in situ spatial distribution of

the microbial community in their natural habitat (Amann *et al.*, 1995). Together with the use of quantitative real time application of the Polymerase Chain Reaction (Q-PCR) the growth of organisms from the ANME clusters and sulfate reducing bacteria under different conditions was monitored. Quantitative PCR is a method for quantitative assessment of a prokaryotic community. With the use of specific 16s rRNA targeted probes the organisms responsible for AOM can be quantified without the use of direct cell counting (Suzuki *et al.*, 2000).

The second experiment (Experiment B) was aimed at determining the long term effect on the AOM rate during incubation with possible co-substrates, this experiment used methane and a co-substrate together with sulfate as electron acceptor. An incubation temperature of 20°C was used during the experiment.

The overall aim was to explore the capabilities of these slow growing organisms to grow under different conditions and develop a specific growth condition which enables growth of organisms capable of AOM.

4.2 Materials and Methods

4.2.1 Sediment sample collection

Eckernförde Bay is a 17 km long and 3 km wide inlet extending southwestward from the Kiel Bight of the western Baltic Sea (Figure 4.1). The seafloor of central Eckernförde Bay is characterized by soft organic-rich muddy sediments that contain free methane gas (Wever *et al.*, 1998). Methane is only accumulated below the sulfate penetration depth because of the anaerobic oxidation of methane which is coupled to sulfate reduction. The Bay's central basin reaches depths of 28 m and is underlain by fine-grained, anoxic mud which can exceed a thickness of 7 m. A summary description of the physical processes controlling water column salinity, density structure, and sedimentation processes in Eckernförde Bay is provided by Friedrichs and Wright (1994). Measured sediment accumulation rates in Eckernförde Bay range from 0.3 to 1.1 cm yr^{-1} (Nittrouer *et al.*, 1998) with an average value of 0.6 cm yr^{-1} at the NRL site utilized in this study. Bioturbation has only been observed to influence sediment properties within the upper few millimeters of the sediment column at the NRL site (Martens *et al.*, 1999).
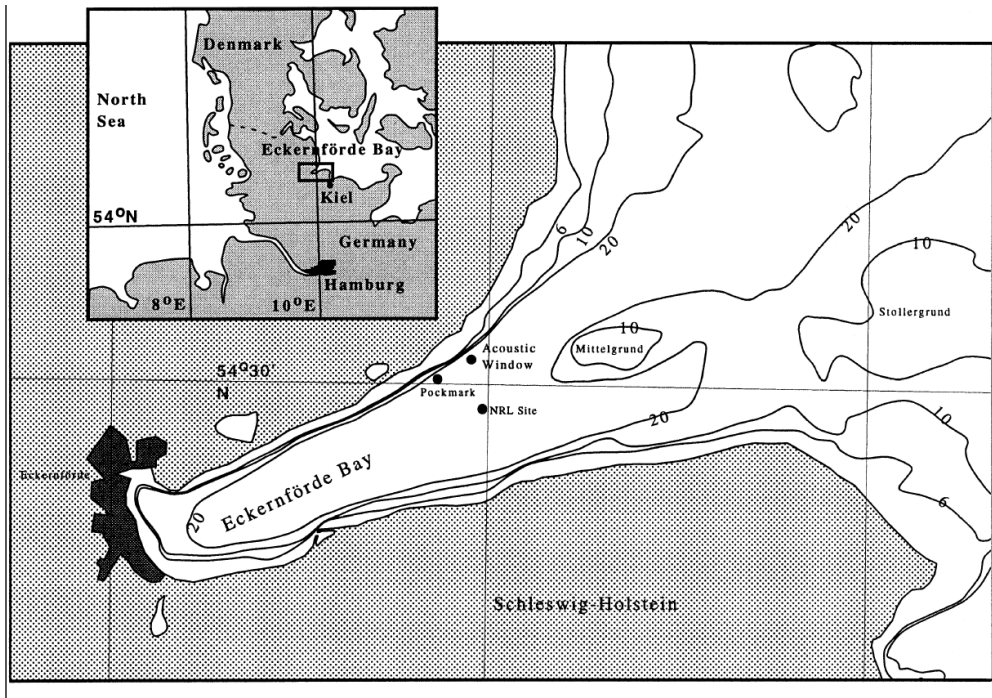
Figure 4.1: Eckernförde Bay, western Baltic sea, samples used in this thesis were taken near the NRL study site (Martens et al., 1999)

During a one day cruise in June 2005 on the RV *Littorina* sediment samples were taken with a small multiple corer based on the construction described by Barnett *et al.* (1984). This MiniMuc takes four sediment cores of up to 50-cm length, with an inner diameter of 10 cm within an area of 0.25 m^2. Sampling depth was about 28 m and the sediment temperature during the sampling period was 8°C. The cores taken reached a length of 30-40 cm. Cores were collected in a glass jar and closed without inclusion of air with a rubber stopper. In the home laboratory the sediment was divided in 500 ml batches in 1 liter glass bottles in an anaerobic tent providing anaerobic conditions. The bottles were closed with butyl rubber stoppers and the gas phase was changed by flushing 8 times with pure methane (99.9995%, Methane 5.5 Scientific, Linde Gas Benelux). Methane 5.5 Scientific compared to the more

commonly used Methane 4.5 Research does not contain methanogenesis inhibitors (Tugtas *et al.*, 2007; Oremland *et al.*, 1975) which could also affect anaerobic methane oxidation. The bottles were stored in the dark at 4°C .

4.2.2 Nucleic acid extraction and purification

DNA was extracted using a FastDNA SPIN for Soil kit (MP Biomedicals, Ohio, USA) and purified with the Zymo research DNA clean and concentrator-5 kit. The DNA was quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Del.).

4.2.3 Quantitative PCR

Quantitative real-time PCR amplification was performed with universal primers for bacteria and archaea and specific primers for ANME-1, ANME-2c and *D*SRB using the Bio-Rad iCycler system. All reactions were carried out with Bio-Rad iQ SYBR green supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The details of the primers can be found in Table 4.1. Each of the primer sets was optimized with respect to the annealing temperature and time required for extension. DNA samples for standard curves were prepared by amplifying group-specific cloned 16S rRNA genes using vector-targeted primers and purifying the products with the Zymo research DNA clean and concentrator-5 kit. DNA standards were quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Del.) and a serial dilution of our quantified plasmid DNA was then prepared for subsequent use as Q-PCR standards. The ANME-1, ANME-2c and *D*SRB standards were checked against a known universal standard with universal archaeal or bacterial primers.

Table 4.1: Primer sequences. Along with the primer name, the position on the rRNA gene is given. f= forward; r=reverse primer of the primer set. Primers for ANME-1, ANME-2c and DSRB (Desulfosarcina and Desulfococcus sp. Related to AOM) were derived from (Girguis et al. 2003; Girguis et al. 2005). Archaea specific primers were derived from Yu et al. (2005) and eubacterial primers were derived from Smits et al. (2004).

Target organism	Primer + position on SSU rRNA gene	Sequence
Archaea	Arch-787f	5' ATTAGATACCC(G/C)(G/T/C)GTAGTCC 3'
Archaea	Arch-1059r	5' GCCATGCACC(A/T)CCTCT 3'
Eubacteria	Eub-341f	5' CCTACGGGAGGCAGCAG 3'
Eubacteria	Eub-534r	5' ATTACCGCGGCTGCTGGC 3'
ANME-2c	AR-468f	5' CGCACAAGATAGCAAGGG 3'
ANME-2c	AR-736r	5' CGTCAGACCCGTTCTGGTA 3'
*D*SRB	*D*SRB-213f	5'CTGTTGTTTGGAGATGAGCCC 3'
*D*SRB	*D*SRB-658r	5' ATTCCACTTCCTTCTCCCATA 3'
ANME-1	ANME-1 337f	5' AGGTCCTACGGGACGCAT 3'
ANME-1	ANME-1 724r	5' GGTCAGACGCCTTCGCT 3'

4.2.4 PCR Quantification of ANME-1, ANME-2c, and DSS from batch incubations

Real-time PCR amplification of methane oxidizing archaea from the ANME-1 and ANME-2c cluster and *D*SRB phylotypes found in association with anaerobic

methanotrophs was performed in a Bio-Rad iCycler as previously described (Girguis *et al.*, 2003, Girguis *et al.*, 2005). Results are expressed in SSU rRNA copies per ml medium. The dry weight of the mixed Eckernförde bay sediment we used for inoculation was determined at 0.28 ± 0.04 g/g wet weight.

Detection limits ranged from $5.6x10^2$, $1.3x10^3$, and $4.0x10^2$ SSU rRNA copies ml $^{-1}$ for ANME-1, ANME-2c, and *D*SRB, respectively. DNA standards were quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Del.).

4.2.5 PCR Quantification of archaea and bacteria from batch incubations

Quantitative real-time PCR amplification was performed with universal primers for bacteria and archaea (Table 4.1) using the Bio-Rad iQ SYBR green supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Real-time PCR amplification was performed in a Bio-Rad iCycler programmed for 10 min at 95°C for initial heat activation, followed by 45 cycles of denaturation for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. For archaea, the annealing and extension steps were combined (30 s at 60°C).

4.2.6 Quantitative FISH

Samples were fixed overnight at 4°C with 3% formaldehyde, centrifuged and washed twice with PBS and finally stored in PBS/EtOH (1:1) at -20 °C. Stored samples were diluted and treated by 1 s pulsed sonication for 20 s (Branson sonifier B-12, probe from Heinemann, Germany) at an amplitude of 40% of the maximum power of 70W. Dilution series of samples were prepared in order to determine the optimal cell concentration for counting with the different probes. 10 µl of the fixed sample was spotted on the well of a gelatin coated slide (8 mm well, 10 well Multitest slide, MP Biomedicals) and dried for 10 min at 46 °C. The cells were dehydrated for 2 to 3 min in a graded ethanol series with the ethanol concentration increasing from 50 to 80% and finally in 96% ethanol in H_2O. 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) was added to each well, and 1 µl of each probe (50 ng/µl) was added to the wells and this was followed by incubation at 46°C for 2-3 h. After hybridization the slides were washed in 50 ml of pre-warmed (48°C) washing buffer with SDS for 10 min. For total counts 4',6-diamidino-2-phenylindole (DAPI) was added to the washing buffer at a final

concentration of 100 ng/ml. After the slides were rinsed in water, they were immediately air dried, mounted in Vectashield (Vector Labs, Burlingame, USA) and covered with a cover slide (42x60mm, Menzel-Glaser, Germany) Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMR epifluorescence microscope, were taken with a Leica DFC 340FX camera. The oligonucleotide probes with CY3- and carboxyfluorescein- (FLUOS-) labels were obtained from Eurogentec (Belgium).

The following probes were used in this study: ANME-1-350: 5'-AGTTTTCGCGCCTGATGC-3' (Boetius et al., 2000), EelMS932 (targeting ANME-2): 5'-AGCTCCACCCGTTGTAGT-3' (Boetius et al., 2000). DSS658: 5'-ATTCCACTTCCTTCTCCCATA-3' (Manz et al., 1998)

4.2.7 Chemical analyses

The total dissolved sulfide was measured photometrically according to the copper sulfide formation method by Cord-Ruwisch (1985). CH_4 in the headspaces of batches was measured by gas chromatography with a Shimadzu GC-14B gas chromatograph equipped with a thermal-conductivity detector and molecular sieve 13x (60/80 mesh). The column temperature was 50°C, and the carrier gas was argon at a flow rate of 30 ml/min. Additionally, rates of AOM in some experiments (methane oxidation with alternative electron acceptors and addition of electron capture substances) were also determined via the conversion of $^{13}CH_4$ to $^{13}CO_2$. $^{13}CO2$ was measured with a Thermo GC-MS on a Plot Column ((30 m by 0.32 mm; Ritek, USA).

4.2.8 Experiment A

Eckernförde bay sediment was incubated in 120-ml serum flasks filled with 50 ml bicarbonate-buffered medium based on the medium described by Stams *et al.* (1993). No yeast extract was added. Eckernförde bay sediment was added as a 10% inoculum (5 ml sediment in 45 ml medium) or 1% inoculum (0.5 ml sediment in 49.5 ml medium) in order to see the possible effect of dilution. All incubations were done on a rotating shaker. The headspace composed of 1.7 bar N_2-CO_2 (80:20). Acetate, butyrate, pyruvate, methanol and sulfate/thiosulfate were added from sterile anaerobic stock solutions to a concentration of 20 mM. Methane was added to the controls as a gas from a sterile 100% CH_4 stock. Sterile controls were prepared by sterilizing the batches twice for 20 min at 120°C after addition of the sediment. The

batches were incubated in duplicate or triplicate at 20°C or 30°C in the dark. After 650 days of incubation DNA was extracted, Q-PCR analyses and FISH imaging was performed. Bottles were left closed during the incubation period in order not to disturb the setup of the experiment, the only adaptations were the sampling of gas and liquid for the different analyses. Sampling was done on the same day as DNA extraction, DNA extraction and purification was done in duplicate. Extracted DNA was stored in -20°C until further analysis.

Liquid samples for sulfide and substrate measurements were taken regularly, and gas phase samples were used to determine the methanogenic activities. When the concentrations of sulfide in certain incubations reached about 15 mM, liquid samples of all incubations were taken for Q-PCR and FISH analysis. Quantitative PCR results were repeated three times to ensure a reproducible result. Q-PCR reaction products were checked with the built-in program for melting curves and the data that corresponded to products with a different melt curve than the positive control were not used in further data analysis.

Total cell counts (with 4_,6_-diamidino-2-phenylindole [DAPI]) and fluorescence in situ hybridization (FISH) with specific probes (chapter 4.2.6) were carried out to both visualize the spatial distribution and interactions of the ANME organisms with the bacteria from the DSS cluster, and also to quantify the relative abundance of the targeted organisms as a parallel method to the Quantitative PCR method.

4.2.9 Experiment B

In this experiment the rate of methane oxidation of the samples was determined in incubations with ^{13}C-labeled methane. The effect of incubation with co-substrates together with methane was determined by measuring the $^{13}CO_2$ which is formed during anaerobic oxidation of methane. The production of methane during the experiment was also measured. 10 ml mixed Eckernförde bay sediment was incubated in 60-ml serum flasks filled with 10 ml bicarbonate-buffered medium. No yeast extract was added. The headspace was composed of 1.7 bar N_2-CO_2 (80:20). Sulfate and a co-substrate (acetate, butyrate, pyruvate, or methanol) were added from sterile anaerobic stock solutions to a concentration of 20 mM. ^{13}C-labeled methane was added as a gas from a sterile 100% $^{13}CH_4$ stock. Sterile controls were prepared by sterilizing the batches twice for 20 min at 120°C after addition of the

sediment. The experiment was done *in duplo* at 20°C in the dark. Measurements for $^{13}CO_2$ and $^{13}CH_4$ were done in duplo on a gas chromatograph- mass spectrometer (GC-MS) from Thermo Scientific (Waltham, USA). The GC-MS was equipped with a Plot column (30 m by 0.32 mm; Ritek, USA). Helium was used as carrier gas at a flow rate of 1.7 ml min^{-1}. The column temperature was 30°C. The fractions of CH_4 and CO_2 in the headspace were derived from the peak areas in the gas chromatograph. The fractions of ^{13}C-labeled CH_4 ($^{13}CH_4$) and ^{13}C-labeled CO_2 ($^{13}CO_2$) were derived from the mass spectrum as done by Shigematsu *et al.* (2004), the method was checked using standards with known mixtures of $^{12}CO_2$, $^{13}CO_2$, $^{13}CH_4$ and $^{12}CH_4$. After 650 days of incubation DNA was extracted, Q-PCR analyses were performed with primers for ANME-1, ANME-2c, *D*SRB, archaea and eubacteria (Table 4.1). The final concentrations of sulfate, thiosulfate and the added methanogenic substrates were also determined as well as the final concentration of sulfide.

4.3 Results

4.3.1 Experiment A

Q-PCR results show a clear effect of the addition of alternative substrates on the ANME-1 cell numbers over a long incubation period of 650 days (Figure 4.2). Under specific combinations of incubation conditions the relative number of ANME-1 cells compared to the total number of archaea reached almost 80% (Fig 4.2b). In incubations without addition of substrate or electron acceptors the relative number of ANME-1 cells compared to the total archaeal counts was maximal 6%. Only the combination of a substrate (methanol, acetate, pyruvate or butyrate) with thiosulfate and the 1% sediment inoculum yielded a relative increase of ANME-1 cells.
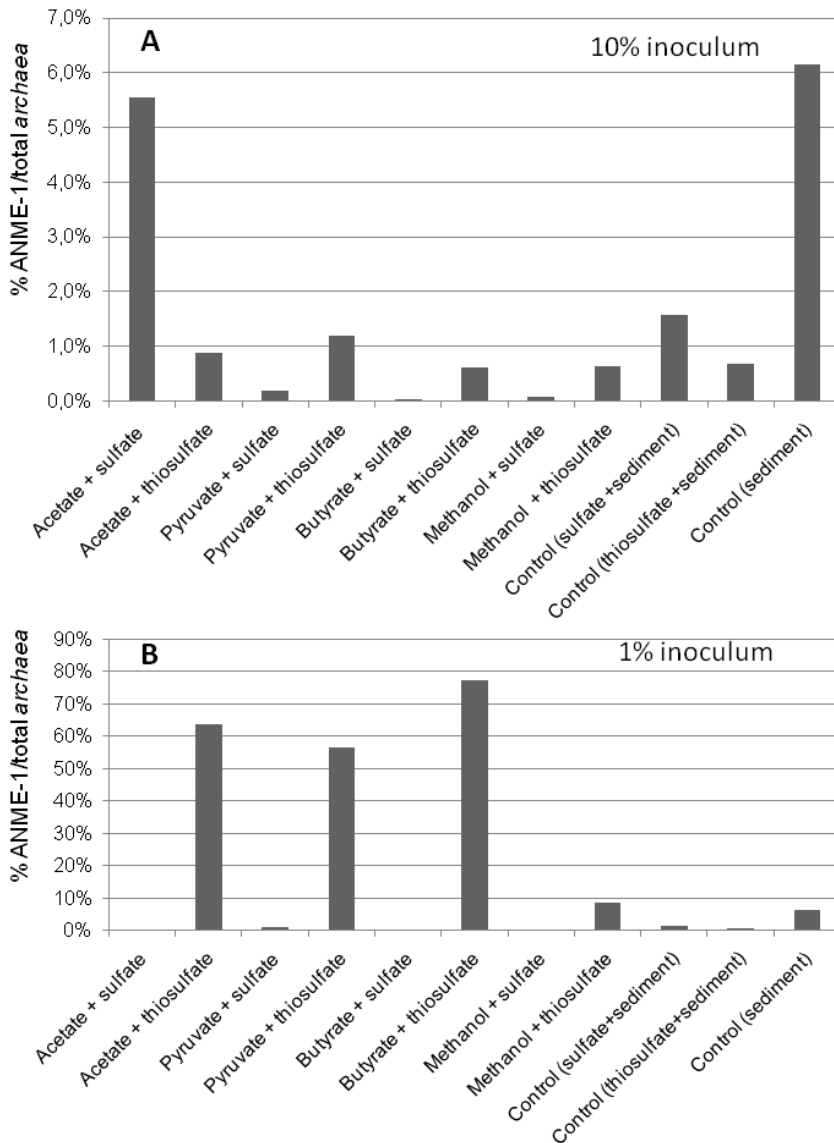
*Figure 4.2: Quantitative PCR measurement expressing the percentage of ANME-1 archaea compared to the total number of archaea growing on different electron donors with sulfate and thiosulfate. All cultivations were done at 30 ^{o}C. **A** shows the 10% inoculum (marine medium with 10% sediment) and **B** shows the 10 times diluted inoculum (marine medium with 1% sediment). Controls are 10% inoculations without addition of co-substrates.*

ANME-2c signals could not be detected in this substrate experiment with Q-PCR, probably the detection limit for ANME-2c of 1.3×10^3 SSU rRNA copies . gram sediment^{-1}, was too low to detect the ANME-2c signal in the diluted samples. The number of DSS signals did not show a correlation with ANME-1 signals and also no clear effect of the different incubation conditions could be detected (Figure 4.3).
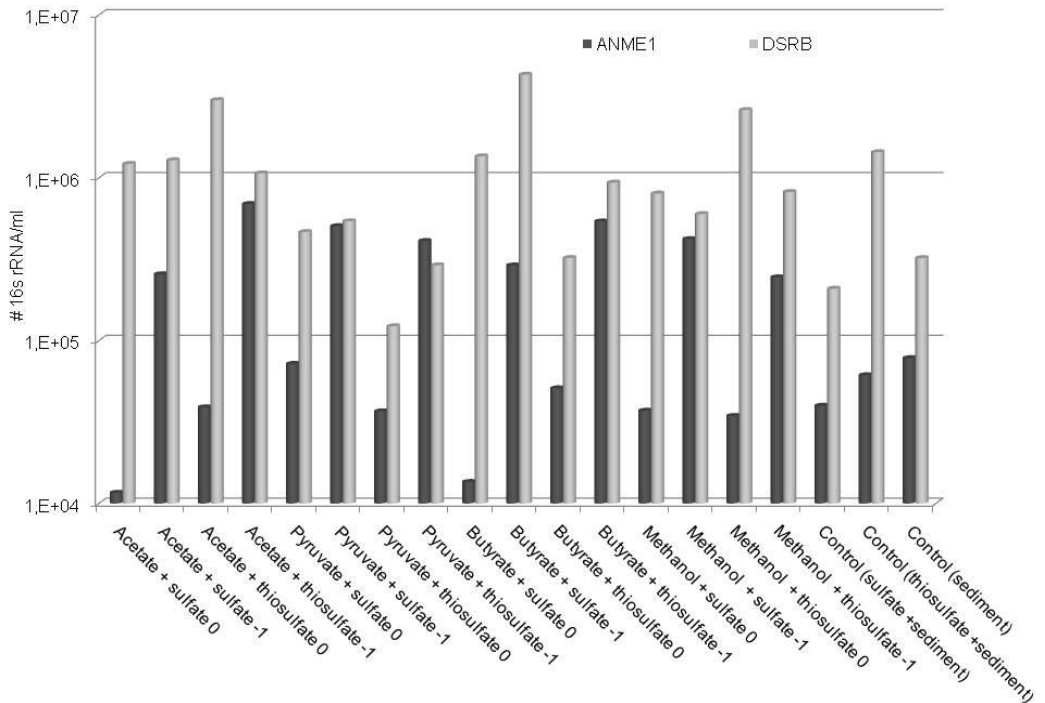
Figure 4.3: Quantitative PCR measurement expressing the absolute numbers of 16s rRNA of ANME-1 and DSRB in incubations of Eckernförde bay sediment with different combinations of sulfate/thiosulfate and electron donors. '0' is the 10% inoculum (marine medium with 10% sediment) and '-1' shows the 10 times diluted inoculum (marine medium with 1% sediment). Controls are 10% inoculations without addition of co-substrates.

Quantitative FISH was performed on samples of all incubated bottles, the counting of signals from specific probes for ANME-1 and ANME-2/DSS was done manually.

137

Table 4.2 shows the relative abundance of three selected probe signals compared to the signal of the universal DAPI stain. Because of the relative low abundance of the cells hybridizing with the ANME-1 and ANME-2/DSS probes, the counting was done according to the relative amount of hybridized cells to 1000-2000 DAPI signals. FISH images of the ANME-1 enrichments (figure 4.4) show single cells or small aggregates of a maximum of 15-20 cells.
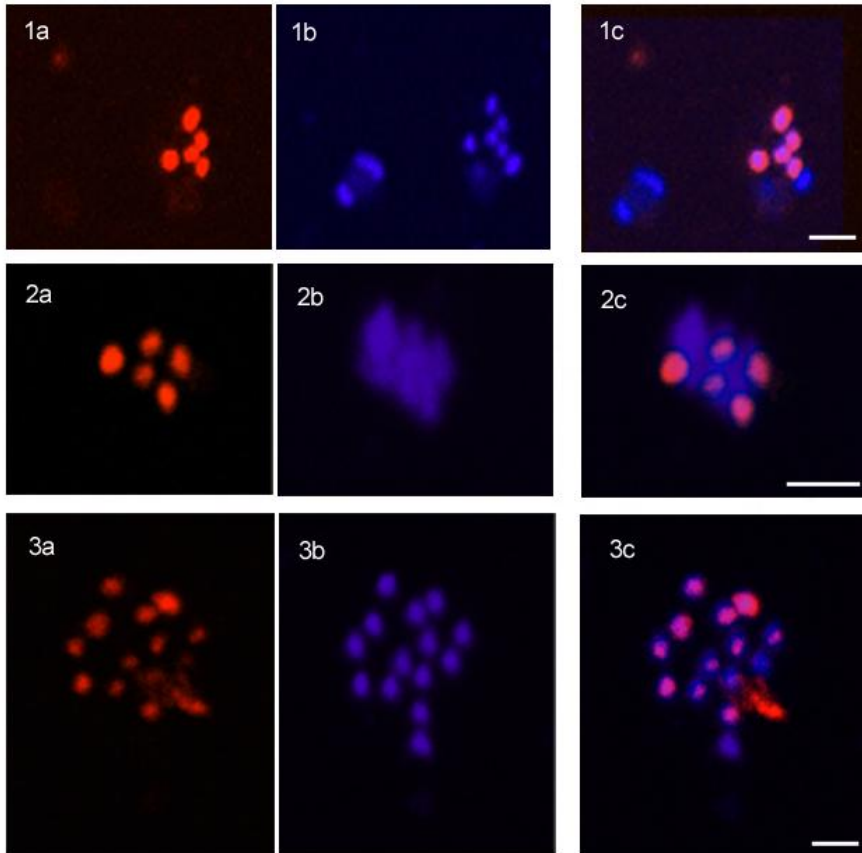
Figure 4.4: Whole-cell fluorescent in situ hybridization of an inoculation with Eckernförde bay sediment specifically enriched on acetate (1 and 2) and pyruvate (3) with ANME-1 aggregates. Red stained cells correspond to ANME-1 subgroup (ANME-1-350 (Boetius et al., 2000), 1a, 2a, 3a) and blue-stained cells correspond to nonspecific stain for DNA (DAPI, fig 1b, 2b, 3b). Separate images were overlaid to represent the structure of the aggregate (1c,2c and 3c). Scale Bar, 2 μm.

Table 4.2 Quantitative FISH results showing relative abundance compared to nonspecific stain for DNA (DAPI).

Incubation	Inoculum	Relative abundance for different probe signals		
		ANME-1-350	*EelMS932*	*DSS658*
Acetate +sulfate	10%	3.1 % (±1.3)	n.d.	n.d.
	1%	6.5 % (±2.7)	2.7 % (±0.9)	7.5 % (±2.8)
Acetate +thiosulfate	10%	2.9 % (±1.2)	n.d.	n.d.
	1%	14.5 % (±6.6)	n.d.	n.d.
Pyruvate +sulfate	10%	2.5 % (±1.3)	0.7 % (±0.3)	1.5 % (±0.8)
	1%	5.0 % (±2.8)	4.5 % (±1.3)	3.4 % (±1.5)
Pyruvate +thiosulfate	10%	3.5 % (±1.3)	n.d.	n.d.
	1%	13.5 % (±3.2)	4.2 % (±1.5)	4.0 % (±1.2)
Butyrate + sulfate	10%	4.5 % (±1.5)	n.d.	2.1 % (±1.0)
	1%	2.1 % (±1.1)	n.d.	3.0 % (±1.7)
Butyrate + thiosulfate	10%	3.6 % (±0.8)	n.d.	1.7 % (±0.3)
	1%	7.5 % (±4.1)	n.d.	3.7 % (±2.0)
Methanol +sulfate	10%	2.5 % (±1.3)	2.5 % (±1.5)	3.2 % (±1.2)
	1%	2.2 % (±1.2)	2.9 % (±0.8)	3.6 % (±1.5)
Methanol +thiosulfate	10%	2.9 % (±1.3)	2.1 % (±1.0)	1.6 % (±0.8)
	1%	6.5 % (±2.2)	n.d.	n.d.
Control (sediment+sulfate)	10%	3.0 % (±0.9)	2.5 % (±1.1)	3.9 % (±1.6)
Control (sediment+thiosulfate)	10%	3.1% (±1.5)	3.2 % (±1.3)	3.2% (±2.2)
Control (sediment)	10%	2.8 % (±1.2)	4.9 % (±1.7)	2.8 % (±1.0)

n.d. not detectable

4.3.2 Experiment B

During the incubation period of 200 days the amount of $^{13}CO_2$ was determined and expressed as percentage of the total CO_2 concentration (Fig 4.5). Only in the incubation with methanol added to the incubation with methane and sulfate there is a relatively larger production of $^{13}CO_2$ when compared to the control incubation with only ^{13}C-labeled methane and sulfate.
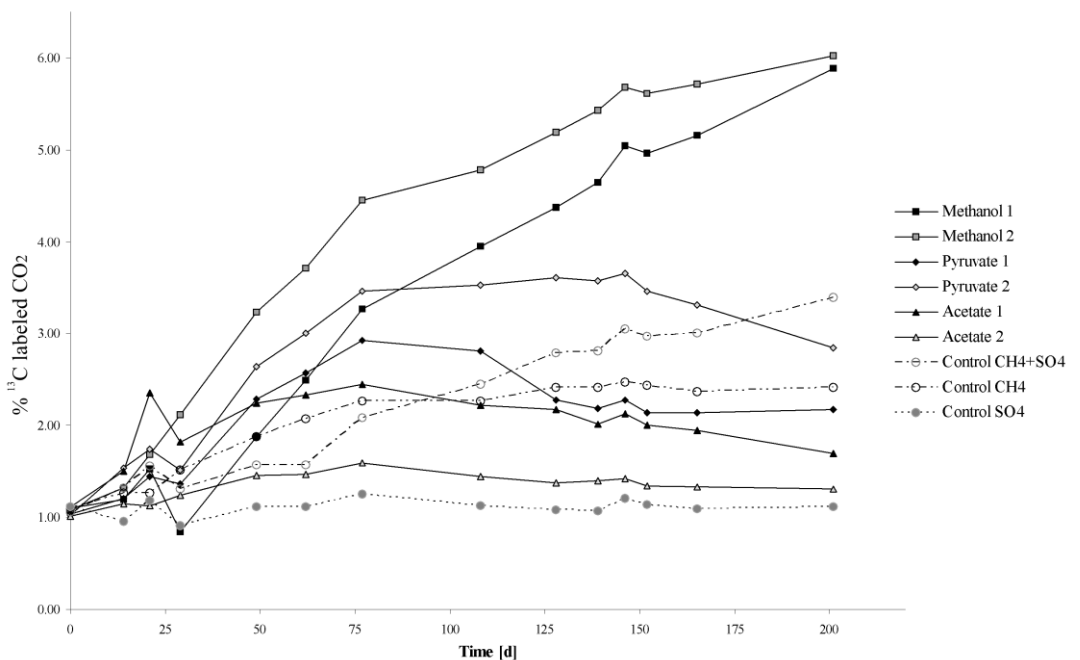
Figure 4.5. Change in relative concentration of ^{13}C-labeled CO_2 expressed as percentage of ^{12}C-labeled CO_2 in time.

The addition of other substrates together with methane had a negative effect on the relative production of $^{13}CO_2$, probably because the added methanogenic substrates were used for methanogenesis or sulfate reduction, resulting in $^{12}CO_2$ production. To check this, we measured the total methane concentration and only in the first 10 days of incubation there was an increase in the total methane concentration in all the incubations with added methanogenic substrates.

The control incubation with sediment, CH_4 and SO_4 was regarded as reference for the natural occurring AOM rate in Eckernförde Bay sediment. As seen in figure 4.5 only the incubations with methanol showed a higher $^{13}CO_2$ production than the reference (control CH4+SO4). The control with CH_4 and the control with SO_4 show the natural $^{13}CO_2$ component of atmospheric CO_2 (1,1%).

None of the methanogenic substrates were detectable after the incubation period with HPLC measurements, indicating that they have been used as metabolic substrates. The final sulfate and thiosulfate concentrations were also very low without correlation of the incubation conditions (data not shown). The sulfide concentration in the bottles reached an average of 6.6 (± 3.1) mM with a maximum of 15 mM in the incubations with 10% inoculum and an average of 3.8 mM (± 2.2) mM and a maximum of 7.0 mM in the incubations with 1% inoculum. Also here, no clear correlation between the different substrate combinations could be shown.

Discussion

Eckernförde bay sediment is a sediment capable of anaerobic oxidation of methane both *in situ* and *in vivo*. The sediment is different from other methane-oxidizing sediments because of the presence of ANME-2 consortia without a bacterial partner (Treude *et al.*, 2005). Sediment from the Eckernförde bay region shows generally a lower methane oxidation rate than other sediments known for AOM capability (Krüger *et al.*, 2005). This was also clear in the low formation of hydrogen sulfide during the incubation process and the stable methane concentration in the serum vials where methane was added (data not shown).

In experiment A we did not add methane to the gas phase and the addition of a methanogenic substrate to a marine sediment capable of anaerobic methane oxidation does not immediately stimulate growth of anaerobic methanotrophs. Under the incubation conditions growth of methanogens seems more likely and methane formation from the substrates was indeed observed. The aim of the experiment was not focused on substrate consumption but on growth of anaerobic methanotrophs under specific conditions during a long period of time.

To our knowledge this is the first report of a specific batch enrichment of ANME-1 methanotrophs. Both FISH and Q-PCR results show a clear enrichment of ANME-1 cells relative to the total number of archaea during prolonged incubation with thiosulfate and acetate, pyruvate or butyrate. Because ANME-2c/DSS signals were

not detected in the Q-PCR analysis and the FISH analysis did not show an increase of consortia compared to the original Eckernförde bay sediment it is clear that from the targeted organisms only ANME-1 organisms are able to proliferate under the specific incubation conditions used in this experiment. Because of the relative increase of ANME-1 cells compared to the total number of archaea it can be concluded that ANME-1 cells can be enriched in a highly diluted sample by using a combination of specific substrates (acetate, pyruvate, butyrate) combined with thiosulfate. The increase of ANME-1 cells is low and far form practical use in bioreactors or other growth experiments but this research can provide a novel strategy to address the problems occurring with the growth of these extreme slow growing organisms.

In experiment B we tested the possibility of using methanogenic substrates as co-substrates during the oxidation of methane. Because methane oxidation yields a relatively low free Gibbs energy the use of other substrates present could stimulate the growth rate of anaerobic methanotrophs when the substrate is used as alternative carbon or energy source.

Our experiments with bioreactor enrichments from the Eckernförde Bay sediment showed that AOM coupled to sulfate reduction stopped at 2.4mM sulfide (Meulepas *et al.*, 2009). In the batch experiments described in this paper the sulfide concentrations went up to 15 mM suggesting that the organisms enriched in batch have a higher sulfide tolerance. The Q-PCR and FISH results of the specific batch incubations showed an increase in the relative number of ANME-1 methanotrophs. In the bioreactor enrichments the archaeal community was dominated by ANME-2a cells and no ANME-1 could be detected using DGGE, FISH and a clone library (Chapter 2). These findings suggest that ANME-1 related archaea from the Eckernförde Bay are less sensitive to sulfide and capable of growth on other substrates than methane. Possibly they can switch between methanotrophy and methanogenesis. ANME-2 from Eckernförde Bay sediment could be obligate methanotrophs because they can only be found in incubations with methane present in the gas phase and only under relatively low sulfide concentrations.

Further experiments could use the described incubation methods to enrich and hopefully isolate one of the methanotrophic archaea. Also the pathway of the co-metabolic use of methanol and methane during the net anaerobic oxidation of methane should be examined.

Acknowledgements

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Chapter 5

Effect of methanogenic substrates on anaerobic oxidation of methane by a methane-oxidizing sulfate-reducing enrichment.

5.1 Abstract

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is assumed to be a syntrophic process, in which methanotrophic archaea (ANME) produce an interspecies electron carrier (IEC), which is subsequently utilized by sulfate-reducing bacteria (SRB). In this paper, six methanogenic substrates are tested as candidate IECs by assessing their effect on AOM and SR rates during four-days incubations with a CH_4-oxidizing sulfate-reducing Eckernförde Bay enrichment. The presence of acetate (1.0 mM), formate (1.0 mM) or hydrogen (7.2 kPa) enhanced SR, but did not inhibit AOM, nor did these substrates trigger methanogenesis. Carbon monoxide (7.2 kPa) also enhanced SR but slightly inhibited AOM. Any additional SR could be coupled to the oxidation of the added candidate IEC, although AOM was always the dominant oxidation process. Methanol (1.0 mM) did not enhance SR nor did it inhibit AOM. Methanethiol (1.0 mM) did inhibit both SR and AOM completely. Based on thermodynamic consideration it can be predicted that the conversion of CH_4 to one of the candidate IECs is only possible when the IEC concentration is extremely low; the actual acetate, formate, methanol, carbon monoxide, methanethiol and hydrogen concentrations in the bulk liquid during the experiment were at least 1000 times too high . As AOM was not or hardly inhibited, this work shows that acetate, formate, methanol, carbon monoxide and hydrogen can be excluded as sole IEC in AOM coupled to SR. The reported experiments do not exclude methanethiol as IEC.

5.2 Introduction

5.2.1 Anaerobic oxidation of methane coupled to sulfate reduction

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is assumed to be a syntrophic process, in which methanotrophic archaea (ANME) produce an interspecies electron carrier (IEC), which is subsequently utilized by sulfate-reducing bacteria (SRB) (Zehnder and Brock, 1980; Alperin and Reeburgh, 1985; Hoehler *et al.*, 1994; Boetius *et al.*, 2000; DeLong, 2000). There is evidence that AOM is a form of reversed methanogenesis (Krüger *et al.*, 2003; Hallam *et al.*, 2004) and methanogenic substrates were proposed to act as IECs (Sørensen *et al.*, 2001). The Gibbs free energy change at standard condition ($\Delta G°'$) of the production of these IECs from CH_4 is positive (Table 5.1). However, when the IEC concentration is kept low enough by

the sulfate reducing partner, the $\Delta G'$ will become negative and the overall reaction could still proceed.

Table 5.1: Candidate interspecies electron carriers and their conversions. The standard Gibbs free energy changes were obtained from Thauer et al. (1977).

Candidate IEC	Potential sub-conversions in AOM coupled to SR	The standard Gibbs free energy changes	
Acetate	$CH_4 + HCO_3^- \rightarrow CH_3COO^- + H_2O$	$\Delta G^{o'}_{ANME}$	$+31$ kJ mol^{-1} CH$_4$
	$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$	$\Delta G^{o'}_{SRB}$	-47 kJ mol^{-1} SO$_4^{2-}$
Formate	$CH_4 + 3HCO_3^- \rightarrow 4HCO_2^- + H^+ + H_2O$	$\Delta G^{o'}_{ANME}$	$+128$ kJ mol^{-1} CH$_4$
	$4HCO_2^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^-$	$\Delta G^{o'}_{SRB}$	-144 kJ mol^{-1} SO$_4^{2-}$
Methanol	$CH_4 + {}^1/_3HCO_3^- + {}^1/_3H^+ + {}^1/_3H_2O \rightarrow {}^4/_3CH_3OH$	$\Delta G^{o'}_{ANME}$	$+104$ kJ mol^{-1} CH$_4$
	${}^4/_3CH_3OH + SO_4^{2-} \rightarrow HS^- + {}^4/_3HCO_3^- + {}^1/_3H^+ + {}^4/_3H_2O$	$\Delta G^{o'}_{SRB}$	-120 kJ mol^{-1} SO$_4^{2-}$
Carbon monoxide	$CH_4 + 3HCO_3^- + 3H^+ \rightarrow 4CO + 5H_2O$	$\Delta G^{o'}_{ANME}$	$+196$ kJ mol^{-1} CH$_4$
	$4CO + SO_4^{2-} + 4H_2O \rightarrow HS^- + 4HCO_3^- + 3H^+$	$\Delta G^{o'}_{SRB}$	-212 kJ mol^{-1} SO$_4^{2-}$
Methane-thiol	$CH_4 + {}^1/_3HCO_3^- + {}^5/_3H^+ + {}^4/_3HS^- \rightarrow {}^4/_3H_3CSH + H_2O$	$\Delta G^{o'}_{ANME}$	$+55$ kJ mol^{-1} CH$_4$
	${}^4/_3H_3CSH + SO_4^{2-} \rightarrow {}^7/_3HS^- + {}^4/_3HCO_3^- + {}^5/_3H^+$	$\Delta G^{o'}_{SRB}$	-71 kJ mol^{-1} SO$_4^{2-}$
Hydrogen	$CH_4 + 3H_2O \rightarrow 4H_2 + HCO_3^- + H^+$	$\Delta G^{o'}_{ANME}$	$+136$ kJ mol^{-1} CH$_4$
	$4H_2 + SO_4^- + H^+ \rightarrow HS^- + 4H_2O$	$\Delta G^{o'}_{SRB}$	-152 kJ mol^{-1} SO$_4^{2-}$

This study investigates whether methanogenic substrates act as IEC by assessing the effect of the presence of candidate IECs, at relative high concentrations, on AOM and SR by a CH_4-oxidizing sulfate-reducing enrichment. In addition, the concentration of each candidate IEC is calculated at which no more energy can be obtained from their production from CH_4, according to the reactions in Table 5.1. If AOM still occurs at IEC concentrations far above the theoretical maximum, the AOM does not proceed via the production of that particular IEC.

5.3 Material and methods

5.3.1 Eckernförde Bay enrichment

The biomass used for this research was taken from a 1 L submerged-membrane bioreactor, in which anaerobic methanotrophs were enriched (Jagersma *et al.*, 2009) The reactor was inoculated with 10 $g_{dry\ weight}$ Eckernförde Bay sediment (Baltic Sea), operated at 15ºC and fed with sulfate as electron acceptor and CH_4 as electron donor and carbon source. During 884 days, the volumetric conversion rate increased exponentially from 0.002 to 0.6 mmol L^{-1} day^{-1} (Chapter 3). The activity of the obtained CH_4-oxidizing sulfate-reducing enrichment was 1.0 mmol g_{VSS}^{-1} day^{-1}. To ensure homogeneous sampling, liquid recirculation (0.5 L min^{-1}) and gas sparging (2 L min^{-1}) were applied prior to and during sampling.

5.3.2 Standard incubation procedure

Experiments were done in 35-ml serum bottles closed with butyl rubber stoppers and caps. After determining the exact weight and volume, the bottles were flushed eight times with nitrogen gas and made vacuum. Subsequently, 30 ml undiluted reactor suspension (0.59 g_{VSS} L^{-1}) was transferred from the bioreactor to the bottles by syringe. The headspace of each bottle was made vacuum again and filled with 0.16 (±0.01) MPa ^{13}C-labeled CH_4 ($^{13}CH_4$) with a purity of 99% from Campro (Veenendaal, the Netherlands). Subsequently, candidate IECs were added from stock bottles. Control incubations without IEC and incubations with 1.0 mM acetate, 1.0 mM formate, 1.0 mM methanol, 7.2 kPa (=1.0 mmol L_{liquid}^{-1}) carbon monoxide, 1.0 mM methanethiol or 7.2 kPa (=1.0 mmol L_{liquid}^{-1}) hydrogen as IEC were done in duplicate. However, one bottle with methanethiol and one bottle with hydrogen were leaking, these duplicates could not be repeated due to a limited biomass stock. The bottles were incubated at 15°C and shaken in an orbital shaker at 100 rpm. The gas composition, pH and pressure were determined once or twice a day. The carbon monoxide and hydrogen fraction in the headspace, the sulfate and formate concentration, the dissolved sulfide concentration and the concentration of fatty acid and alcohols were analyzed immediately after inoculation and after four days. Sampling was done at incubation temperature (15°C).

5.3.3 Analysis

The headspace composition ($^{13}CH_4$, $^{12}CH_4$, $^{13}CO_3$ and $^{12}CO_3$), headspace pressure, sulfide concentration, sulfate concentration, acetate concentration, methanol concentration and pH were analyzed as described by Meulepas *et al.* (2009).
Formate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA). The used columns were IonPac AG17 and AS17 4 mm operated at a temperature of 30°C and a flow rate of 1.5 ml min^{-1}. The injection volume was 25 µl. The eluent was made on-line using the EG40 Eluent Generator (Dionex) equipped with a KOH cartridge (Dionex P/N 053921) and deionized water as the carrier. Prior to analysis, samples were centrifuged and diluted 20 times.
Hydrogen and carbon monoxide were measured on a gas chromatograph HP 5890 (Hewlett Packard, Palo Alto, USA) as described previously (Sipma *et al.*, 2004). Methanethiol was measured on a HP 6890 gas chromatograph equipped with a Supelco sulfur SPB-1 column (Bellefonte, PA, USA) according to van den Bosch (2008).

5.3.4 Calculations

The $\sum^{13}CO_2$ (^{13}C-labeled CO_2 and ^{13}C-labeled bicarbonate) and $\sum^{12}CO_2$ per bottle were calculated according to the equation given by Meulepas *et al.* (2009) (Chapter 3) The volumetric AOM, $\sum^{12}CO_2$ production, methanogenesis, sulfate reduction and candidate IEC removal rates are estimated from, respectively, the $\sum^{13}CO_2$ production, $\sum^{12}CO_2$ production, $^{12}CH_4$ production, sulfide production and candidate IEC consumption over the four-day incubation period.

The concentration of each candidate IEC at which no more energy can be obtained ($\Delta G_{ANME} = 0$) from their production from CH_4 (Table 5.1) was calculated. This is done according to equation 15.

$$(15) \qquad \Delta G'_{anme} = \Delta G°_{anme} + RTLn \frac{\prod \gamma [products]}{\prod \gamma [substrates]}$$

Nomenclature
R = gas constant = 8.314 J mol^{-1} K^{-1}
T = temperature in °K = 288.15 °K

γ = activity coefficient, at a salinity of 35‰: $\gamma(HS^-)$ = 0.410, $\gamma(SO_4^{2-})$ = 0.104, $\gamma(HCO_3^-)$ = 0.532 and $\gamma(CH_4)$ = 1.24 (Millero and Schreiber, 1982; Davison, 1980)

5.4 Results

5.4.1 Incubations

Figure 5.1 shows the accumulation of $^{13}CO_2$ for each incubation. The presence of acetate, formate, methanol and hydrogen did not inhibit CH_4 oxidation, the rates (0.13-0.19 mmol L^{-1} day^{-1}) were comparable with the rates of the incubations without IEC (0.13 and 0.14 mmol L^{-1} day^{-1}). In the incubations with carbon monoxide, the CH_4 oxidation was slightly lower (0.09 and 0.10 mmol L^{-1} day^{-1}) and methanethiol completely inhibited CH_4 oxidation.
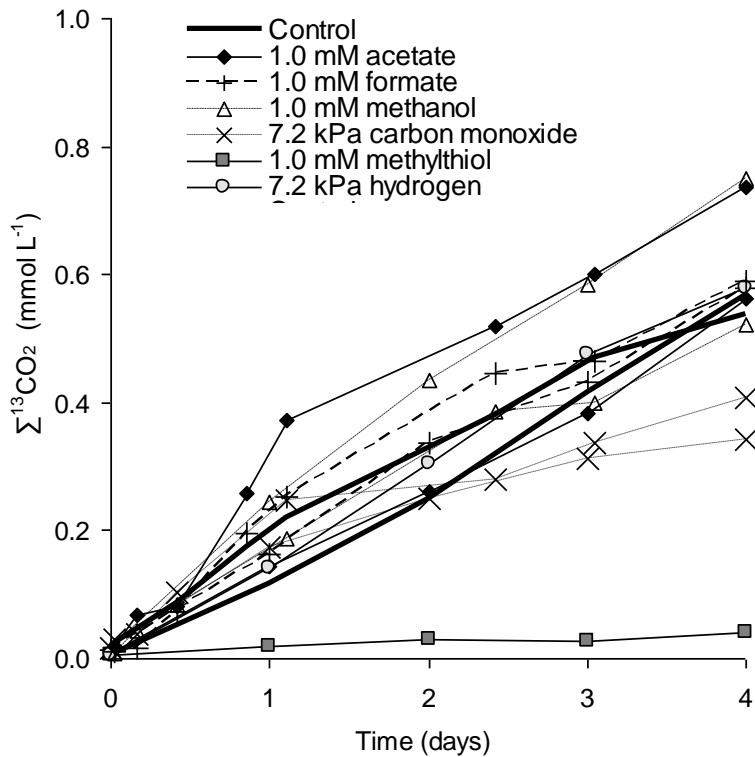
Figure 5.1: $\sum^{13}CO_2$ *production in time, during four-day batch incubations, in the absence (control) or in the presence of one candidate IEC, at an initial concentration of 1 mM or 7.2 kPa (=1.0 mmol L_{liquid}^{-1}). The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa $^{13}CH_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.*

Of the initial 1 mmol L^{-1} IEC; 0.15 and 0.14 mM acetate, 0.27 and 0.28 mM formate, 2.1 and 2.6 kPa (0.30 and 0.36 mmol L^{-1}) carbon monoxide, and 4.0 kPa (0.55 mmol L^{-1}) hydrogen were consumed. Figure 5.2 shows that the consumption of acetate, formate and carbon monoxide was mainly coupled to the production of $^{12}CO_2$ and not to acetate or CH_4 production, indication a complete oxidation. Methanol (0.03 and 0.03 mM) and methanethiol (0.01 mM) were hardly consumed. All incubations showed some background $^{12}CO_2$ production, possibly released from the unlabeled biomass.
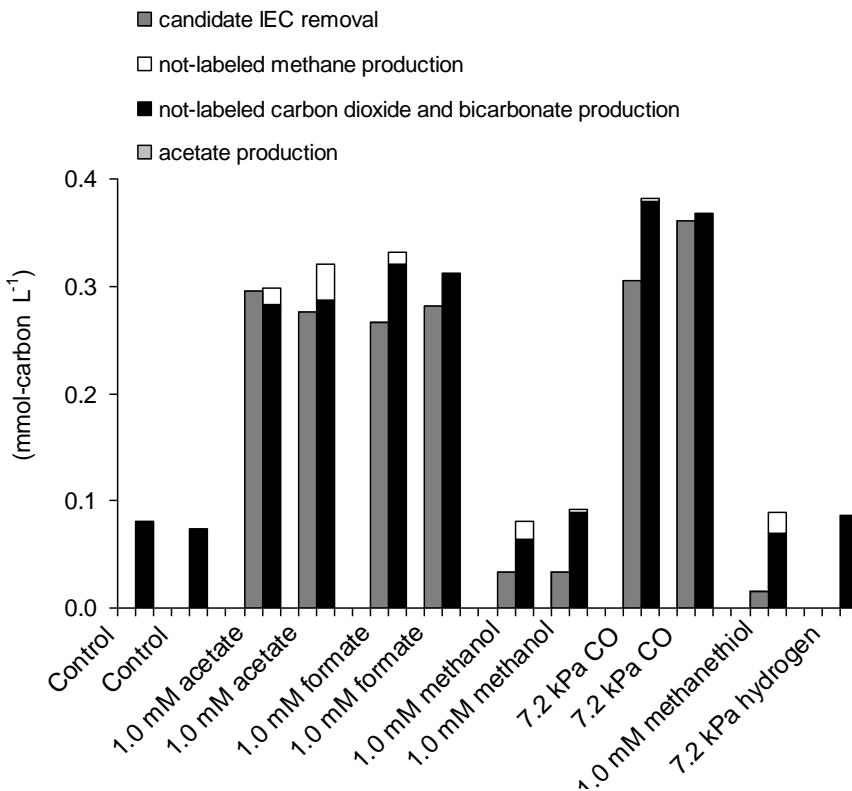
Figure 5.2: Candidate IEC removal compared to the $^{12}CH_4$ production, $\sum^{12}CO_2$ production and acetate production after four days of incubation in batch, in the absence (control) or in the presence of one of the candidate IEC at an initial concentration of 1mM or 7.2 kPa (=1.0 mmol L_{liquid}^{-1}). The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa $^{13}CH_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.

Figure 5.3 compares oxidation reactions with reduction reactions. In the control incubations, CH_4 oxidation was coupled to SR. In the presence of acetate, formate, carbon monoxide and hydrogen more sulfate was reduced than CH_4 oxidized, the differences were 0.13 and 0.16; 0.09 and 0.13; 0.11 and 0.12; and 0.11 mmol L^{-1}, respectively. This additional SR was coupled to the oxidation of candidate IECs. Therefore, CH_4, acetate, formate, carbon monoxide and hydrogen were all used as electron donor for sulfate reduction by the Eckernförde Bay enrichment, although CH_4 oxidation was in all incubations dominant over candidate IEC oxidation. Both SR and CH_4 oxidation were inhibited by the presence of methanethiol.
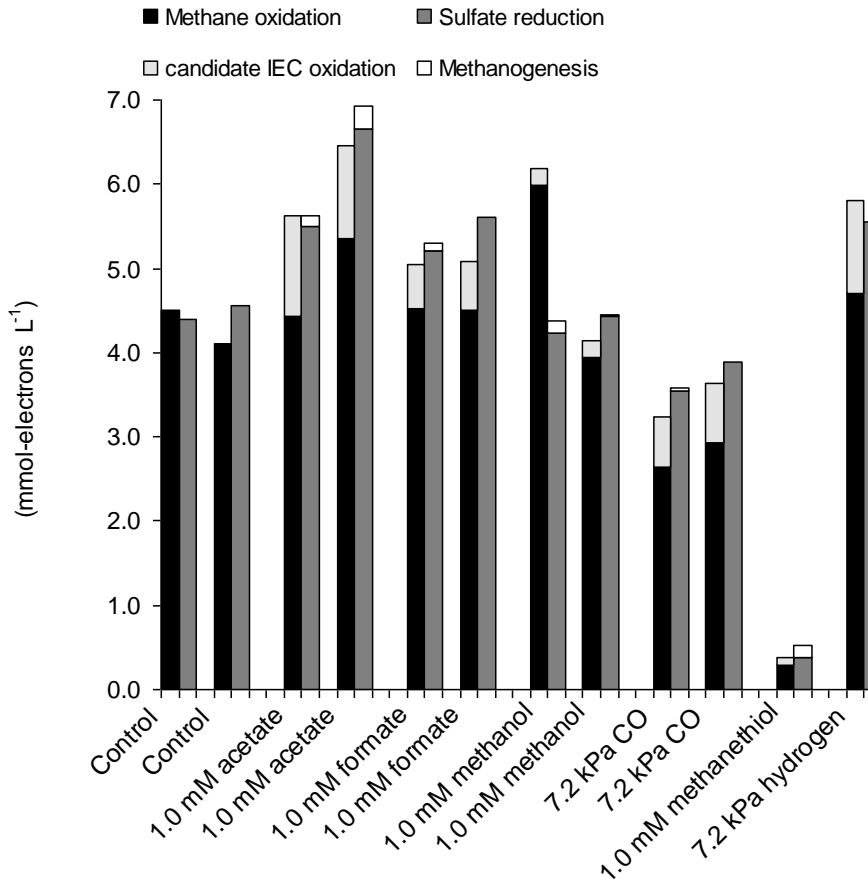
Figure 5.3. CH$_4$ and candidate IEC oxidation compared to SR and methanogenesis after four days of incubation in batch, in the absence (control) or in the presence of one candidate IEC, at an initial concentration of 1mM or 7.2 kPa (=1.0 mmol L$_{liquid}^{-1}$). The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa ^{13}CH$_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.

5.4.2 Thermodynamic calculations

Table 5.2 presents the concentrations of candidate IECs at which their production, under the applied experimental conditions, is no longer thermodynamically possible.

Table 5.2: The concentration of candidate IECs at which their production from CH$_4$ is no longer thermodynamically possible (ΔG'=0), at 1.4 atm CH$_4$, 1 mM HCO$_3^-$, 1 mM HS$^-$ and a pH of 7.

IEC	IEC concentration at which $\triangle G_{ANME}$ = 0 (mM)	Lowest actual IEC concentration (mM)
Acetate	$3.4.10^{-6}$ mM	0.85 and 0.86
Formate	$9.7.10^{-6}$ mM	0.73 and 0.72
Methanol	$1.7.10^{-12}$ mM	0.93 and 0.93
Carbon monoxide	$8.0.10^{-12}$ atm.	4.9 and 4.4 kPa / 0.048 and 0.043 atm.
Methanethiol	$7.6.10^{-9}$ mM	0.99
Hydrogen	$4.2.10^{-6}$ atm.	3.2 kPa / 0.032 atm.

To obtain maximum concentrations, the lowest measure CH$_4$ partial pressure (0.14 MPa) and the highest measured HS$^-$ and HCO$_3^-$ concentrations (both 1 mM) were used for the calculations. The theoretical maximum concentration for the production of each candidate IEC was always at least 1000 times lower than the actual concentration measured at day 4.

5.5 Discussion

5.5.1 Exclusion of candidate IECs

This research shows that acetate, formate, methanol, carbon monoxide and hydrogen could not have been produced from CH$_4$ during AOM by the Eckernförde Bay enrichment. The AOM rates in the presence of these compounds was between 61 and 139% of the rates obtained in the controls. During the 4-days incubations, the concentrations of acetate, formate, methanol, carbon monoxide and hydrogen were at least 0.85 mM, 0.72 mM, 0.93 mM, 4,4 kPa and 3.2 kPa, respectively (Table 5.2). While thermodynamics predict that the production of these compounds from CH$_4$, at the experimental conditions, can no longer proceed when the concentration of these compounds exceeds $3.4.10^{-6}$ mM, $9.7.10^{-6}$ mM, $1.7.10^{-12}$ mM, $8.0.10^{-12}$ atm. and $4.2.10^{-6}$ atm. for acetate, formate, methanol, carbon monoxide and hydrogen,

respectively (Table 5.2). Therefore, these compounds can be excluded as IEC in AOM coupled to SR.

Both carbon monoxide and methanethiol are toxic for many archaea and sulfate reducers. Carbon monoxide hampered SR by sulfate-reducing sludge at a concentration of 5% onwards (van Houten *et al.*, 1995), and sulfate reducers used only methyl sulfides as substrate at low concentrations (< 10 µM) (Kiene *et al.*, 1986). If these compounds would be produced *in situ*, the concentrations would remain much lower due to simultaneous consumption, therefore toxic effects would be less profound. At a concentration of 1.0 mM, methanethiol inhibited AOM and can therefore not be excluded as IEC in AOM coupled to SR. Moran *et al.* (2007) also reported an inhibition of AOM by methanethiol. If electrons would be transferred via methanethiol, sulfate reducers would be able to utilize these compounds, which did not occur (Figure 5.3). However, SR could have been inhibited as well, due to the toxic effect of methanethiol.

Many of the candidate IECs tested were consumed (Figure 5.2), which can result in a concentration gradient within the biomass flocks. Therefore, the concentration near the responsible organism can be lower than in the bulk liquid. A big difference between the concentration in the bulk liquid at the concentration near the organism mediating AOM is not expected though, because the reactor suspension was well-mixed (orbital shaker at 100 rpm), the biomass flocks were extremely small (0.1mm; Chapters 2 and 3) and the IEC consumption rates were low (<0.6 mmol L^{-1} day^{-1}).

5.5.2 Syntrophy between ANME and SRB

Our findings are in agreement with the thermodynamic calculations reported by Sørensen *et al.*, (2001). That study excluded hydrogen, acetate and methanol as IEC in AOM coupled to SR, because the maximum diffusion distances of those compounds at *in situ* concentrations and rates were smaller than the thickness of two prokaryotic cell walls, for formate this was not the case though. Recent research demonstrated that the SRB involved in AOM, from three different sites, incorporate carbon derived from carbon dioxide into their lipids, rather than carbon from CH_4 (Wegener *et al.*, 2008). It is therefore unlikely that these SRB take up an IEC containing the carbon from CH_4, which is in agreement with our findings that acetate, formate, methanol and carbon dioxide can be excluded as the sole IEC in AOM coupled to SR.

Alternative theories for the shuttling of electrons between ANME and SRB are that reduction equivalents are transferred via extracellular redox shuttles (Widdel and Rabus 2001; Wegener *et al.*, 2008), or via membrane bound redox shuttles or so called "nanowires" (Reguera *et al.*, 2005; Stams *et al.*, 2006; Thauer and Shima, 2008; Wegener *et al.*, 2008, Stams *et al.*, 2009). The extracellular redox shuttle theory requires the shuttle to be transported back to the ANME after donating the electrons to the SRB, giving rise to an additional loss in Gibbs free energy change, available for the microorganisms, due to the concentration gradients between the syntrophic partners. The membrane bound redox shuttles or nanowire theories require the ANME and SRB to make physical contact, which is not always the case (Michaelis *et al.*, 2002; Knittel *et al.*, 2005; Orphan *et al.*, 2002; Treude *et al.*, 2005; Chapters 2 and 3).

At *in situ* conditions there is only -22.35 kJ mol^{-1} available for AOM coupled to SR (Harder, 1997). Methanogenic archaea and sulfate reducing bacteria have been shown to require a free energy change under physiological conditions of at least -10 kJ mol^{-1} and -19 kJ mol^{-1}, respectively, to support their metabolism *in situ* (Hoehler *et al.*, 2001; Dale *et al.*, 2006). Therefore, the *in situ* free energy change of AOM coupled to SR is probably not sufficiently large to fuel the energy metabolism of two microorganisms in tandem (Schink, 1997; Thauer and Shima, 2008). Further research should consider the possibility that one microorganism is responsible for AOM coupled to SR.

5.5.3 Alternative electron donors

The Eckernförde Bay enrichment was able to utilize acetate, formate, methanol, carbon monoxide and hydrogen as electron donor for SR (Figure 5.3), although the enrichment was not fed with any other electron donor and carbon source than CH$_4$ for 512 days (Chapter 3). Prior to this, the enrichment was fed solely CH$_4$ and acetate (70 µmol L^{-1} day^{-1}) for a period of 330 days. Possibly, the sulfate reducers involved in AOM coupled to SR are capable of utilizing acetate, formate, methanol, carbon monoxide and hydrogen as alternative electron donors for the IEC or CH$_4$. If this would be the case, those microorganisms could be enriched on those alternative substrates instead of on CH$_4$. Another explanation is that other SRB, not involved in AOM coupled to SR, survived this enrichment period. This hypothesis would require inactive SRB to become active within the four-days duration of the experiment.

SR with any of the added candidate IECs would yield more Gibbs free energy change than AOM coupled to SR (Table 5.1). However, AOM was the dominant oxidation process, within the four-day incubation period, the microorganisms involved in AOM coupled to SR were not able to switch completely from CH_4 to acetate, formate, methanol or hydrogen as the preferred electron donor. Nauhaus *et al.* (2002; 2005) found that SR with hydrogen, formate, acetate, methanol, carbon monoxide and methanethiol by AOM sediment (Hydrate Ridge) was much slower than with CH_4. In addition, the authors showed that in the presence of CH_4, additions of hydrogen, formate, acetate, methanol neither stimulated nor inhibited SR. The finding that hydrogen, formate and acetate did not stimulate SR is in contrast to the findings of this study with the Eckernförde Bay enrichment. However, incubations with a microbial mat from the Black sea sediment showed comparable SR rates with acetate and CH_4, and higher SR rates on hydrogen and formate (Nauhaus *et al.*, 2005). This shows that, like the Eckernförde Bay enrichment used in this study, also a natural AOM enrichment was able to use other electron donors than CH_4 for sulfate reduction.

Acknowledgements

Nomenclature

eff = effluent
f = fraction
HRT = Hydraulic retention time
inf = influent
k = Henry's law constant for CO_2 at sampling temperature(20°C): 0.0388 mol L^{-1}
K_z = dissociation constant of H_2CO_3: $4.5 \cdot 10^{-7}$
P = pressure
t = time
V_{gas} = gas volume in serum bottle for activity assay
$V_{gaswash}$ = liquid volume in gas wash bottle
$V_{inoculum}$ = volume reactor suspension used for inoculation
V_{liquid} = liquid volume in serum bottle for activity assay
[X] = molar concentration of compound X

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Chapter 6

General discussion

General discussion

Anaerobic oxidation of methane coupled to sulfate reduction is thought to be performed by syntrophic communities, that are in a direct physical contact. These so called consortia have been found in very diverse marine habitats where AOM and SR occur. The sediment from the Eckernförde Bay region that acted as inoculation material for most of the incubation experiments in this thesis shows generally a lower methane oxidation rate than other sediments known for AOM capability (Krüger *et al.*, 2005). The Eckernförde bay sediment also differs from other AOM sediments in the occurrence of aggregates consisting of only ANME archaea. (Treude *et al.*, 2005b). Our results show that it is possible for non-aggregated cells to perform AOM coupled to SR. This thesis describes the enrichment of a sediment capable of AOM in novel submerged-membrane bioreactors (Chapters 2 and 3). The doubling time of the responsible micro-organisms was 3.8 months and the enrichment reached an AOM and SR rate of 1.0 mmol g_{VSS}^{-1} day^{-1} (286 µmol $g_{dry\ weight}^{-1}$ day^{-1}) after 884 days which is the highest specific AOM activity reported so far. The enrichment consisted of loose flocks. The dominance of ANME-2a sequences in the archaeal clone library and the increase in single ANME-2a cells in the FISH analysis, suggest that archaea from the ANME-2a subgroup of the anaerobic methanotrophs are responsible for the exponential increase in AOM rate in the bioreactor. Because no other ANME sequences were detected by denaturing gradient gel electrophoresis (DGGE) (Chapter 3), the clone library analysis and FISH we can conclude that ANME-2a cells and bacteria occurring in non-aggregated form without direct contact with other cells perform AOM with SR. This finding shows that it is possible to enrich for a very specialized microbial community with submerged membrane bioreactors. The growth rates are obviously slow and the sulfide tolerance of the enrichment is relatively low (around 2.4 mM, Meulepas *et al.*, 2009a) whereas sulfide levels in marine sediments can reach far higher values.

The known ANME clusters are associated with specific SRB that belong to the *Desulfosarcina/Desulfococcus* (DSS) group (Boetius *et al.*, 2000; Michaelis *et al.*, 2002; Knittel *et al.*, 2003) and the *Desulfobulbus* group (Treude *et al.*, 2007) of the *Deltaproteobacteria*. Despite several investigations, the exact mechanism of metabolic interaction between the syntrophic partners is still unclear (Hoehler *et al.*, 1994; Nauhaus *et al.*, 2002; Moran *et al.*, 2008, Orphan 2009). The sequences in the

clone library of our Eckernförde bay enrichment confirm the presence of sulfate-reducing bacteria related to *Desulfotignum* sp. and of uncultured environmental clones also found in other anaerobic methanotrophic sediments (Musat *et al.*, 2008; Heijs *et al.*, 2005).

The other dominant group of sequences found in the bacterial clone library belongs to the phylum *Bacteroidetes* and form a cluster within the order of *Flavobacteriales*. The novel cluster is phylogenetically distantly related to *Blattabacteria*, isolated from cockroach hindgut. The physiology of these intracellular endosymbionts of insects is not yet fully understood, but it is reported to be linked to the conversion of inorganic sulfate to organic sulfur compounds (Wren *et al.*, 1987) or the nitrogen-metabolism (Cruden *et al.*, 1987). Recent findings also indicate a much larger role of bacteria not related to known SRB in AOM like *Betaproteobacteria*, most similar to members of the *Burkholderiaceae*, and *Alphaproteobacteria*, related to *Sphingomonas*, (Pernthaler *et al.*, 2008). Other clones from the bioreactor enrichment can be linked to known marine micro-organisms and because of their low abundance after more than 800 days of continuous incubation, they are most probably residual micro-organisms from the original Eckernförde bay sediment.

The presence of single cells which hybridize with the ANME-IIa-647 FISH probe without a directly associated bacterial partner does not correspond with the idea that AOM is a syntrophic process that requires a close physical interaction of the micro-organisms involved (Boetius *et al.*, 2000; Schink, 2002). In some sediments highly structured ANME-2/*Desulfosarcina* consortia are not the sole entities responsible for AOM, but also monospecific consortia and single cells were found (Orphan *et al.*, 2002). Lipid analysis of the enrichment biomass showed that bacterial lipids were dominating over those of archaea, in agreement with the FISH results which showed a dominance of bacteria over archaea. ^{13}C-label from methane was substantially incorporated in both archaeal and bacterial lipids during batch incubation with bioreactor sludge. Our results are different from those of Blumenberg *et al.* (2005), who showed that the ^{13}C-label from methane can mainly be found in bacterial lipids rather than archaeal lipids. The difference can be explained by the much higher AOM rates observed here and the much more active archaea in the AOM consortium studied. Interestingly, the degree of labeling of the bacterial lipids observed in our study is much larger than that of Blumenberg *et al.* for the same lipids and after the same period of incubation (e.g. 44% versus 0.2% for the C16:1 fatty acid), suggesting that the SRB were also much more active. The

reason why the ^{13}C-label is taken up by bacteria in this and previous studies (Blumenberg et al., 2005) is yet unclear. Possibly they have taken up [13]CO$_2$ or organic compounds produced by ANME-2a. However, the direct uptake of methane by bacteria cannot be excluded. Raghoebarsing et al. (2006) found low uptake rates of ^{13}C-labelled methane in archaeal lipids in batch reactors in which AOM was performed by a consortium of archaea and denitrifying bacteria and methane oxidation coupled to denitrification was later found to be a bacterial process not involving archaea (Ettwig et al., 2008).

An explanation of the possible syntrophic interaction between ANME archaea and SRB suggest the formation of an interspecies electron carrier (IEC) by the ANME archaea and the subsequent utilization of this IEC by the SRB. Gene analogues coding for many of the enzymes involved in methanogenesis were found in archaea that belong to ANME groups, but not for enzymes required for dissimilatory SR. In Chapter 4 and 5, multiple methanogenic substrates are reviewed as candidate IEC's by assessing their effect on AOM and SR rates during short four day incubations (Chapter 5) and one long term 200 day incubation (chapter 4) with a methanotrophic sulfate-reducing enrichment from Eckernförde Bay. In Chapter 5 the presence of acetate (1.0 mM), formate (1.0 mM) or hydrogen (7.2 kPa) enhanced SR, but did not inhibit AOM, nor did it trigger methanogenesis. Carbon monoxide (7.2 kPa) also enhanced SR but slightly inhibited AOM. Any additional SR could be coupled to the oxidation of the added potential IEC, although AOM was always the dominant oxidation process. Methanol (1.0 mM) did not enhanced SR nor did it inhibit AOM. Methanethiol (1.0 mM) did inhibit both SR and AOM completely. Thermodynamics predict that the conversion of methane to one of the potential IEC's is only possible when the IEC concentration is extremely low: the concentrations of the potential IEC's in the bulk liquid were at least 1000 times too high during the period from which AOM and SR rates were obtained. Even considering concentration gradients within biomass flocks due to IEC consumption, it is unlikely that one of the tested potential IEC's was produced from methane. Therefore, and because AOM was not or hardly inhibited, the work in Chapter 5 shows that acetate, formate, methanol, carbon monoxide and hydrogen are excluded as interspecies electron carriers in AOM coupled to SR. These experiments did not exclude methanethiol as IEC, however the inhibitory effect on AOM could also be caused by toxicity rather than by thermodynamics. The fact that methanethiol was not utilized as electron donor for sulfate reduction could be an indication for this.

A 200 day incubation with methane and methanogenic substrates acting as possible co-substrates during AOM showed that only methanol caused a positive effect on the AOM rate for our specific enrichment. The other substrates: acetate, butyrate, pyruvate and yeast extract even lowered the AOM rate possibly due to the competition for these methanogenic substrates between ANME archaea and methanogens still present in the enrichment.

From batch enrichments of Eckernförde bay sediment without methane incubated at 30°C it was possible to enrich for ANME-1 archaea, the sulfide levels reached 15 mM and it is clear that by using different incubation conditions different microbial communities could be enriched. In enrichments without methane ANME-1 archaea could be enriched and this suggests that ANME-1 are methanogens capable of reversing their methanogenic pathway to methanotrophy if needed. This can also explain why ANME-1 and ANME-2 archaea can co-occur in the same sediments without outcompeting each other.

The application of the AOM process for sulfate removal from industrial wastewater is hampered by the extreme slow growth rates. To achieve the same sulfate removal rates the enrichment should have a high density in cell numbers and capable of high rate removal of sulfate. Currently the growth rates in a membrane bioreactor or any other enrichment method are too low for developing up-scale experiments. Future research should aim for the improvement of the current enrichment methods. Genomic data that can be gathered from a pure culture of an ANME archaea or an associated SRB can give insight in the metabolic pathways and could give an indication of possible novel enrichment methods. Unraveling the possible syntrophic interaction between the ANME archaea and associated SRB could also make way for increasing the growth rates of AOM communities.

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Summary

Anaerobic methane oxidation (AOM) coupled to sulfate reduction (SR) is a process that occurs in anaerobic marine sediments. A biotechnological application of this process is the removal of metals and sulfate from industrial wastewater. To apply this process on a large scale, an enrichment of a highly active methane-oxidizing sulfate-reducing community is necessary. Chapter 3 describes the development of a novel well-mixed ambient-pressure submerged-membrane bioreactor which was inoculated with a known methane-oxidizing sediment from Eckernförde bay (German Baltic). The bioreactor operated continuously at 15 °C with artificial marine medium, methane and sulfate. An active enrichment was obtained with an AOM rate of 1.0 mmol G_{vss}^{-1} day^{-1}. The rate of AOM doubled every 3.8 months. Chapter 2 describes the molecular analyses of the enrichment consisting of a community with ANME-2a archaea and a diverse group of bacteria, mainly sulfate reducing bacteria (SRB) and a subgroup of *Blattabacteria*. None of the microbial groups showed a direct physical association, but were found in very loose aggregates. Carbon derived from methane was incorporated in both archaeal and bacterial lipids proving that both groups of organisms were involved in the oxidation of methane. AOM coupled to SR is suggested to be a syntrophic process, in which methanotrophic archaea produce an interspecies electron carrier (IEC), which is subsequently utilized by SRB. To investigate this syntrophic pathway the effect of methanogenic substrates were tested in a long term (200 day) experiment (Chapter 4) and short (4-day) experiments both with ^{13}C labeled methane (Chapter 5). Acetate, formate, carbon monoxide and hydrogen can be excluded as sole IEC in AOM coupled to SR because these substrates do not inhibit AOM (Chapter 5). Methanol does not inhibit AOM nor does it enhance SR in a short experiment, in a long term experiment methanol clearly stimulates AOM suggesting that methanol is used as co-substrate during methane oxidation (Chapter 4). The apparent slow growth rate remains an important bottleneck in the scale-up of the process but the novel bioreactor design developed in this thesis enables the enrichment of a microbial community capable of high rate sulfate reduction with methane as sole electron donor.

Samenvatting

Anaerobe oxidatie van methaan (AOM) gekoppeld aan sulfaat reductie (SR) is een proces dat in anaerobe marine sedimenten voorkomt. Een biotechnologische toepassing van dit proces is het verwijderen van metalen en sulfaat uit industrieel afvalwater. Om het AOM proces op grote schaal toe te kunnen passen is een ophoping nodig van hoog actieve methaanoxiderende en sulfaatreducerende micro organismen. Hoofdstuk 3 beschrijft de ontwikkeling van een nieuwe goed gemengde membraan bioreactor die continue gevoed wordt met methaan en sulfaat bij 15 °C. Een actieve ophoping werd verkregen met een AOM activiteit van 1.0 mmol G_{vss}^{-1} dag^{-1}. De AOM snelheid verdubbelde elke 3.8 maand. Hoofdstuk 2 beschrijft de moleculaire analyses van de ophoping. De microbiële samenstelling van de verrijking was een gemeenschap van ANME-2a archaea en een diverse groep van bacteriën met voornamelijk sulfaat reducerende bacteriën en bacteriën uit een *Blattabacteria* subgroep. Geen van de organismen waren direct met elkaar verbonden maar zaten in losse aggregaten (vlokken). Een lipide analyse geeft aan dat koolstof afkomstig van methaan werd opgenomen door zowel bacteriën als archaea. Dit geeft aan dat beide groepen betrokken zijn bij de anaerobe oxidatie van methaan. Methaanoxidatie gekoppeld aan sulfaatreductie wordt beschouwd als een syntroof proces waarin de methanotrofe archaea een "*interspecies* elektronen drager" (IED) produceren die vervolgens door de sulfaat reducerende bacterie kan worden opgenomen. Om dit syntrofe proces te onderzoeken zijn er experimenten gedaan met methanogene substraten en ^{13}C gelabeld methaan in een lange termijn experiment (hoofdstuk 4) en korte termijn incubaties van 4 dagen (hoofdstuk 5). Acetaat, formaat, koolstofmonoxide en waterstof kunnen worden uitgesloten als IED in AOM met SR aangezien deze substraten het AOM proces niet remmen (hoofdstuk 5). Methanol remt in het 4 dagen experiment de AOM snelheid niet en ook stimuleert het niet de SR snelheid maar in een lange termijn experiment (200 dagen) stimuleert methanol de AOM snelheid wat aangeeft dat methanol mogelijk gebruikt kan worden als cosubstraat tijdens anaerobe methaan oxidatie (hoofdstuk 4).

De extreem lage groeisnelheid blijft een belangrijke drempel voor het ontwikkelen van een toepassing voor het AOM proces voor industriële afvalwaterzuivering. De nieuw ontwikkelde membraan bioreactor heeft voor een verrijking gezorgd van een methaanoxiderend sediment met een hoge activiteit en is een goede mogelijkheid voor het ophopen van zeer langzaam groeiende micro-organismen.

List of publications

Jagersma, C.G., Meulepas, R.J.W., Heikamp-de Jong, I., Gieteling, J., Klimiuk, A., Schouten, S., Sinninghe Damsté, J.S., Lens, P.N.L., Stams, A.J.M. (2009) Microbial diversity and community structure of a highly active anaerobic methane oxidizing sulfate-reducing enrichment. Environmental Microbiology, Published online 24 august 2009 (Epub ahead of print)

Jagersma*, C.G., Meulepas*, R.J.W., Khadem, A.F., Buisman, C.J.N., Stams A.J.M., Lens, P.N.L. Effect of methanogenic substrates on anaerobic oxidation of methane by a methane-oxidizing sulfate-reducing enrichment. Submitted.

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C.G. Jagersma, R.J.W. Meulepas, A.M. Szperl, P.N.L. Lens, A.J.M. Stams. The effect of possible co-substrates on the rate of anaerobic methane oxidation with sulfate. manuscript in preparation.

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Christian Gerard Jagersma was born on the 5th of September 1978 in Leeuwarden. After graduating in 1997 from the Comenius College in Leeuwarden he received a bachelor's degree in Biotechnology in 2001 from the Noordelijke Hogeschool Leeuwarden/Van Hall Institute. He did a practical internship at the University of Parma, Italy with the department of Food Safety, This work resulted in a paper published in 2002. The bachelors thesis was performed at the Utrecht University within the Department of the Science of Food of Animal Origin. After this he moved to Wageningen for a Masters degree in Food technology with a specialization in Food Fermentation and Enzymology. He was actively involved with the study association for Food Technology "Nicolas Appert" as a board member and he joined the board of the studentpub "Annies".

He obtained the Masters degree in May 2004 and started a PhD study at the Laboratory of Microbiology in Wageningen in June 2004. The research was funded by the Dutch government in the EET (*Economie, Ecologie en Technologie*) program. The research was conducted in close collaboration with the technology development company Paques BV in Balk, the Netherlands and NyrStar Budel Zink a large Zinc smelter in the South of the Netherlands. During this period he was involved in the Wageningen PhD counsel as a member and in the WIMEK PhD counsel as a chair (2005-2008). In 2006 he joined the WIMEK board as a PhD representative and in 2007 he became the PhD representative for all PhDs of Wageningen University in the central co-management counsel of WUR. He was also involved in the evaluation committee that looked at the Training and Supervision Plan (TSP) for PhD students of Wageningen University.

From August 2009 Christian is working as Advisor/National Contact Point for the largest research funding program of the European Community (Framework Programme 7) at EG-Liaison/SenterNovem. In this context he is advising researchers to apply for a European grant within this FP7 programme. He is currently living in The Hague with his wife Marieke.

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Christian

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

CERTIFICATE

The Netherlands Research School for the
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Place: *Wageningen* Date: *20 November 2009*

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SENSE board

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Research and Management Skills:

°   **Training in Q-FISH Technology**
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°   **Microbial aspects of anaerobic methane oxidation for sulfate reduction, SENSE symposium "Sensible Water Technology", 12 – 13 April 2007, Leeuwarden, The Netherlands**
°   **Anaerobic methane oxidation for sulfate reduction, SENSE symposium, 20 February 2009, Wageningen, The Netherlands**
°   **Molecular detection of anaerobic methanotrophic communities in a high rate sulfate reducing reactor, First international AOM symposium, 22 February 2008, Anselage, Germany**
°   **Anaerobic methane oxidation for sulfate reduction, Multiple oral and poster presentations during a PhD trip in California (San Diego, LA, San Francisco, etc), Oktober – November 2006, Berkeley, UCLA, USC, Stanford, UCSD, USA**
°   **Microbial aspects of anaerobic methane oxidation for sulfate reduction, Labtrip Max-Planck institute Konstanz en Marburg, May 2006, Konstanz/Marburg, Germany**

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°   **Supervising 4 thesis students as principal supervisor (periods ranging from 4 to 10 months)**

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