Biotechnological aspects of Anaerobic Oxidation of Methane coupled to Sulfate Reduction

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“My commitment is to truth not consistency.”

Mohandas K. Gandhi

Abstract

Sulfate reduction (SR) can be used for the removal and recovery of metals and oxidized sulfur compounds from waste streams. Sulfate-reducing bacteria reduce oxidized sulfur compounds to sulfide. Subsequently, sulfide can precipitate dissolved metals or can be oxidized to elemental sulfur. Both metal sulfides and elemental sulfur can be reused in various applications. SR with hydrogen or ethanol as electron donor is an established biotechnological process. However, the costs of these electron donors limit the application possibilities. Methane would be a cheaper and more attractive electron donor. SR coupled to the anaerobic oxidation of methane (AOM) occurs in marine sediments. Uncultured archaea, distantly related to methanogens, and bacteria are involved in this process. The *in vitro* demonstration of SR coupled to AOM gave rise to this research, which aims to develop a biotechnological process in which methane is used as electron donor for SR.

Three types of anaerobic granular sludge were screened for the ability to reduce sulfate with methane as electron donor. To do so, incubations were done with ^{13}C-labeled methane. All three sludge types anaerobically oxidized ^{13}C-labeled methane to ^{13}C-labeled carbon dioxide. Moreover, the presence of methane enhanced the SR rate. However, AOM by sludge was not coupled to SR, but coincides with net methanogenesis. The methane-dependent SR was caused by the inhibitory effect of methane on methanogens competing (possibly in syntrophic consortia with acetogenic bacteria) with sulfate reducers for the same endogenous substrate. Therefore, anaerobic granular sludge does not form a suitable inoculum for sulfate-reducing bioreactors fed with methane.

Well-mixed ambient-pressure submersed-membrane bioreactors, fed with sulfate and methane, were inoculated with sediment from Eckernförde Bay (Baltic Sea). Initially AOM rates were extremely low (0.004 mmol L^{-1} day^{-1}), but at 15ºC AOM and SR rates increased over the course of 884 days to 0.60 mmol L^{-1} day^{-1} or 1.0 mmol g_{VSS}^{-1} day^{-1}. The AOM rate doubled approximately every 3.8 months. Molecular analyses revealed that the archaea in the obtained enrichment belonged predominately to the anaerobic methanotroph ANME-2a. Both bacteria and archaea incorporated carbon derived from ^{13}C-labeled methane into their lipids, indicating that both were involved in AOM coupled to SR. To investigate which kind of waste streams can be treated by the methane-oxidizing sulfate-reducing enrichment, the effect of environmental conditions and alternative substrates on AOM and SR 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 ($K_m \leq 1.0$ mM), a low affinity for methane ($K_m > 75$ kPa) and AOM was completely inhibited by 2.4 (±0.1) mM sulfide. The enrichment utilized sulfate, thiosulfate and sulfite as electron acceptors for methane oxidation, and methane, formate, acetate and hydrogen as electron donors for SR.

This study shows that methane can be used as electron donor for sulfate reduction in bioreactors. However, the low growth rate of the responsible microorganisms still forms a major bottleneck for biotechnological applications.

Contents

Chapter 1

General Introduction

1.1 Carbon and sulfur cycling

1.1.1 Physical and chemical properties of methane

Methane (CH_4) is a tetrahedral shaped molecule, and a colorless, nontoxic and odorless gas (above 109°K at 1 atm.). CH_4 gas is only flammable when the concentration in the air is between 5 and 15%. It has a relatively low solubility product in water (1.44 mM in distillated water at 20ºC and 0.101 MPa CH_4) (Yamamoto *et al.*, 1967). 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). CH_4 is the simplest and most stable hydrocarbon. Compared with other alkanes, CH_4 has an unusually high C-H bond strength, making it chemical rather stable. The dissociation energy of the C-H bond in CH_4 is +439 kJ mol^{-1} (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). Therefore, methane is only a good substrate for specialized microorganisms.

CH_4 is the most reduced form of carbon (oxidation state -4), carbon dioxide (CO_2) being the most oxidized form (oxidation state +4). CH_4 is also the main component of natural gas (70-90%) 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 per kWatt during the complete oxidation of CH_4.

1.1.2 Methane production

Biogas, with CH_4 as the major reduced component, is produced during the biological degradation of organic matter when respiration is not possible. In the presence of inorganic electron acceptors like oxygen, nitrate, iron (III), manganese (IV) and sulfate, microorganisms oxidize organic compounds completely to CO_2. During these respiratory processes, microorganisms conserve energy for their metabolism. At standard conditions the reduction of oxygen is most favorable and the reduction of CO_2 to CH_4 is the least favorable. Sulfate reduction (SR) is only slightly more favorable than CO_2 reduction. Because less energy can be conserved by methanogenic processes, compared to processes in which inorganic electron acceptors are reduced, organic matter degradation will in general only result in CH_4 production after these electron accepters are depleted.

Methanogenesis occurs in marine and freshwater sediments that are rich in organic matter, in wetlands and in the intestinal tract of insects (e.g. termites). Engineered methanogenic systems, e.g. digesters, upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB) reactors, are widely applied for the treatment solid wastes and waste waters rich in organic matter. Such waste streams are produced in agriculture, households, the food and beverage industry and the paper industry (Frankin, 2001). The produced biogas is recovered and can be used as fuel (Lettinga and Haandel, 1993). Anthropogenic CH_4 emissions arise from agriculture and waste disposal, including enteric fermentation, animal and human wastes, rice paddies, biomass burning and landfills.

Anaerobic degradation of organic matter in absence of inorganic electron acceptors proceeds via a number of microbial processes; during hydrolyses, acidogenesis and acetogenesis complex organic matter is degraded to hydrogen and CO_2, formate, acetate and ammonium (Figure 1.1) (Harper and Pohland, 1986; Stams, 1994; Muyzer and Stams, 2008). The final step is methanogenesis. Methanogens are strict anaerobes and belong to the archaea. Methanogenesis proceeds via a number of unique coenzymes (Figure 1.5), which were exclusively found in methanogenic archaea (Blaut, 1994), methylotrophic bacteria (Vorholt, 2002) and anaerobic methanotrophs (Hallam, 2004). Three methanogenic pathways can be distinguished: the hydrogenotrophic pathway, in which hydrogen and CO_2, formate or carbon monoxide (Daniels *et al.*, 1977; O'Brien *et al.*, 1984) are utilized for CH_4 production; the aceticlastic pathway, in which acetate is converted to CH_4 and CO_2; and the methylotrophic pathway, in which methanol or other methylated compounds (methanethiol, dimethyl sulfide, or methylated amines) are partly oxidized and partly converted to CH_4 (Deppenmeier, 1996). Some methanogens are able to use pyruvate as carbon and energy source and some are able to utilize ethanol or isopropanol as electron donor for CO_2 reduction (Stams, 1994).

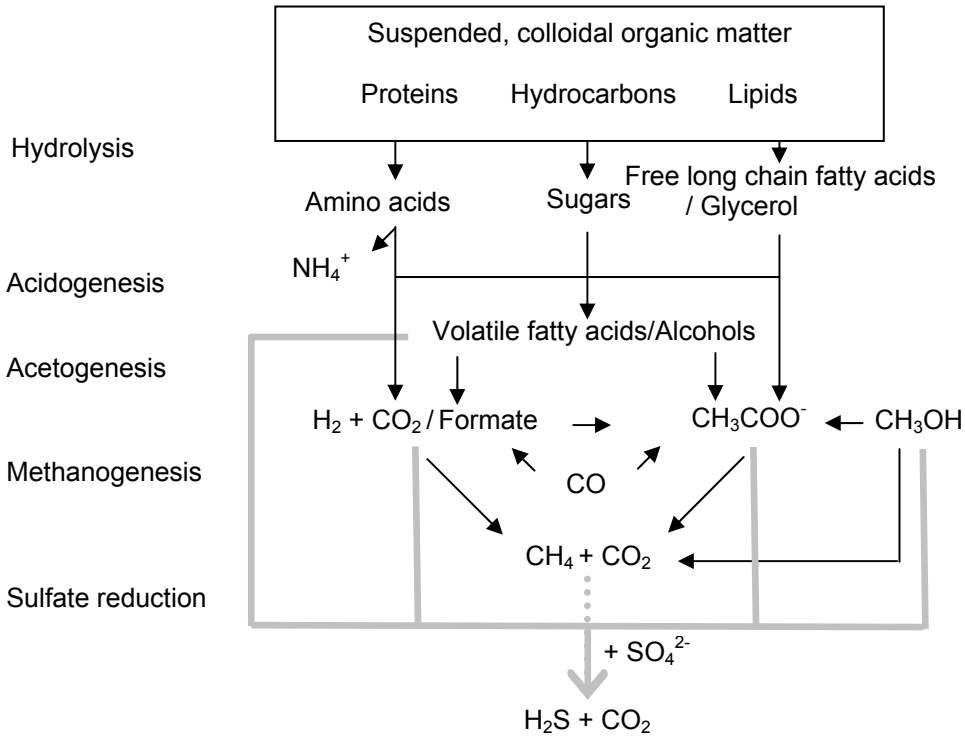

Figure 1.1. Simplified schematic representation of the anaerobic degradation process in the absence (in black) and in the presence (in grey) of sulfate.

1.1.3 Sulfate reduction

Many organisms assimilate sulfur originating from sulfate into their biomass (assimilatory SR), because sulfur is an essential element for all living organisms (Fauque, 1995). Dissimilatory sulfate reduction, on the other hand, is the reduction of sulfate to sulfide to obtain energy for growth and maintenance. This metabolic feature is exclusively done by sulfate-reducing archaea and bacteria (SRB). 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 (Muyzer and Stams, 2008). Typically SRB occur in anoxic marine and freshwater environments (Postgate, 1984). Eight electrons are needed for the reduction of one sulfate to one sulfide. The reduction equivalents are obtained by the oxidation of an organic electron donor or hydrogen. The different SRB are

able to utilize a wide range of electron donors, including hydrogen, ethanol, formate, lactate, pyruvate, fatty acids, carbon monoxide, methanol, methanethiol and sugars (Figure 1.1) (Widdel *et al.*, 2007; Muyzer and Stams, 2008). SRB have a higher affinity for hydrogen than methanogens, and therefore outcompete methanogens at low hydrogen partial pressures. It has often been observed that acetate is predominately degraded by methanogens in presence of sulfate though (Bodegom and Stams, 1999; Stams *et al.*, 2005). Acetate-degrading sulfate reducers have only slightly better growth kinetic properties than *Methanosaeta* (dominant in anaerobic sludge), therefore it may take years before aceticlastic methanogens are outcompeted by acetate-degrading sulfate reducers, when the relative cell number of the acetate-degrading sulfate reducers is initially low (Stams *et al.*, 2005).

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, in which the penetration of oxygen is limited. Sulfide produced in the anoxic compartment will be partly transported to the aerobic compartment where sulfide is oxidized to sulfate, and visa versa (Bottrell *et al.*, 2006; Holmer *et al.*, 2001). SR and sulfide oxidation form the main routes of the biological sulfur cycle (Figure 1.2).

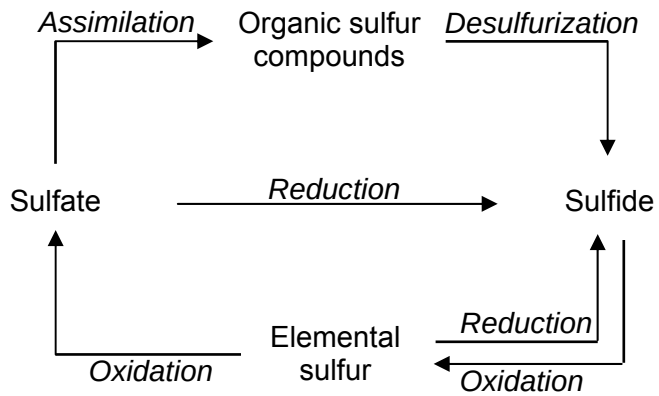

Figure 1.2. The main biological processes of the Sulfur-cycle.

1.1.4 Sources of methane in marine sediments

Seawater contains approximately 28 mM sulfate, therefore organic matter oxidation in marine sediments is for a large part coupled to 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. 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 is largest in those relative shallow waters. This CH_4 production by organic matter degradation results in a very diffuse source for CH_4.

There are also some less diffuse sites where CH_4 is passing 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 niches with large CH_4 inputs. These seeps can occur in many forms, e.g. as mud volcano's (Figure 1.3C) or brine pools. In addition to cold seeps and vents there are hydrothermal vents where mainly CH_4 is being vented (Boetius, 2005). These are different from the "black smokers", in which mainly sulfide is vented.

The CH_4 from these vents and seeps can be produced biologically, but can also be produced geochemically or thermogenically from organic matter (Sibuet and Olu, 1998). CH_4 seeps and vents occur above fossil fuel fields or gas hydrates. Gas hydrates are ice-like structures in which a gas, mostly CH_4, is incorporated (Figure 1.3A). The earth's gas hydrates contain more energy than all other known oil, natural gas and coal reservoirs combined (Kvenvolden, 1995). These hydrates are stable at low temperatures (<15°C), high pressures (>5.0 MPa) and in the presence of dissolved CH_4 (Sultan *et al.*, 2003), but the hydrates will dissociate when they come in contact with warm fluids or when dissolved CH_4 is depleted (Boetius and Suess, 2004).

1.1.5 Aerobic methane oxidation

Aerobic methanotrophs are bacteria that can use CH_4 as electron donor and carbon source (Anthony, 1982; Amaral and Knowles, 1995). Aerobic CH_4 oxidation proceeds according to reaction 1. Aerobic methanotrophs are found in samples from muds, swamps, rivers, rice paddies, oceans, ponds, soils from meadows, deciduous woods and sewage sludge (Hanson and Hanson, 1996). The methane oxidation occurs via a linear pathway, in which CH_4 is first converted to methanol by a NADH-dependent monooxygenase. 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 and Hanson, 1996).

(1) $CH_4 + 2\ O_2 \rightarrow CO_2 + 2H_2O$ $\Delta G^{\circ\prime} = -773\ kJ.mol^{-1}$

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 SR has thus far not been described, although some sulfate reducers can tolerate the presence of oxygen (Muyzer and Stams, 2008).

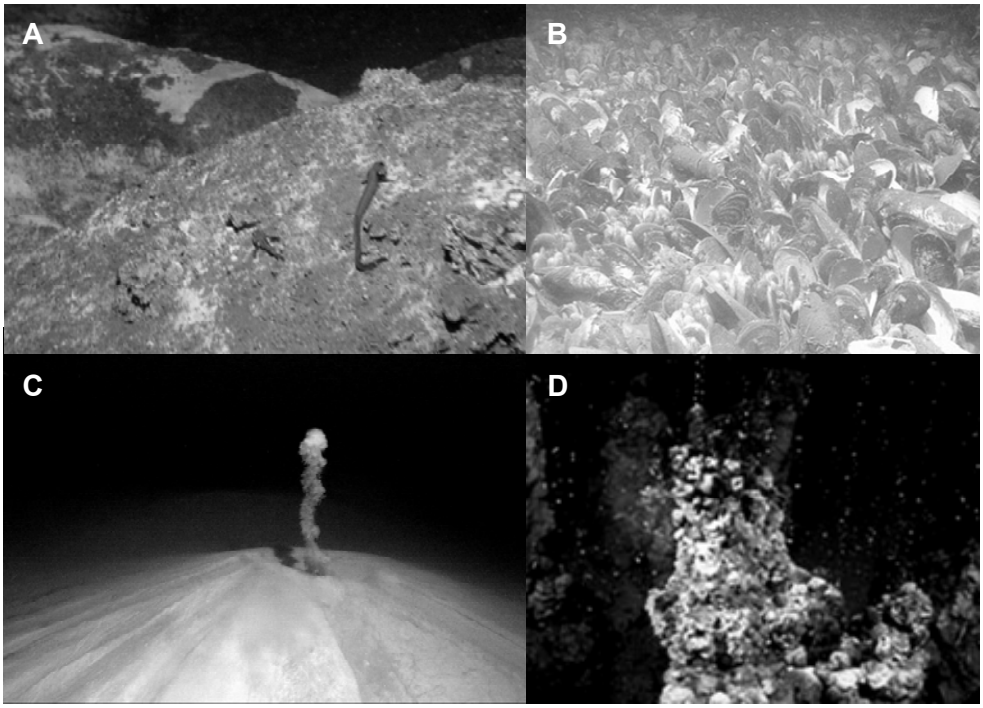

Figure 1.3. Hills of frozen hydrate and bacterial mats at Hydrate Ridge in the Pacific Ocean (A; Yancey, 2008), a chemosynthetic community on a cold seep at Black Ridge in the Atlantic Ocean (B; Ocean Explorer, 2008), a mud volcano in the golf of Mexico (C; National Marine Sanctuary, 2008) and a chimney-like reef structure in the Black Sea (D; Michaelis *et al.*, 2002).

1.1.6 Anaerobic oxidation of methane

For many years anaerobic oxidation of methane (AOM) by microbes was thought to be impossible (Thauer and Shima, 2008), until in the seventies of the last century AOM was discovered during geochemical *in situ* studies in anaerobic marine sediments and waters (Martens and Berner, 1974; Barnes and Goldberg, 1976; Reeburgh, 1976). CH_4 diffusing upwards from deeper sediment layers was oxidized before reaching oxic zones. The consumption of CH_4 was coupled to the consumption of sulfate, diffusing downward from the seafloor (Figure 1.4; Martens and Berner, 1974; 1977; Barnes and Goldberg, 1976; Reeburgh, 1976; Alperin and Reeburgh, 1985). Where CH_4 and sulfate meet (the sulfate to methane transition zone), radioisotope tracer experiments with ^{14}C-labeled CH_4 and ^{35}S-labeled sulfate, showed a maximum AOM and SR rate (Reeburgh, 1980; Iversen and Blackburn, 1981; Iversen and Jørgensen, 1985; Iversen *et al.*, 1987; Alperin, 1989; Reeburgh *et al.*, 1991; Joye *et al.*, 1999). In addition, at the sulfate to methane transition zone shifts in the isotopic composition (^{13}C and ^{12}C content) of CH_4, which was heavier above the transition zone, and inorganic carbon, which was lighter above the transition zone, were found (Oremland and Des Marais, 1983; Whiticar *et al.* 1986; Oremland *et al.*, 1987; Alperin *et al.*, 1988; Blair and Aller, 1995; Martens *et al.*, 1999). These studies showed a stoichiometry according to reaction 2.

(2) $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ $\Delta G^{\circ\prime} = -16.6\ kJ.mol^{-1}$

The bicarbonate and alkalinity production by AOM has resulted in the formation of chimney-like structures from calcium carbonate above CH_4 vents (Figure 1.3D) (Michaelis *et al.*, 2002; Stadnitskaia *et al.*, 2005). These CH_4 seeps or vents can also drive chemotropic ecosystems (Figure 1.3B). The sulfide produced by AOM is, at least partly, transported upwards and aerobically oxidized to sulfur or sulfate, e.g. in tube worms or in microbial mats of *Beggiatoa*.

Table 1.1 lists most of the AOM sites that have been studied. The AOM rate depends on a variety of conditions including the organic content of the sediment, CH_4 supply rate, sulfate penetration in the sediment, temperature and pressure (Valentine, 2002). Because of the higher supply rates, the AOM rates at CH_4 seeps and vents are higher than in sediments where CH_4 is just supplied by organic matter degradation.

AOM has also been observed in non-marine environments. Iversen *et al.* (1987), Panganiban *et al.* (1979) and Eller *et al.* (2005) observed AOM in lakes and Grossman *et al.*

(2002) in a landfill. In these cases AOM was probably coupled to SR. Islas-Lima *et al.* (2004) demonstrated for the first time denitrification with CH_4 as electron donor in absence of oxygen. Raghoebarsing *et al.* (2006) demonstrated AOM coupled to nitrite and nitrate reduction by freshwater sediment from Twente kanaal (the Netherlands), this AOM process is mediated by bacteria via a completely other pathway than AOM coupled to SR (Ettwig *et al.*, 2008; Thauer and Shima, 2008). From AOM coupled to nitrate or nitrite reduction more energy can be conserved than from AOM coupled SR. The same would be true for AOM coupled to iron (III) or manganese (IV) reduction, but thus far these processes have not been discovered.

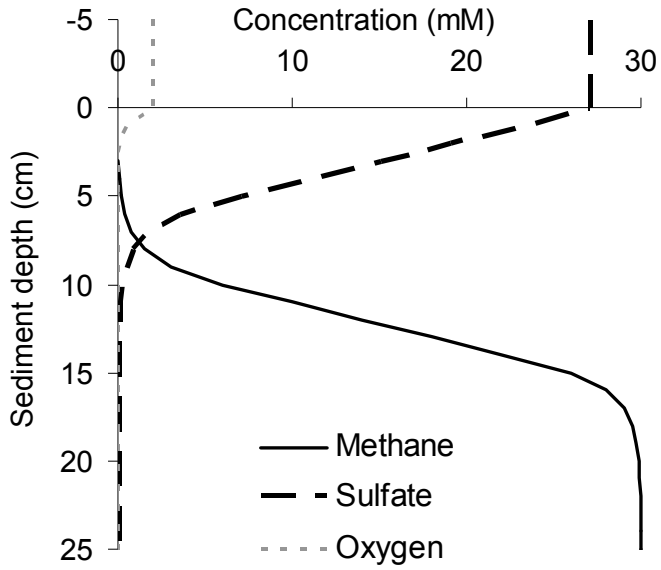

Figure 1.4. Typical CH_4, sulfate and oxygen concentration profiles in deep-sea AOM sediments where no convection takes place.

1.1.7 Relevance of the anaerobic oxidation of methane for global warming

Estimates of the current human-activity-related CH_4 emissions range from 340 to 420 Tg CH_4 $year^{-1}$, while the total natural terrestrial sources are estimated to be between 160 and 270 Tg CH_4 $year^{-1}$ (Khalil *et al.*, 2000; Lelieveld *et al.*, 1998; Houweling *et al.*, 1999). The annually CH_4 production in anoxic marine sediments is probably more than 85 Tg (Hinrichs and Boetius, 2002). CH_4 is after CO_2 the most important greenhouse gas, responsible for 20% of the infrared radiation trapping in the atmosphere (Mackenzie, 1998). The lifetime of CH_4 in the atmosphere is shorter than that of CO_2, but the strong global warming effect is due to the fact that a relative high fraction of the CH_4 occurs in the troposphere. Atmospheric CH_4 is mainly oxidized in the troposphere, by the reaction with a hydroxyl radical (OH·), this accounts for a removal of 445-530 Tg CH_4 per year. Just 40 Tg CH_4 $year^{-1}$ is transported to the stratosphere. In aerated soils, about 30 Tg CH_4 is annually oxidized by aerobic methanotrophs (Khalil *et al.*, 2000; Lelieveld *et al.*, 1998; Houweling *et al.*, 1999). Initial AOM was estimated to be responsible for 75 Tg CH_4 removal per year (Reeburgh, 1996). Later estimates suggested that 300 Tg CH_4 was annually removed by AOM (Hinrichs and Boetius, 2002), which would make AOM the second most important process for removal of the greenhouse gas CH_4.

Table 1.1. Overview of AMO sites and rates reported in the literature.

Location	Depth (m)	CH_4 source	AOM (µmol g_{dw}^{-1} day^{-1}) radiotracers	*in vitro*	Reference
Eckernförde Bay, Baltic Sea	28	Organic matter decomposition	0.03-0.06	0.1-0.3	Treude *et al.*, 2005a
Kattegat, Baltic Sea	0.5	Organic matter decomposition	0.05-0.2	0.05-1	Krüger *et al.*, 2005
Spiekeroog, North Sea	0-5	Organic matter decomposition	n.d.	0.01-0.2	Krüger *et al.*, 2005
Aarhus Bay, Denmark	16	Organic matter decomposition	n.d.	n.d.	Thomsen *et al.*, 2001
Black Sea	250	Fossil methane	0.2-7.5 8-21	0.5-3.5	Krüger *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	400-3000	Mud Volcano	n.d.	n.d.	Niemann *et al.*, 2006 Stadnitskaia *et al.*, 2006
Namibiaan 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	800-1000	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

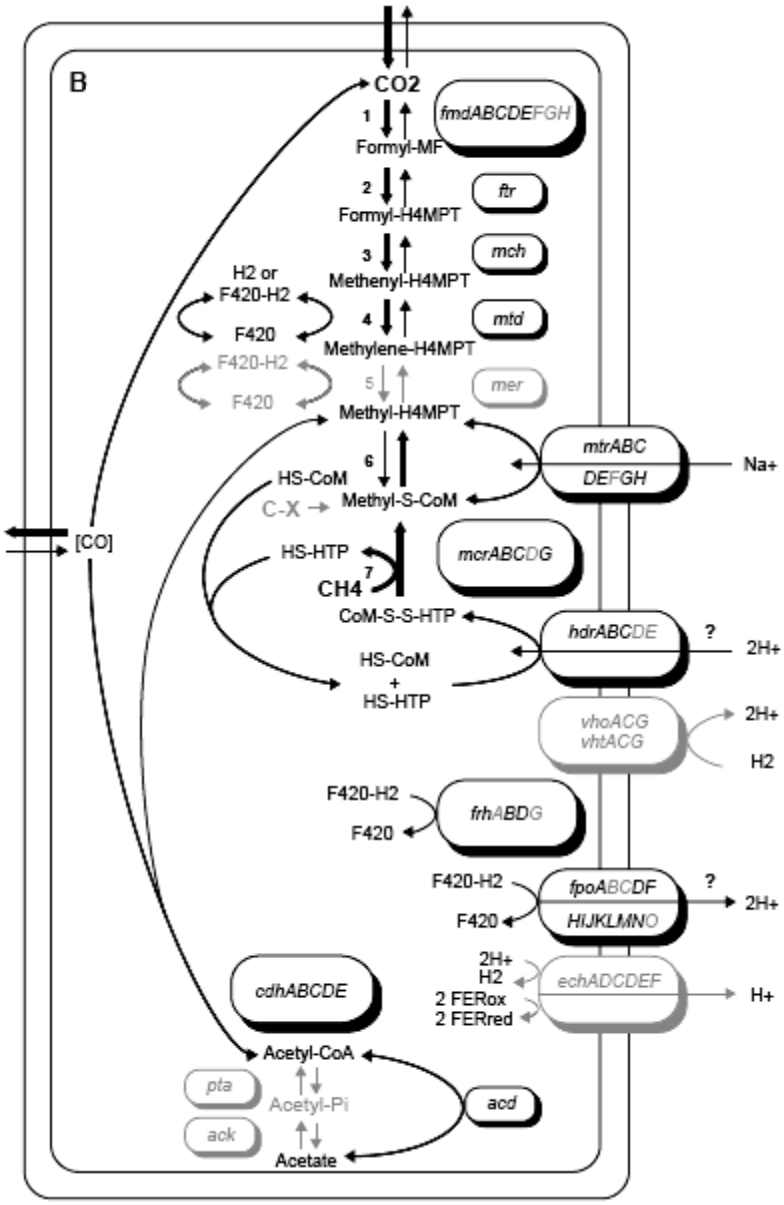

Figure 1.5. The combined hydrogenotrophic and aceticlastic pathway for methanogenesis, with genes indentified in methanogens. The black colored genes have also been indentified in ANME-1, the grey colored genes not. (Hallam *et al.*, 2004).

1.2 Microbial aspects of anaerobic oxidation of methane coupled to sulfate reduction

1.2.1 Anaerobic methanotrophs

In contrast to aerobic CH_4 oxidation, the biochemistry of AOM coupled to SR is not completely understood. AOM is mediated by uncultured Archaea, called anaerobic methanotrophs (ANME). Specific archaeal lipids (biomarkers), from *in situ* samples, are highly depleted in ^{13}C (Elvert *et al.*, 1999; 2001; Hinrichs *et al.*, 1999; 2000; Thiel *et al.*, 1999; 2001; Pancost *et al*; 2000). This is evidence that the isotopically light CH_4 (biologically produced CH_4 is depleted in ^{13}C) was the preferred carbon source for these microorganisms rather than other "heavier" carbon sources. Phylogenetic analysis of AOM sediments identified three novel groups of archaea, putatively called ANME-1, ANME-2 and ANME-3. ANME-1 and ANME-2 are most abundant and geographically widespread. 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). Orphan *et al.*, (2001a, 2002) combined isotopic and phylogenetic analysis and showed that cells belonging to ANME-1 and ANME-2 assimilated carbon from CH_4 during AOM.

1.2.2 Reversed methanogenesis

AOM is a form of reversed methanogenesis: AOM is like methanogenesis inhibited by bromoethanesulfonate (BES) (Nauhaus *et al.*, 2005), ANME-1 cells were found to contain most of the genes typically associated with CH_4 production (Figure 1.5; Hallam *et al.*, 2003; 2004) and an analogue of the methyl-coenzyme M reductase was found to make up 7% of the extracted soluble proteins from an AOM mediating microbial mat from the Black Sea (Krüger *et al.*, 2003). The $\Delta G^{\circ\prime}$ of the reduction of methyl-coenzyme M to produce CH_4 is -30 (±10) kJ mol^{-1}, the back reaction becomes exogenic when the product to substrate concentration ratio is approximately 10^5, such a ratio is physiologically not unrealistic (Thauer and Shima, 2008). In addition, pure cultures of methanogenic archaea also oxidize CH_4 to CO_2 in the absence of oxygen, but in low amounts and during net methanogenesis (Zehnder and Brock, 1979; Harder, 1997; Moran *et al.*, 2004; Moran *et al.*, 2007a). SRB did not show any CH_4 oxidation during SR (Harder, 1997).

Thus far, there is no direct evidence that ANME are capable of methanogenesis. However, AOM and CH_4 production occur simultaneously in microbial mats from the Black Sea (Seifert

et al., 2006), in sediments from Cape Lookout Bight (North Carolina; Hoehler *et al.*, 1994) and in sediments from the Golf of Mexico (Orcutt *et al.*, 2005). CH_4 production by Hydrate Ridge sediment on hydrogen, formate, acetate and methanol, in absence of CH_4, was an order of a magnitude lower than the AOM rate though (Nauhaus *et al.*, 2002), and microbial mats from the Black Sea did not show any CH_4 production in presence of hydrogen and absence of sulfate (Treude *et al.*, 2007). In addition, growth of ANME on solely methanogenic substrates has not been reported.

1.2.3 SRB associated with AOM

Some archaea (belonging to the Euryarchaeota or Crenarchaeota) are capable of SR (Muyzer and Stams, 2008). However, in the archaea belonging to the ANME groups, no gene analogues for enzymes involved in SR were found (Thauer and Shima, 2008). In addition, methyl-coenzyme M reductase was shown to be inhibited by sulfite, an intercellular intermediate of SR (Mahlert *et al.*, 2002). Therefore, it is unlikely that AOM and SR take place in the same cell (Shima and Thauer, 2005). At AOM sites, ANME co-occur with SRB belonging taxonomically to the delta group of proteobacteria and associated with the *Desulfosarcina*/*Desulfococcus* cluster (Boetius *et al*., 2000; Orphan *et al*., 2001b; Michaelis *et al*., 2002; Elvert *et al.*, 2003; Knittel *et al*., 2003). During incubations of AOM sediment with ^{13}C-labeled CH_4, ^{13}C was incorporated both in archaeal lipids associated with ANME and bacterial lipids associated with 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). It has frequently been suggested that an archaeon produces an electron carrier compound from CH_4 that is utilized by a sulfate-reducing partner (Zehnder and Brock, 1980; Alperin and Reeburgh, 1985; Hoehler *et al.*, 1994 and DeLong, 2000). In sediment from Hydrate Ridge, Eel River Basin and the Golf of Mexico, ANME-2 and SRB live in consortia with a diameter of up to circa 20 µm (Figure 1.6) (Boetius *et al.*, 2000; Hinrichs *et al.*, 2000; Knittel *et al.*, 2005). Moreover, both microorganisms were growing in consortia with CH_4 and sulfate as sole substrates (Nauhaus *et al.*, 2007), confirming the involvement of the SRB in AOM coupled to SR.

These ANME/SRB aggregates are not dominant in all AOM sites though. In Black sea microbial mats, SRB mainly occur in microcolonies surrounded by bulk ANME-1 cells clusters (Michaelis *et al.*, 2002; Knittel *et al.*, 2005). The distances between ANME and SRB in those microbial mats are larger than in the consortia from Hydrate Ridge (Figure 1.6). In samples from Eel River Basin ANME-1 archaeal group frequently existed in monospecific

aggregates or as single filaments, apparently without a bacterial partner (Orphan *et al.*, 2002). In Eckernförde Bay sediment, clusters of ANME-2a cells were found without sulfate-reducing partners (Treude *et al.*, 2005a).

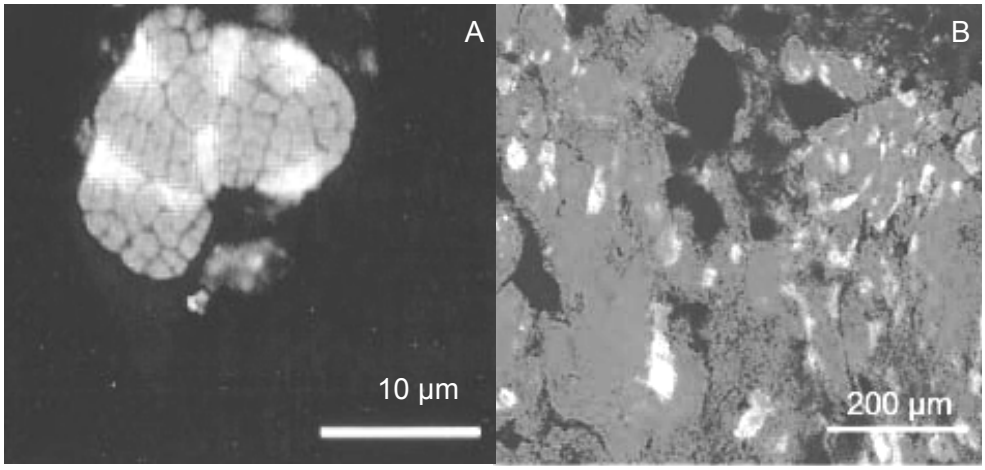

Figure 1.6. An ANME-2/SRB aggregate from Hydrate Ridge sediment (A; Knittel *et al*., 2005) and microcolonies of SRB are surrounded by bulk ANME-I in a microbial mat from the Black Sea (B; Michaelis *et al*., 2002), visualized with fluorescently labeled rRNA-targeted oligonucleotide probes. The grey microorganisms are ANME and the white cells are SRB.

1.2.4 Possible syntrophic routes

Given the evidence for reversed methanogenesis, hydrogen (reactions 3 and 4) and acetate (reactions 5 and 6) were initially proposed to act as interspecies electron carrier (IEC) (Hoehler, 1994; DeLong, 2000). The standard Gibbs free energy change at pH 7 ($\Delta G°'$) of the production of these IECs from CH_4 is positive, however when the IEC concentration is kept low enough by the sulfate-reducing partner, the ΔG will be negative.

(3) $CH_4 + 3H_2O \rightarrow 4H_2 + HCO_3^- + H^+$ $\Delta G°' = +136$ kJ mol^{-1}

(4) $4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^-$ $\Delta G°' = -152$ kJ mol^{-1}

(5) $CH_4 + HCO_3^- \rightarrow CH_3COO^- + H_2O$ $\Delta G°' = +31$ kJ mol^{-1}

(6) $CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$ $\Delta G°' = -47$ kJ. mol^{-1}

There are some thermodynamic concerns about this theory though. At *in situ* conditions there is only -22 kJ mol^{-1} available for AOM coupled to SR (Harder, 1997). This energy would need to be shared between the syntrophic partners. Methanogenic archaea have been shown to require a free energy change of at least -10 kJ mol^{-1} and SRB of at least -19 kJ mol^{-1} to support their metabolism *in situ* (Hoehler *et al.*, 2001; Dale *et al.* 2006). The *in situ* free energy change is therefore probably not sufficiently large to fuel the energy metabolism of two microorganisms (Schink, 1997; Thauer and Shima, 2008). Moreover, for diffusive transport between the syntrophic partners a concentration gradient is needed. Therefore the IEC concentration near the SRB will be lower than the concentration near the ANME and the actual available energy for the microorganisms will be even lower. The bigger the distance between the syntrophic partners the greater the loss (Sørensen *et al.*, 2001). Thermodynamic calculations excluded hydrogen, acetate and methanol as IEC, because the maximum diffusion distances of those compounds at *in situ* concentrations and rates were smaller than the thickness of two prokaryotic cell walls (Sørensen *et al.*, 2001). Also activity assays provided evidence against potential IECs; SR activity of Hydrate Ridge sediment with hydrogen, formate or acetate was lower than SR activity on CH_4, indicating that SRB involved in AOM, were not adapted to these substrates (Nauhaus *et al.*, 2002; 2005). It therefore remains unclear if and how reducing equivalents are transferred from the ANME to a sulfate-reducing partner.

1.2.5 *In vitro* studies

Nauhaus *et al.* (2002) demonstrated *in vitro* AOM coupled to SR by Hydrate Ridge sediment. *In vitro* studies of AOM coupled to SR have mostly been done with non-enriched sediment or microbial mats, because the responsible microorganisms are extremely difficult to cultivate. Thus far, the microorganisms responsible for AOM coupled to SR have not been isolated, nor has any isolated organism been shown to be capable of net AOM.

The SR rates of Hydrate Ridge sediment, Black Sea microbial mats and Eckernförde Bay sediment were highest between 5 and 16°C, 16 and 24°C, and 20 and 28°C, respectively (Nauhaus *et al.*, 2005; Treude *et al.*, 2005a). Other findings of Nauhaus *et al.* (2005) were that CH_4 driven SR was positively affected by the CH_4 partial pressures and that besides sulfate no other electron acceptor (nitrate, Mn (IV), Fe (III), S° and fumarate) was used for AOM. Estimates of the doubling time of the microorganisms mediating AOM coupled to SR vary from 1 to 7 months (Girguis *et al.*, 2005; Nauhaus *et al.*, 2007; Krüger *et al.*, 2008).

1.3 The potential of methane as electron donor for sulfate reduction in biotechnological applications

1.3.1 Environmental problems related with the sulfur cycle

Sulfur compounds are cycled between the earth's soils, oceans, atmosphere and living matter in the so-called "natural sulfur cycle". However, due to human activities the emissions of sulfur compounds to surface waters and the atmosphere have increased largely. The earth's crust contains large amounts of immobilized sulfides. During mining and processing of ores and fossil fuels, sulfide minerals are oxidized and have been emitted to the surface waters, soils and the atmosphere. This has caused major environmental problems like the acidification of surface waters, the mobilization of heavy metals, the increasing salinity of freshwaters and the production of toxic sulfide in anaerobic soils (Morin *et al*. 2006).

Here three important sources of anthropogenic sulfur emissions are distinguished. The first are waste streams of the mining and metallurgical industry. During the mining of metal ores, minerals like pyrite are biologically oxidized (Johnson, 2000), resulting in the production of sulfuric acid and the mobilization of heavy metals. Heavy metals are toxic for humans and have a devastating effect on ecosystems. This mining wastewater is called acid mine drainage. Also during the processing of these minerals at metallurgical plants, waste streams with sulfuric acid, sulfur dioxide and residual metals are produced. The second source of sulfurous emissions is the combustion of fossil fuels. Fossil fuels (like coal, oil and gas) contain hydrogen sulfide or organic S-compounds; their combustion results in the emission of sulfur dioxide, a major compound in the acid rain formation. Therefore, sulfur dioxide has to be removed from the off-gas (flue gas desulfurization) or sulfur compounds have to be removed from fuels prior to combustion, both processes result in the generation of a waste stream containing the sulfur compounds. A third source are wastewaters contaminated with oxidized sulfur compounds (sulfate, sulfite and thiosulfate) that are produced in industries that use sulfuric acid or sulfate-rich feedstock, e.g. tannery, pulp and paper, textiles, fermentation and the sea food processing industry (Lens *et al.*, 1998), 136 Tg sulfuric acid is annually used in the industry (Kirk-Othmer, 2000).

1.3.2 Biological sulfate reduction as a treatment technique

SR in anaerobic bioreactors treating organic wastes has long been regarded as an unwanted side process due to the loss of electron donor and inhibition of the methanogenic process by sulfide (Colleran *et al.*, 1995; Oude Elferink *et al.*, 1994). Currently, biological SR

is an established biotechnological process for the treatment of inorganic waste streams containing sulfur compounds and/or metals (Weijma *et al.*, 2002; Lens *et al.*, 2002). Oxidized sulfur compounds can be converted to elemental sulfur by applying subsequently SR and partial sulfide oxidation (Janssen *et al.*, 1999; van den Bosch, 2008). The insoluble sulfur can be recovered by means of a settler and is a safe, storable and reusable product. The hydrophilic nature of biologically produced sulfur makes it an ideal soil fertilizer, in addition, sulfur can be used to produce sulfuric acid (van den Bosch, 2008). Dissolved metals can be removed by precipitation with biologically produced sulfide, the formed insoluble metal sulfides can be separated from the water phase in a settler and reused in the metallurgical industry (Huisman *et al.*, 2006; Veeken *et al.*, 2003). These biological treatment techniques allow the recovery of sulfur and metals; they can be used for the treatment of AMD, groundwater leachate, industrial wastewaters and industrial waste gases (containing SO_2 or H_2S). In addition, SR can be applied *in situ*, in order to immobilize metals as metal sulfides in soils and sediments.

Biological SR forms a relative new alternative to remove sulfate from liquid streams for the widely-applied chemical precipitation, in which sodium sulfate or gypsum is produced. Gypsum can be reused as construction material. However, the sulfate containing waste streams from the mining and metallurgical industry are polluted with metals, the produced gypsum will therefore be polluted as well and needs to be stored as chemical waste. For chemical precipitation, large amounts of chemicals are needed, per kg sulfate about 0.8 kg slaked lime is needed. During slaked lime production from limestone CO_2 is released, additional to the CO_2 produced related to the energy consumption of the process (the process requires a temperature of 900ºC). Because of a lower CO_2 emission and the production of a reusable product, biological treatment of wastewaters containing sulfate and metals is more sustainable than treatment by chemical precipitation.

1.3.3 Electron donors for sulfate reduction

An electron donor is required for SR. A wide range of electron donors can be used by sulfate reducers, including alcohols, fatty acids, hydrogen and carbon monoxide (Widdel *et al*., 2007; Muyzer and Stams, 2008). In syntrophic mixed-cultures, complex organic matter can also fuel SR. The costs of the electron donor forms a major part of the running cost of a SR process and therefore limit the application of biological SR as it cannot always economically compete with chemical precipitation. Cheap electron donors like organic waste streams are

not easily degradable and often contain some inert material, which would need to be removed by pre or post treatment. In addition, undesired byproducts can be formed and the quantity and quality of these waste streams is not constant. Fully degradable electron donors are therefore a better option. Such electron donors include hydrogen, synthesis gas, methanol, ethanol, acetate, lactate, propionate, butyrate, sugar, and molasses (Liamleam and Annachhatre, 2007), many of which have been extensively investigated as electron donor for SR in bioreactors (Table 1.2). According to van Houten (1996) hydrogen is the best electron donor at large scale (>5-10 kmol SO_4^{2-} h^{-1}), while ethanol is an interesting electron donor at smaller and middle scale.

1.3.3.1 Hydrogen

Two advantages of gaseous electron donors are that the wastewater is not diluted and that the electron donor can not wash-out with the effluent. A disadvantage of gaseous electron donors is that they are voluminous and therefore need to be compressed during transportation. High rate SR with H_2 as electron donor and carbon dioxide (CO_2) as carbon source has been demonstrated at both mesophilic and thermophilic conditions (Table 1.2). A maximum SR rate of 30 g $SO_4^{2-}.L^{-1}.day^{-1}$ was reached. Van Houten (2006) showed that in a H_2 and CO_2 fed gas-lift bioreactor, SRB do not take CO_2 as sole carbon source, instead they depend on the acetate produced by homoacetogens. Hydrogenotrophic methanogens compete with SRB for the available H_2, using CO_2 as terminal electron acceptor. In a well-mixed stable-performing bioreactor, the consortium of hetrotrophic SRB and homoacetogens outcompetes methanogens, because of a higher affinity for H_2. At elevated H_2 concentrations (e.g. during startup, in poorly mixed systems or after a disturbance) methanogens are able to grow, resulting in a loss of electron donor due to methanogenesis (van Houten *et al.*, 2006).

Hydrogen is commonly produced by steam reforming from natural gas or by gasification of oil or coal (Armor, 1999; Bartisch *et al.*, 1978). Steam reforming takes place at high temperatures (750-800°C) and pressures (0.3-2.5 MPa) in the presence of a nickel-based catalyst, the efficiency ranges from 60% to 80%. The gas produced by steam reforming or gasification (synthesis gas) contains, besides hydrogen, between 6 and 60% carbon monoxide (CO) (Bartisch *et al.*, 1978). CO can be removed via the so called water-gas-shift reaction, in which CO and water react over a chemical catalyst at 360°C to form carbon dioxide and hydrogen. To limit methanogenic and homoacetogenic activity the carbon dioxide can subsequently be removed from the gas (e.g. using an alkaline scrubber). More

sustainable ways to produce hydrogen are emerging, e.g. gasification of organic waste or biomass (van der Drift *et al.*, 2001), electrolysis using "green" electricity, hydrogenogenic phototrophic microorganisms (Hoekema *et al.*, 2002), dark fermentation (Nath *et al.*, 2004) and biocatalyzed electrolyses in a fuel cell (Rozendal *et al.*, 2006).

Table 1.2. Effect of the electron donor, pH, temperature and reactor concept on the volumetric sulfate-reducing activity.

e-donor	pH	Temp (^oC)	Reactor concept	Volumetric activity (gSO_4^{2-} L^{-1} day^{-1})	Reference
Hydrogen	8.0	30	GLB	25	van Houten, 2006
Hydrogen	7.0	30	GLB	30	van Houten *et al*., 1994
Hydrogen	7.0	55	GLB	8	van Houten *et al*., 1997
Hydrogen	6.0	30	GLB	13	van Houten *et al*., 1995a
Synthesis gas	7.0	30	GLB	7[a]	van Houten *et al*., 1995b
Synthesis gas	-[b]	35	Anaerobic packet bed reactor	1.2	du Preez *et al*., 1994
CO	-[b]	35	Anaerobic packet bed reactor	2.4	du Preez *et al*., 1994
CO	6.9	50-55	GLB	0.2	Sipma *et al*., 2007
Formate	6.0	30	MBR	29	Bijmans *et al*., 2008
Methanol	7.5	65	EGSB	15	Weijma *et al*., 2000
Ethanol	8	35	FBR	5	Kaksonen *et al*., 2004
Ethanol	7	8	FBR	0.6	Sahinkaya *et al*., 2007
Ethanol	7.2	33	MBR	0.6[c]	Vallero *et al*., 2005
Acetate	8	35	Fixed bed bioreactor	65	Stucki *et al*., 1993
Acetate	8	33	EGSB	10	Dries *et al*., 1998

[a] 80% H_2 and 20% CO. [b] uncontrolled and varying. [c] bioreactor operated at 50 g l^{-1} NaCl

1.3.3.2 Synthesis gas

The chemical water-gas-shift reaction has two disadvantages. Firstly, the chemical catalysts become polluted by hydrogen sulfide which is also present in synthesis gas and secondly, energy is needed to reach the required temperature. Alternatively the untreated synthesis gas, including the CO, could be fed to the SR bioreactor. Van Houten (1995b) found that the

SR rate dropped from 12-14 g SO_4^{2-} L^{-1} day^{-1} to 6-8 g SO_4^{2-} L^{-1} day^{-1} when adding 5% CO to the H_2/CO_2 feed gas. Increasing the percentage CO to 20% did not further deteriorate the SR rate. Sipma *et al*. (2004) showed that some SRB were able to tolerate up to 100% CO. At thermophilic conditions, the responsible microorganisms could convert CO and H_2O to H_2 and CO_2 and simultaneously use the H_2 for SR. Although CO is inhibitory for methanogenesis, methanogens could only be eliminated at a short hydraulic retention time (3 hours) in a synthesis gas fed gas-lift bioreactor (Sipma *et al*., 2007).

1.3.3.3 Methane

Another alternative would be the use of natural gas or biogas directly as electron donor for biological SR. This would have three advantages. Firstly, the steam reforming and the carbon monoxide removal are avoided. These processes contribute to the additional costs of hydrogen over CH_4 (Table 1.3). The chemical catalysts used for steam reforming and the water-gas shift are easily polluted by hydrogen sulfide, present in the natural gas or biogas. Sulfide forms no problem when the CH_4 containing gas would be fed directly to the bioreactor. Secondly, energy needed for the transfer of the gas to the liquid can be saved. Four times less gas needs to be transferred from the gas to the liquid phase, as one CH_4 can donate eight electrons, and one hydrogen only two. In addition, the solubility of CH_4 (1.44 mM in distillated water at 0.101 MPa CH_4 and 20ºC) is higher than of hydrogen (0.817 mM at 0.101 MPa hydrogen and 20ºC). The volumetric conversion rates in bioreactors fed with a gaseous substrate are, in general, limited by the transfer of the gas to the liquid phase.

A third advantage is that substrate losses due to unwanted methanogenesis and acetogenesis (from hydrogen and CO_2) can be avoided, only microorganisms involved in AOM coupled to SR are able to grow in a methane-fed sulfate-reducing bioreactor.

Table 1.3. Prices and costs of electron donors for sulfate reduction.

Electron donor	Industrial market price (January 2008)	Required amount per kg sulfate reduced	Electron donor cost [$\$.kg_{sulfate}^{-1}$]
Ethanol	0.60 $\$.L^{-1}$ [a, b]	0.40 L	0.24
Hydrogen [c]	0.21 $\$.m^{-3}$ [d]	0.934 m^3	0.20
Natural gas [e]	0.24 $\$.m^{-3}$ [f]	0.292 m^3	0.07

[a] Ethanol Market, 2008; [b] California Energy Commission, 2008; [c] produced from natural gas; [d] Mueller-Langet *et al.*, 2007; [e] 80% CH_4; [f] Energy Information Administration, 2008

1.3.4 Reactor type

The gas-lift bioreactor (GLB) is the most common bioreactor type for SR with gaseous electron donors. In this system the transfer of gas to the liquid is optimized. A GLB is usually equipped with a three-phase separator (Esposito *et al.*, 2003; van Houten *et al.*, 1994; Weijma *et al.*, 2002) or an external settler (Sipma *et al.*, 2007) to retain the biomass in the system. GLBs can be operated with (van Houten *et al.*, 1994) or without (Sipma *et al.*, 2007) carrier material like pumice and basalt. Metal-sulfides produced in gas-lift bioreactors can also act as carrier material for the microorganisms.

Membrane bioreactors (MBRs) are relatively new in the field of SR. The advantage is that almost complete biomass retention can be obtained, which is especially useful when slow-growing microorganisms are used. MBRs have been applied in research on SR under high saline conditions (Vallero *et al.*, 2005) and SR at low pH (Bijmans *et al.*, 2007a).

1.3.5 The Nyrstar process

At the Nyrstar zinc refinery in Budel (the Netherlands), SR is applied to separate and recover sulfuric acid and zinc from waste streams that also contain other dissolved compounds, e.g. Mg^{2+} and Cl^-. The waste streams are treated in a single-stage hydrogen-fed 500 m^3 GLB. In the GLB, SR and zinc-sulfide precipitation take place (Boonstra *et al.*, 1999; Weijma *et al.*, 2002). The sulfate concentration is reduced from 5-15 g L^{-1} to 0.05 g L^{-1}, while the zinc concentration is reduced to less than 0.3 mg.L^{-1}, recovering about 8.5 tons of zinc-sulfide per day (Boonstra *et al.*, 1999; Weijma *et al.*, 2002). The recovered zinc-sulfide can be directly reused in the zinc smelter. At the Nyrstar zinc refinery, hydrogen produced by steam CH_4 reforming is used as electron donor for biological SR. The relative small steam reformer has a low efficiency, 1.88 mol CH_4 is needed to reduce 1 mol sulfate.

Table 1.4 compares the current SR process at Nyrstar (Figure 1.7A) with the theoretical process if CH_4 would be used as electron donor for biological SR (Figure 1.7B). From the stoichiometry of AOM coupled to SR, a consumption of one mol methane per mol sulfate can be expected. Because less methane is needed and less energy is needed for gas recirculation, the carbon dioxide emission of the process in which methane is used directly is expected to be half of the current CO_2 emission.

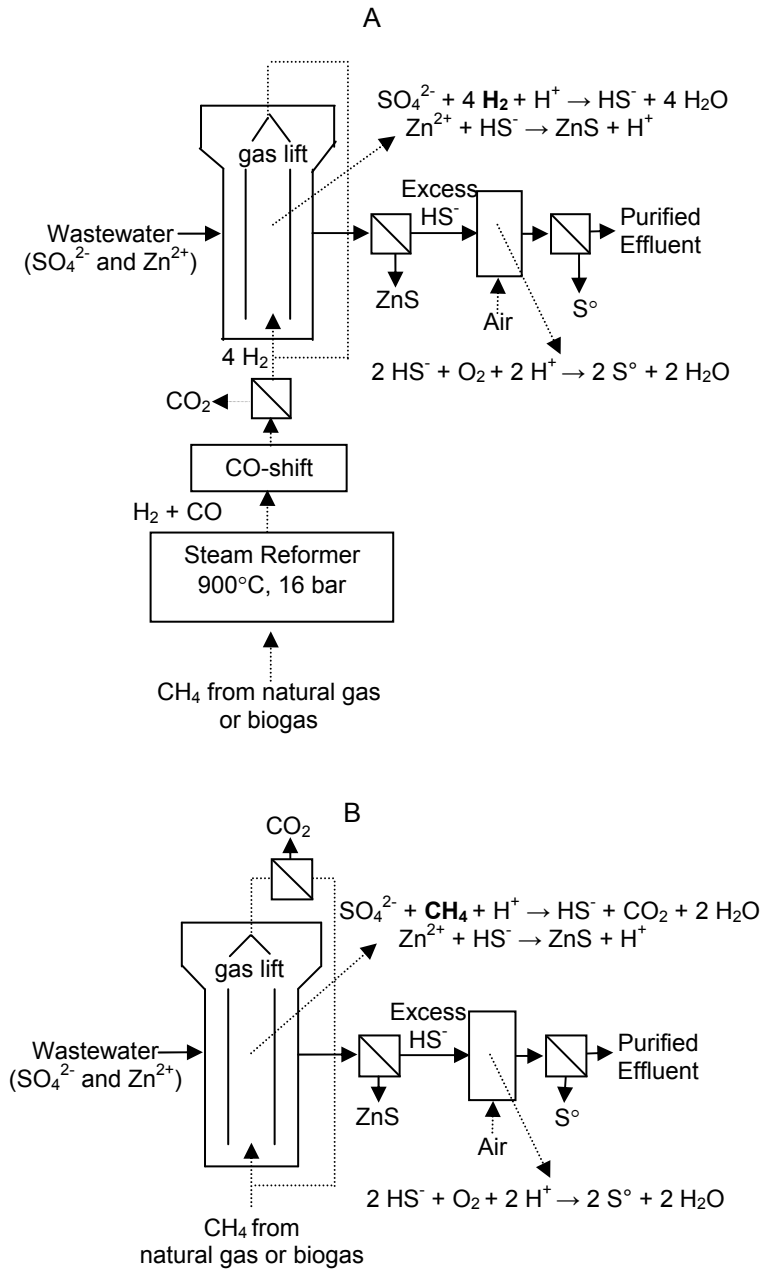

Figure 1.7. Simplified schematic representation of the current wastewater treatment process at the zinc factory of Nyrstar in Budel (the Netherlands). (A). The wastewater treatment process when CH_4 would be used as direct electron donor (B).

Table 1.4. Basic parameters of the current wastewater treatment process at the zinc refinery of Nyrstar and of the wastewater treatment process when CH_4 would be used directly as electron donor for biological SR.

	SR with CH_4 via H_2 production plant	SR with CH_4 directly
	Three step process	One step process
Temperature required	900°C	Wastewater temperature (5-70°C)
Pressure required	1.6 Mpa (16 bar)	0.1 Mpa (1 bar)
CH_4 required	1.88 mol per mol SO_4^{2-}	1 mol per mol SO_4^{2-}
CO_2 emission	0.9 ton per ton SO_4^{2-}	0.45 ton per ton SO_4^{2-}

1.3.6 Challenges

Research described in this thesis aims to investigate the possibilities for biotechnological applications of the AOM coupled to SR. AOM coupled to SR has been extensively studied, however only a few *in vitro* studies have been described. Moreover, AOM has not been studied at all in mixed bioreactors or at conditions typical for bioreactors, e.g. high shear rate, mesophilic temperature and a relative short hydraulic retention time. The reported AOM rates per gram dry sediment (0.001-21 µmol $g_{dry\ weight}^{-1}$ day^{-1}; Table 1.1) are extremely low compared to the SR rates, per gram total suspended solids (TSS), that have been found in sulfate-reducing bioreactors fed with ethanol (23 mmol SO_4^{2-} g_{TSS}^{-1} day^{-1}; Vallero *et al.*, 2005) or formate (40 mmol SO_4^{2-} g_{TSS}^{-1} day^{-1}; Bijmans *et al.*, 2008). In addition, the sludge retention times of GLRs (3-7 days for the GLB of Nyrstar; Houten *at al*., 2006) are much shorter than the doubling time estimates of the microorganisms involved in AOM coupled to SR (1-7 months) (Girguis *et al.*, 2005; Nauhaus *et al.*, 2007; Krüger *et al.*, 2008), which would make it impossible to maintain enough biomass in the reactor system.

1.4 Scope and organization of this thesis

This introduction chapter gives an overview of the current knowledge of both AOM coupled to SR and the biotechnological aspects of SR. Based on that knowledge, the possibilities and limitations for biotechnological applications of AOM coupled to SR are addressed.

AOM coupled to SR has been demonstrated in marine sediments, but not in well-mixed bioreactors or in bioreactor sludge. In Chapter 2, AOM and SR by methanogenic and sulfate-reducing granular sludge are investigated. If granular sludge contains microorganisms capable of AOM coupled to SR, it would form an easily available biomass source for “high rate” bioreactors.

Chapter 3 describes the enrichment of microorganisms from marine sediment, mediating AOM coupled to SR, in a well-mixed MBR. For applications it is crucial that microorganisms mediating AOM coupled to SR can be grown in well-mixed bioreactors. Two incubation temperatures are compared, and the growth and conversion rates are determined.

In Chapter 4, the obtained enrichment is microbially characterized and the microorganisms involved in AOM coupled to SR are identified. A clone library is constructed, the distribution of different phylogenetic groups is visualized with Fluorescent *In Situ* Hybridization and the incorporation of carbon, derived from CH_4, in archaeal and bacterial lipids is presented.

Chapter 5 deals with the physiological characterization of the enrichment. The effect of environmental parameters (temperature, pH, salinity, CH_4 partial pressure, sulfate concentration, inorganic carbon concentration and sulfide concentration) on AOM and SR is assessed. In addition, the ability of the enrichment to use other electron acceptors than sulfate for the oxidation of CH_4 is investigated. From these results the operational window for possible applications of AOM coupled to SR, is extracted.

Chapter 6 investigates AOM coupled to SR in a fed-batch bioreactor operated at a CH_4 partial pressure of 10.1 MPa.

Chapter 7 focuses on the possible syntrophic interaction between ANME and SRB. It is tested whether methanogenic substrates act as IEC in AOM coupled to SR by the obtained enrichment. In addition, the ability of the enrichment to utilize alternative electron donors is addressed.

Chapter 8 concludes this thesis, the implications of the obtained results for biotechnological applications of the AOM coupled to SR are discussed and suggestions for further research are given.

Chapter 2

Methane-dependent sulfate reduction and trace methane oxidation by anaerobic granular sludge

Abstract

Anaerobic oxidation of methane (AOM) in anoxic marine sediments is mediated by methanotrophic archaea and is coupled to the reduction of sulfate. In this study, AOM and the possible coupling to sulfate reduction (SR) by methanogenic and sulfate-reducing microbial communities from anaerobic bioreactors are investigated. Three different types of granular sludge oxidized ^{13}C-labeled methane anaerobically to ^{13}C-labeld carbon dioxide, during net methane production. AOM rates followed methane production rates, and AOM and methanogenesis were both hampered by the presence of sulfate or bromoethanesulfonate, an inhibitor for methanogenesis. AOM by granular sludge was therefore a site effect of methanogenesis and not coupled to SR. This process is referred to as trace methane oxidation. The methane partial pressure positively affected SR and negatively affected methanogenesis. The inhibition of methanogenesis at elevated methane partial pressures gave an advantage to the sulfate reducers competing with methanogens (possibly in syntrophic consortia with acetogenic bacteria) for the same substrates, resulting in the observed methane dependence of SR. These substrates were released from endogenous material, no other substrate than methane and sulfate was added. Trace methane oxidation and the competitive advantage that sulfate reducers have over methanogens at elevated methane partial pressures should be considered when investigating AOM coupled to SR in samples that show endogenous methane production.

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2.1 Introduction

2.1.1 Anaerobic methanotrophs

Anaerobic oxidation of methane (AOM) in marine sediments is an important process in the global carbon cycling and is coupled to the reduction of sulfate, according to reaction 1 (Valentine and Reeburgh, 2000; Hinrichs and Boetius, 2002; Nauhaus *et al.*, 2002).

(1) $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ $\Delta G^{\circ\prime} = -16.6\ kJ.mol^{-1}$

AOM is mediated by uncultured archaea, called anaerobic methanotrophs (ANME), distantly related to cultivated methanogenic members from the orders *Methanosarcinales* and *Methanomicrobiales* (Hinrichs *et al.*, 1999; Orphan *et al*, 2002; Knittel *et al.*, 2005). ANME often occur in consortia with, or in the proximity of, sulfate-reducing bacteria (SRB) (Boetius *et al*., 2000; Orphan *et al*., 2001; Michaelis *et al*., 2002; Elvert *et al.*, 2003; Knittel *et al*., 2003). It has been suggested that an archaeon produces an electron carrier compound from CH_4, which is subsequently utilized by a sulfate-reducing partner (Zehnder and Brock, 1980; Alperin and Reeburgh, 1985; Hoehler *et al*., 1994 and DeLong, 2000). However, it remains unclear how reducing equivalents are transferred from the methanotroph to the sulfate-reducing partner.

There is evidence that AOM is a form of reversed methanogenesis. ANME-1 were found to contain nearly all genes typically associated with CH_4 production (Hallam *et al.*, 2004), and two analogues of the methyl-coenzyme M reductase were found to make up 7 and 3% of the extracted soluble proteins from AOM mediating Black Sea sediment samples (Krüger *et al.*, 2003).

2.1.2 Anaerobic oxidation of methane by methanogens

Pure cultures of methanogenic archaea also oxidize CH_4 to CO_2 anaerobically. As this process is not coupled to sulfate reduction (SR) but occurs during methanogenesis, it is referred to as trace methane oxidation (TMO) (Moran *et al*., 2004). Zehnder and Brock (1979) observed TMO in all of the 9 methanogens tested. The process was found to occur during hydrogenotrophic, methylotrophic and aceticlastic methanogenesis. The amounts of CH_4 oxidized varied between 0.001 and 0.32% of the amount of CH_4 produced. However, the biologically produced ^{14}C-labeled CH_4 used by Zehnder and Brock (2008) was likely contaminated with ^{14}C-labeled carbon monoxide, potentially resulting in an overestimation of

the methane oxidation (Harder, 1997). Using pure ^{14}C-labeled CH_4, TMO by several methanogenic cultures growing on methanol or hydrogen/CO_2 was observed as well (Harder, 1997).

TMO by pure cultures of *Methanosarcina acetivorans* was not affected by the presence of oxygen, nitrate, sulfate, sulfite or hydrogen (Moran *et al*., 2004). During methanogenesis carbon from CH_4 is incorporated in acetate at the methyl position (0.1% of the biogenic CH_4) and in cultures grown on carbon monoxide, carbon from CH_4 is incorporated in methyl sulfide (3.1% of the biogenic CH_4) (Moran *et al.*, 2007a). These results indicate that methyl sulfides are more likely intermediates in TMO than acetate and hydrogen.

Methane oxidation during net methane production was observed in anoxic sediments, digested sewage sludge and stabilized anaerobic sludge, only at higher methane oxidation to methane production ratios than with pure cultures (Zehnder and Brock, 1980; Harder, 1997). The methane oxidation was 90% of the methane production in digested sewage sludge at a CH_4 partial pressure of 2.0 MPa and in presence of 10 mM ferrous sulfate (Zehnder and Brock, 1980). According to Schilov *et al*. (1999) aceticlastic methanogenesis in granular sludge, consisting of mixed cultures dominated by *Methanosarcina* and *Methanosaeta spp.*, can be reversed at a methane pressure of 100 atmosphere.

2.1.3 Current research

In this study, the capacity of granular sludge, from upflow anaerobic sludge bed (UASB) reactors, to anaerobically oxidize methane is investigated. The reduction equivalents formed by the oxidation of methane should end up in another reduced compound or compounds, also during net methane production. Possibly, under the right conditions, the sulfate reducers are able to utilize these reduced compounds for SR. Therefore, it is assessed if methane oxidation by sludge can contribute to SR. This was done by quantifying CH_4 oxidation, CH_4 production and SR rates in the presence and absence of sulfate and in the presence and absence of bromoethanesulfonate (BES, an inhibitor for methanogenesis). In addition, the effect of the CH_4 partial pressure on SR, CH_4 oxidation and methanogenesis was evaluated. Three types of anaerobic sludge were used, two from methanogenic bioreactors and one from a sulfate-reducing bioreactor.

Besides a possible coupling between SR and methane oxidation by methanogens, it might be possible that anaerobic methanotrophs (capable of net CH_4 oxidation) are present in the bioreactor sludge's. Solid retention times of over 200 days can be achieved in UASB reactors (Hulshoff Pol *et al*., 2004), which is in the same range as the doubling times

estimated for anaerobic methanotrophs (between 2 and 7 months; Girguis *et al*., 2005; Nauhaus *et al*., 2007 and Krüger *et al*., 2008). In addition, the microorganisms in these anaerobic reactors have access to both CH_4, being produced, and traces of sulfate from the influent. Roest *et al.* (2005) have constructed an archaeal clone library of Eerbeek sludge, in which no clone was identified as ANME. To check for the presence of more recently submitted ANME sequences, the similarity search against sequences deposited in publicly accessible databases was repeated.

2.2 Material and Methods

2.2.1 Inocula

Granular sludge samples were obtained from three full-scale mesophilic Up-flow Anaerobic Sludge Blanket (UASB) reactors: a methanogenic reactor treating wastewater from paper mills (Industriewater Eerbeek, Eerbeek, the Netherlands, June 2005); a methanogenic reactor treating wastewater from a distillery (Nedalco, Bergen op Zoom, the Netherlands, July 2005) and a sulfate-reducing reactor fed with ethanol (Emmtec, Emmen, the Netherlands, May 2006). Additionally, a mix of crushed methanogenic (Eerbeek) and sulfate-reducing (Emmtec) sludge was used. The granules (2-4 mm) were crushed by pressing granules through needles with diameters of 1.2, 0.8 and 0.5 mm. The sludge was stored anaerobically at 4°C and washed four times prior to inoculation.

2.2.2 Medium

The basal medium consisted of: NaCl (7 g L^{-1}), $MgCl_2.6H_20$ (1.2 g L^{-1}), KCl (0.5 g L^{-1}), NH_4Cl (0.3 g L^{-1}), $CaCl_2$ (0.15 g L^{-1}), Na_2SO_4 (2.8 g L^{-1}), KH_2PO_4 (0.43 g L^{-1}), $K_2HPO_4.3H_2O$ (1.56 g L^{-1}), a trace element solution (1 mL L^{-1}), a 0.5 g L^{-1} resazurin solution (1 ml L^{-1}) and demineralized water. The trace element solution contained: $FeCl_2.4H_2O$ (1500 mg L^{-1}), $CoCl_2.2H_2O$ (190 mg L^{-1}), $MnCl_2.4H_2O$ (100 mg L^{-1}), $ZnCl_2$ (70 mg L^{-1}), H_3BO_3 (62 mg L^{-1}), $Na_2MoO_4.2H_2O$ (36 mg L^{-1}), $NiCl_2.6H_2O$ (24 mg L^{-1}), $CuCl_2.2H_2O$ (17 mg L^{-1}) and HCl 37% (7 mL L^{-1}). The final pH of the medium was 7.2. Resazurin was added to check if conditions were anaerobic, it becomes colorless at a redox under -110 mV and becomes pink at a redox above -51 mV. The medium was boiled, cooled down under a nitrogen (N_2) flow and transferred to stock bottles with a N_2 headspace. For control incubations, a stock was made with medium from which the sodium sulfate was omitted.

2.2.3 Experimental set-up

The effect of the CH_4 partial pressure on SR by methanogenic sludge was investigated by incubating 0.5 g volatile suspended solids (VSS) of Eerbeek sludge under a headspace of 0.17 MPa N_2, 0.17 MPa CH_4, 1.1 MPa N_2 or 1.1 MPa CH_4. The 0.17 MPa incubations were done in 1 L serum bottles closed with butyl rubber stops and the 1.1 MPa incubations were done in 0.60 L pressure vessels (Parr, Moline, CA). After adding the sludge, the bottles or vessels were closed and flushed with N_2. Subsequently, 500 ml medium, from an anaerobic stock, was added. Finally, the headspaces of the bottles were flushed and filled with N_2 or CH_4. The bottles were incubated at 30ºC in an orbital shaker controlled at 100 rpm. The pressure vessels were controlled at a temperature of 30ºC and equipped with a stirrer, operated at 100 rpm. Three times a week, liquid samples (2.5 ml) were taken for pH, sulfate and sulfide analyses.

To assess AOM by anaerobic sludge, ambient pressure incubations were done with ^{13}C-labeled CH_4 ($^{13}CH_4$) in serum bottles of 120 ml. The $^{13}CH_4$ gas was supplied by Campro (Veenendaal, the Netherlands) and had a purity of 99%, 1.0% $^{12}CH_4$ being the sole major impurity. After inoculation, the bottles were closed with butyl rubber stoppers sealed with crimp seals and flushed with N_2. Subsequently, the bottles were made partly vacuum and filled with 90 ml medium from an anaerobic stock using syringes and needles. Finally, the headspaces of the bottles were made vacuum again (to a residual pressure of circa 5 kPa) and filled with 0.17 MPa N_2 or $^{13}CH_4$. The bottles were incubated at 30ºC in an orbital shaker controlled at 100 rpm. Liquid (2.5 ml) and headspace (100 µl) samples were taken weekly for pH, sulfate, sulfide, fatty acids, alcohols and gas composition analyses. In addition, the headspace pressure and the weight of each bottle (as a measure for liquid and headspace volume) were measured.

Incubations were done with Eerbeek sludge (0.05, 0.1, 0.2, 0.3 and 0.4 g VSS), Nedalco sludge, (0.2 g VSS), Emmtec sludge (0.2 g VSS) and a mix with crushed Eerbeek (0.1 g VSS) and crushed Emmtec sludge (0.1 g VSS). Each sludge type was incubated with a N_2 headspace, a $^{13}CH_4$ headspace (in duplicate) or a $^{13}CH_4$ headspace with sulfate-free medium (in duplicate). The following control incubations with $^{13}CH_4$ and sulfate were done: incubations without biomass, with autoclaved Eerbeek sludge (0.2 g VSS) and with Eerbeek sludge and BES.

To assess the effect of the CH_4 partial pressure on CH_4 production and CH_4 oxidation rates, triplicate incubations with Eerbeek sludge (0.02 g VSS) and Nedalco sludge (0.02 g VSS) were done, at atmospheric (0.10 MPa) and high (10 MPa) pressure. Glass tubes of 18 ml

were used, sealed on one site with butyl rubber stops and caps and equipped with a piston on the other site (De Glasinstrumentenmakerij, Wageningen, the Netherlands). The top part of the piston was made from rubber and precisely fitted the tube. The system did not leak, even if air entrapped in the tube was pressurized to 0.5 MPa (by pressing the piston), no gas bubbles left the tube when submersed under water. Because the plunger was able to move freely, the pressure inside the tube was the same as outside. The tubes were filled with sludge, closed, flushed with N_2 and filled with 9 ml medium. After removing the N_2 gas with a syringe, 3 ml $^{13}CH_4$ was added. The tubes were incubated unshaken at 30°C, in a non-pressurized incubator or in a 2.0 L pressure vessel (Parr, Moline, IL) filled with 1.8 L water. The pressure vessel was pressurized with N_2 from a gas bottle. Weekly, the pH, liquid volume, gas volume and gas composition were measured. To do so, the pressure vessel had to be depressurized. Both pressurization and depressurization were done gradually (over a period of two hours).

2.2.4 Analyses

Prior to analysis, liquid samples were filtered over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany). 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-}). Sulfate was measured on a DX-600 ion chromatograph (Dionex Corporation, Salt Lake City, USA) as described previously (Sipma *et al*., 2004). Volatile fatty acids and methanol were 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 GC-MS system was composed of a Trace GC equipped with a GS-GasPro column (30 m by 0.32 mm; J & W Scientific, Folsom, CA) and an 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 (0-0.4 MPa absolute, WAL Mess- und Regelsysteme, Oldenburg, Germany). The pH

was checked with pH paper (Macherey-Nagel, Düren, Germany). The VSS and total suspended solids (TSS) content of the wet sludge were analyzed according to standard methods (American Public Health Association, 1995).

A previously constructed clone library of Eerbeek sludge (Roest *et al.*, 2005) was used to perform a similarity search against sequences deposited in publicly databases. The search was done using the NCBI Blast search tool (BlastN; www.ncbi.nlm.nih.gov/BLAST/)

2.2.5 Calculations

For each time samples were taken, the total amount of SO_4^{2-}, sulfide, $^{13}CH_4$, $^{12}CH_4$, $\sum^{13}CO_2$ ($^{13}CO_2$ and $H^{13}CO_3^-$) and $\sum^{12}CO_2$ ($^{12}CO_2$ and $H^{12}CO_3^-$) per bottle or tube was calculated, according to:

$$SO_4^{2-} = [SO_4^{2-}] * V_{liquid}$$

$$sulfide = [sulfide] * V_{liquid}$$

$$\sum {}^{13}CH_4 = f\,{}^{13}CH_4 \times P \times V_{gas}$$

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

Nomenclature:

V_{liquid} = liquid volume in serum bottle or tube

V_{gas} = gas volume in serum bottle or tube

k = Henry's law constant for CO_2 at sampling temperature (20ºC): 0.0388 mol L^{-1}

K_z = dissociation constant of H_2CO_3 ($CO_2 + H_2O$) = 4.5 10^{-7}

P = pressure at sampling temperature

f = fraction

2.3 Results

2.3.1 Methane dependent sulfate reduction by methanogenic sludge

Figure 2.1 compares the development of the sulfate and sulfide concentration in time for incubations with Eerbeek sludge at different CH_4 and nitrogen partial pressures. All four incubations show sulfate removal coupled to sulfide production at a more or less constant

rate. However, the SR proceeds faster at a higher CH_4 partial pressure. The increased SR was a result of the increased CH_4 partial pressure rather than the increased total pressure since an elevated nitrogen pressure did not result in an increased SR rate.

2.3.2 Anaerobic oxidation of methane by methanogenic sludge

A series of incubations were done to assess the ability of anaerobic sludge to anaerobically oxidize CH_4 (Figure 2.2 and 2.3). The duplicates show a similar pattern (data now shown). In all incubations with sulfate and non-autoclaved sludge (Figure 2.3: A1, A2, B1, B2, C1, C2, D1 and D2), sulfate was removed and sulfide was production. In addition, SR was accompanied by the production of $\sum CO_2$. In all incubations without sulfate (Figure 2.3: A3, B3, C3 and D3), and most incubations with sulfate (Figure 2.3: A1, A2, B1, B2, D1 and D2), $^{12}CH_4$ was produced. This methanogenic activity was also accompanied with the production of $\sum CO_2$.

In all incubations with $^{13}CH_4$ but without sulfate (Figure 2.3: A3, B3, C3 and D3), and most incubations with both $^{13}CH_4$ and sulfate (Figure 2.3: A2, B2 and D2), more than 0.04 mmol $\sum^{13}CO_2$ was produced. The fraction of $^{13}CO_2$ of the total CO_2 in these incubations was 5% or higher. In controls without $^{13}CH_4$ (Figure 2.3: A1, B1, C1 and D1) the amount of $\sum^{13}CO_2$ that was formed remained below 0.01 mmol, the fraction $\sum^{13}CO_2$ of the total $\sum CO_2$ was always equal to the natural abundance of 1.1%. These results show that some $^{13}CH_4$ was oxidized.

Oxidation by molecular oxygen can be excluded, because in all incubations the liquid remained colorless, indicating that the redox was lower than -51 mV (at which resazurin turns pink). In addition, an overpressure of nitrogen or CH_4 was maintained in the bottles. Moreover, no oxygen or intermediates of aerobic CH_4 oxidation, such as methanol and formaldehyde, were detected.

No $\sum^{13}CO_2$ was formed in the incubations without sludge or with autoclaved sludge (Figure 2.2: A and B). From the 10 incubations with different amounts of Eerbeek sludge and with 0.16 MPa $^{13}CH_4$ and 20 mM sulfate, a linear relation between the CH_4 oxidation rate and the biomass concentration was found according to 12.53 $\mu mol.g_{VSS}^{-1}.day^{-1}$ (R^2=0.97, data not shown). These results show that living methanogenic sludge mediates the oxidation of CH_4 to $\sum CO_2$ under anaerobic conditions.

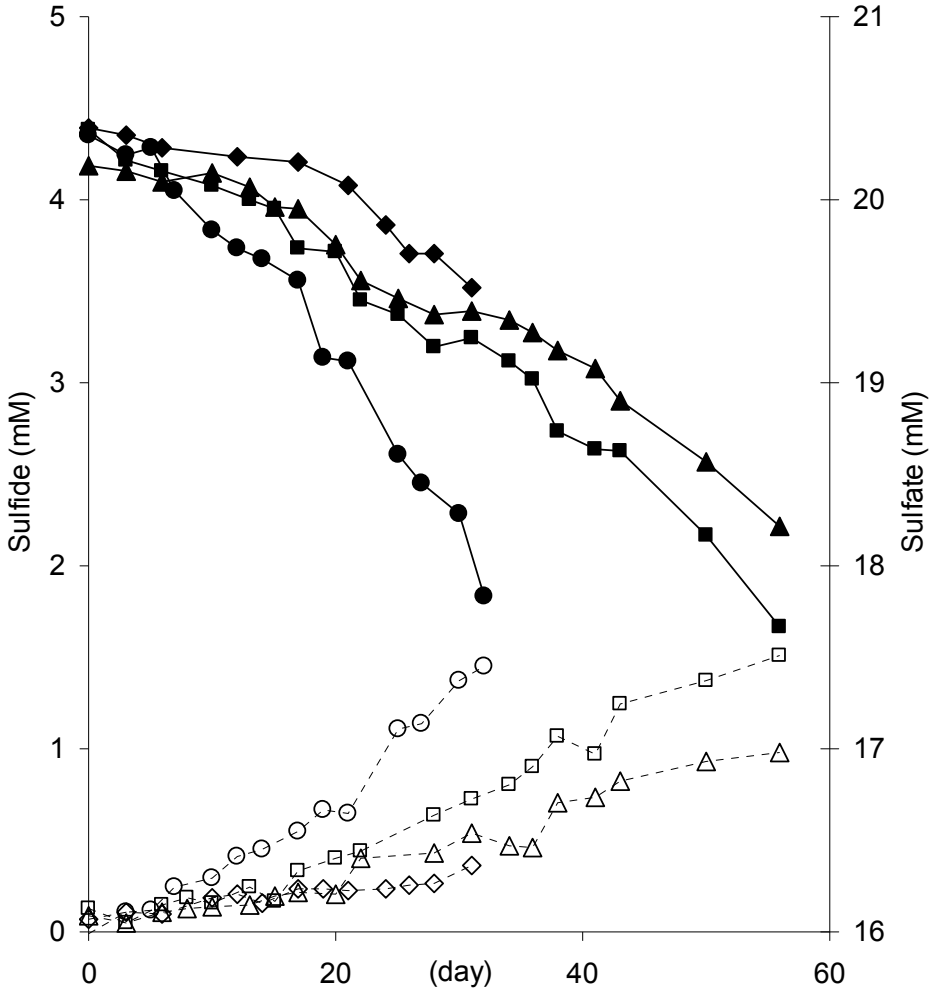

Figure 2.1. The effect of the CH_4 partial pressure on sulfide production (open symbols) and sulfate removal (filled symbols) in batch incubations at 30ºC with 0.5 g_{VSS} Eerbeek sludge in 500 ml medium. The headspace of the different incubations contained: 0.16 MPa N_2 (Δ), 1.1 MPa N_2 (◊), 0.16 MPa CH_4 (□) or 1.1 MPa CH_4 (○).

2.3.3 Endogenous activity

No other substrates than $^{13}CH_4$ and sulfate were added, still $^{12}CH_4$ production took place in almost all incubations (Figure 2.3: A1, A2, A3, B1, B2, B3, C3, D1, D2 and D3). In addition, sulfate was reduced even when no CH_4 was added. Also when $^{13}CH_4$ was added, the $^{13}CH_4$

oxidation was too low to account for all the SR (Figure 2.3: A2, B2, C2 and D2). Moreover, there was no net CH_4 oxidation; the $^{12}CH_4$ production rate was much higher than the $^{13}CH_4$ oxidation rate. Likely, organic compounds present in or released from the inocula were used as substrate. Therefore, VSS measurements were done, the VSS in the bottles with 0.2 g_{VSS} Eerbeek sludge decreased by 23.2 (±3.2) mg (N=4) during 30 days of incubation, indicating that the sludge was slowly decomposing over time. During degradation of particular organic matter, fatty acids, alcohols and hydrogen are produced as intermediates (Stams, 1994). Fatty acids and alcohols can subsequently be further degraded to acetate and hydrogen by acetogenic bacteria or used by sulfate reducers. Acetate and hydrogen form substrates for both sulfate reducers and methanogens (Stams *et al.*, 2005). When complete oxidation of the organic matter (CH_2O) is assumed, 23 mg organic matter would account for 0.39 mmol SR or CH_4 production. This fits reasonably well with the amount of sulfate reduced and $^{12}CH_4$ produced after 30 days added together (Figure 2.3: A1, A2 and A3).

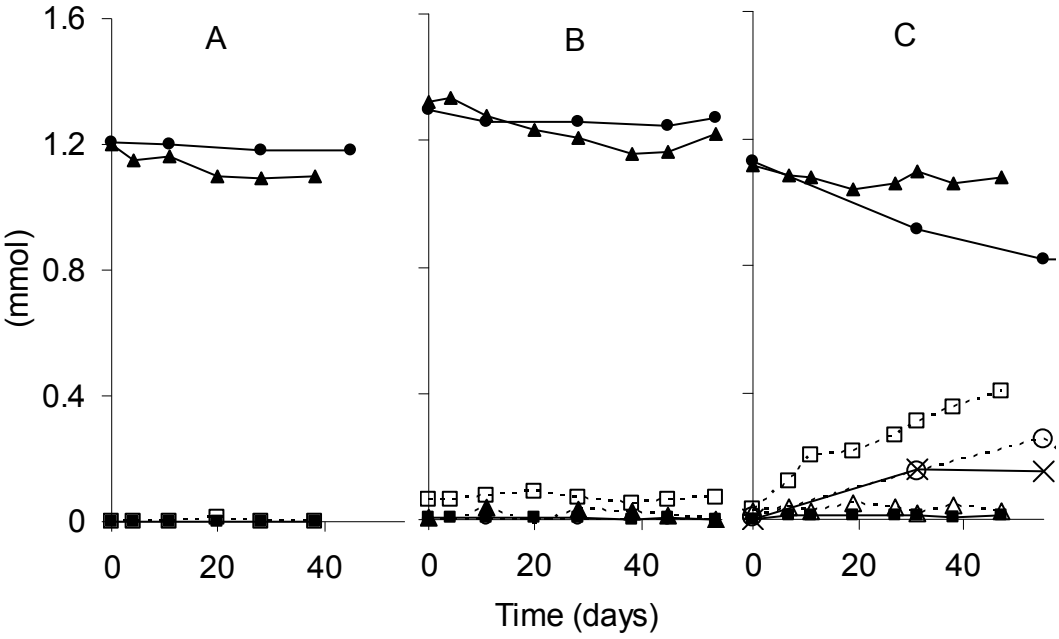

Figure 2.2. $^{12}CH_4$ production, SR and $^{13}CH_4$ oxidation in time, in presence of sulfate and $^{13}CH_4$ at 30ºC, by: blank (A), 0.20 g_{VSS} autoclaved granular Eerbeek sludge (B) and 0.20 g_{VSS} granular Eerbeek sludge in presence of BES (C). Symbols: $^{13}CH_4$ (▲), $^{12}CH_4$ (Δ), $\sum^{13}CO_2$ (■), $\sum^{12}CO_2$ (□), SO_4^{2-} (●), sulfide (○), acetate (X).

2.3.4 Coupling of methane oxidation and sulfate reduction

To find a possible coupling between CH_4 oxidation and SR, incubations with and without sulfate were compared. In the incubations with sulfate; 0.23, 0.33, 0.38 and 0.69 mmol sulfate were reduced during the experiment (Figure 2.3: A2, B2, C2 and D2, respectively). In the incubations without sulfate, no SR could be measured and instead 0.20, 0.46, 0.51, 0.91 mmol additional $^{12}CH_4$ was produced (Figure 2.3: A3, B3, C3 and D3, respectively). In the absence of sulfate, the methanogens were able to utilize the endogenous substrates otherwise utilized by sulfate reducers. Like the $^{12}CH_4$ production, the $^{13}CH_4$ oxidation is also higher in absence of sulfate. Moreover, $^{12}CH_4$ and $\sum^{13}CO_2$ production always proceed simultaneously (Figure 2.3: A2, A3, B2, B3, C3, D2 and D3), suggesting that the CH_4 oxidation was coupled to methanogenesis instead of SR. When 50 mM BES is added (Figure 2.2C) both $^{12}CH_4$ and $^{13}CO_2$ production are completely inhibited, and more sulfate was reduced than when BES was absent (Figure 2.3: A2). Although not all substrate was used for SR, some acetate accumulated.

To assess if CH_4 oxidation can be coupled to SR when methanogenic and sulfate-reducing sludge are crushed and mixed, a series of incubations were done (Figure 2.3: D1, D2 and D3). However, again the $^{13}CH_4$ oxidation was hampered by the presence of sulfate and the ratio between CH_4 oxidation and CH_4 production was not increased (mixed sludge: 0.18; Eerbeek sludge: 0.19; Emmtec: 0.13).

2.3.5 Effect methane partial pressure

Table 2.1 compares incubations conducted at ambient pressure with incubations done at 10 MPa, each time with 100% $^{13}CH_4$ in the headspace. The elevated CH_4 partial pressure slightly inhibited methanogenesis and stimulated CH_4 oxidation. As a result, the CH_4 oxidation to CH_4 production ratio increased from 0.18 and 0.16 at ambient pressure (0.10 MPa) to 0.45 and 0.48 at 10 MPa $^{13}CH_4$ for Eerbeek and Nedalco sludge, respectively.

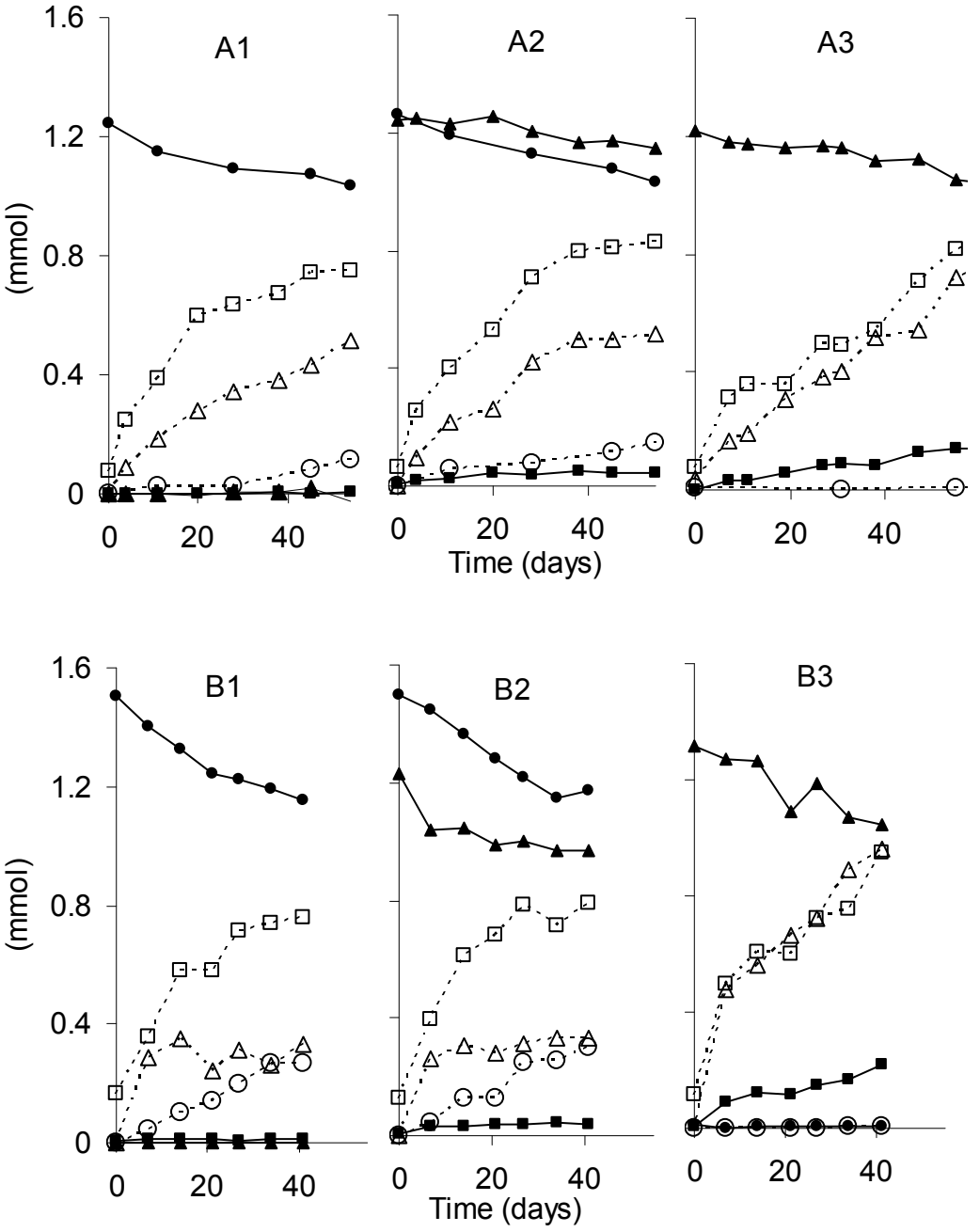

Figure 2.3. $^{12}CH_4$ production, SR and $^{13}CH_4$ oxidation in time at 30ºC by: 0.20 g_{VSS} granular Eerbeek (A), Nedalco (B) and Emmtec (C) sludge, and a mix of crushed Eerbeek (0.10 g_{VSS}) and Nedalco (0.10 g_{VSS}) sludge (D); in the presence of sulfate and in the absence of $^{13}CH_4$ (1), in the presence of sulfate and $^{13}CH_4$ (2) and in the absence of sulfate and in the presence of $^{13}CH_4$ (3). Symbols: $^{13}CH_4$ (▲), $^{12}CH_4$ (Δ), $\sum^{13}CO_2$ (■), $\sum^{12}CO_2$ (□), SO_4^{2-} (●), sulfide (○).

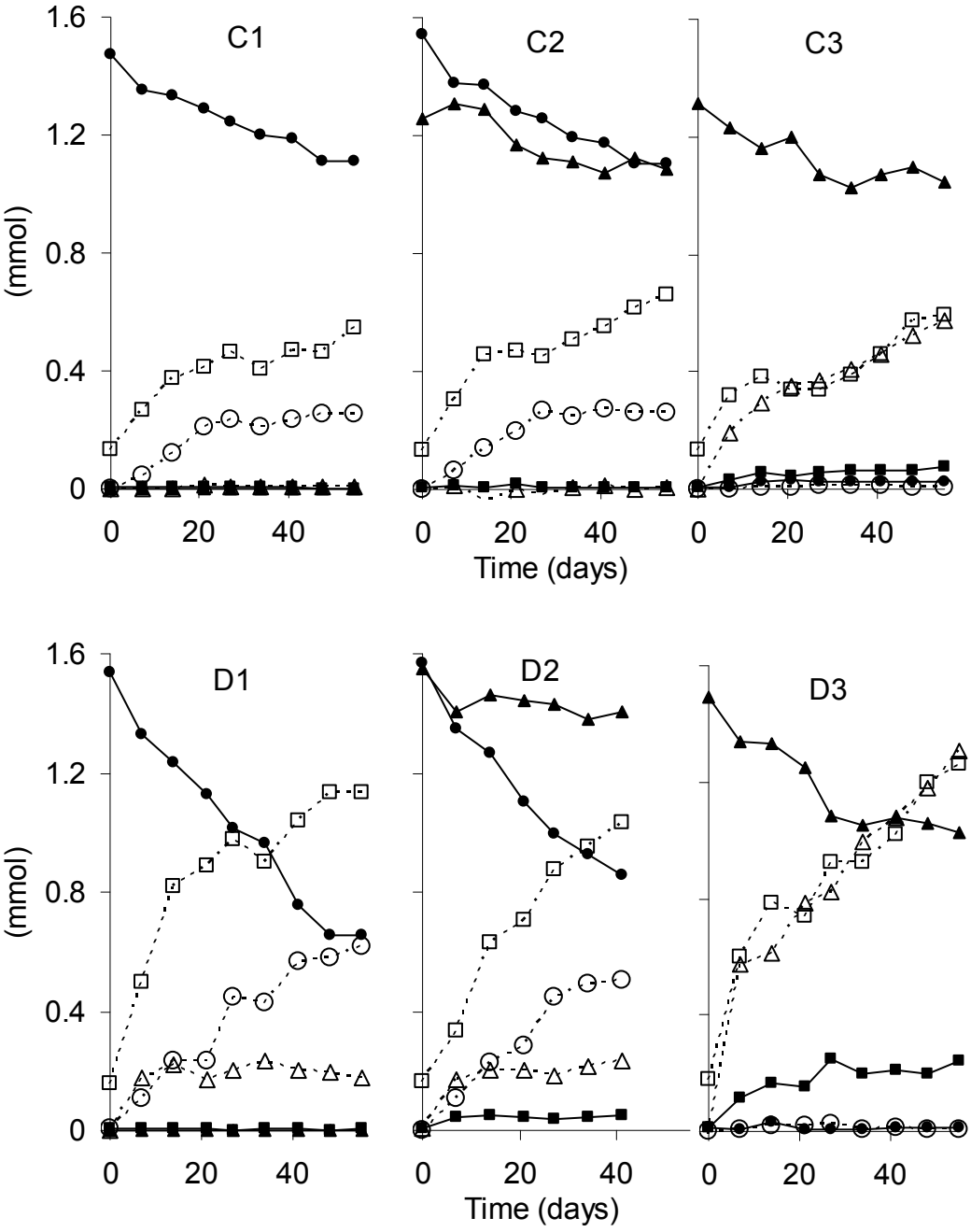

Figure 2.3 continued.

Table 2.1. CH_4 production and CH_4 oxidation rates at 0.10 and 10 MPa CH_4 by Eerbeek and Nedalco sludge. Rates were obtained from a line plotted though five successive data points (N=3).

	Eerbeek sludge		Nedalco sludge	
	0.10 MPa $^{13}CH_4$	10 MPa $^{13}CH_4$	0.10 MPa $^{13}CH_4$	10 MPa $^{13}CH_4$
$^{12}CH_4$ production rate (μmol g_{VSS}^{-1} day^{-1})	47.1 (±1.9)	36.6 (±7.32)	18.9 (±0.4)	15.3 (±2.8)
$\sum^{13}CO_2$ production rate (μmol g_{VSS}^{-1} day^{-1})	8.6 (±0.9)	16.3 (±6.2)	3.0 (±0.24)	7.3 (±2.3)

2.3.6 Clone library Eerbeek sludge

The results presented in Figure 2.1, 2.2 and 2.3A were obtained with Eerbeek sludge. Table 2.2 shows the identity of archaeal cloned 16S rRNA gene amplicons obtained from this sludge. None of the clones was identified as ANME, most were indentified as methanogen.

Table 2.2. Identity of cloned archaeal 16S rRNA gene amplicons retrieved from the anaerobic wastewater treatment system at Eerbeek (the Netherlands), the closest relative and/or closest cultured relative in the NCBI database (BlastN). % = percentage of similarity between 16S rRNA gene sequences of clone and relative.

Clone	Accession number clone	Closest relative and closest cultured relative in database (BlastN)	Accession number relative	%
1A3	AY426474	Uncultured archaeon clone R1A-2 from a denitrifying bioreactor	FJ167430	99
		Methanothrix soehngenii	X51423	99
1A7	AY426475	*Methanosaeta concilii*	X16932	99
1A8	AY426476	Uncultured archaeon 72-18 from municipal wastewater sludge	AF42476	91
1B7	AY426477	Uncultured *Methanosaeta sp.* clone A11 from a propionate-fed UASB reactor	EU888815	94
		Methanothrix soehngenii	X51423	93
1C11	AY426478	Uncultured archaeon clone R2A-4 from a denitrifying bioreactor	FJ167436	99
		Methanobacterium beijingense strain 8-2	AY350742	99
1E4	AY426479	Uncultured archaeon clone R1A-2 from a denitrifying bioreactor	FJ167430	99
1G1	AY426480	Uncultured archaeon clone CG-4 from methanogenic digester sludge	AB233294	99
		Methanothrix soehngenii	X51423	99
1H10	AY426481	Uncultured archaeon clone R1A-2 from a denitrifying bioreactor	FJ167430	93
		Methanothrix soehngenii	X51423	94
2B5	AY426482	Uncultured archaeon clone T64 from manure pit sludge (China)	EU662696	99
2C2	AY426483	Uncultured archaeon clone MP123 from a phenol-degrading methanogenic consortia	EF198036	95
		Methanobacterium beijingense strain 8-2	AY350742	96
2C4	AY426484	Uncultured archaeon clone R2A-4 from a denitrifying bioreactor	FJ167436	99
		Methanobacterium beijingense strain 8-2	AY350742	99
2H1	AY426485	Uncultured bacterium clone HnA32fl from granular sludge from a UASB reactor	AB266905	99
		Methanomethylovorans sp. Z1	EF174501	98

2.4 Discussion

2.4.1 Competition of methanogens and sulfate reducers for endogenous substrates

In incubations with anaerobic sludge and sulfate, methanogenesis and SR occur simultaneously, even when no electron donor is added (Figure 2.3: A2, B2, C2 and D2). Both processes must have been fueled by substrates released from an endogenous source. Methanogenesis increased when sulfate was omitted (Figure 2.3: A3, B3, C3 and D3) and SR increased when methanogenesis was inhibited (Figure 2.2C). This indicates that sulfate reducers in anaerobic sludge compete with methanogens, or with syntrophic consortia of methanogens and acetogenic bacteria, for the same substrate. Although sulfate reducers can obtain more energy from the utilization of acetate, hydrogen and methanol than methanogens at standard conditions (Table 2.3), methanogenesis was not suppressed during the 41 or 55 days of incubation with Eerbeek sludge (Figure 2.3: A1 and A2). With Nedalco sludge SR became dominant after two weeks of incubation (Figure 2.3: B1 and B2). With Emmtec, SR was dominant from the start (Figure 2.3: C1 and C2). Probably, the sulfate-reducing microbial community in Eerbeek sludge is not abundant and versatile enough to win the competition for the endogenous substrate. This is supported by the inability of sulfate reducers in Eerbeek sludge to utilize the acetate that accumulated when BES was added (Figure 2.2C). It has often been observed that acetate is predominately degraded by methanogens in presence of sulfate (Visser *et al.*, 1993; Bodegom and Stams, 1999; Stams *et al.*, 2005). Acetate-degrading sulfate reducers have only slightly better growth kinetic properties than *Methanosaeta* (dominant in anaerobic sludge), therefore it may take years before aceticlastic methanogens are outcompeted by acetate-degrading sulfate reducers, when the relative cell number of the acetate-degrading sulfate reducers is initially low (Stams *et al.*; 2005).

Sulfate reduction by Eerbeek sludge was positively influenced by the CH_4 partial pressure (Figure 2.1). Given the findings that CH_4 oxidation by Eerbeek sludge was not coupled to SR, it is unlikely that the additional SR at elevated pressure was due to the utilization of CH_4 as electron donor. A more likely explanation is that the CH_4 partial pressure gives a competitive advantage to the sulfate reducers due to product inhibition of the methanogens. An inhibition of methanogenesis by the methane partial pressure was indeed observed in incubations with sulfate (Table 2.1).

Table 2.3. Stoichiometry and Gibbs free energy changes of conversions that play a role in sulfate-reducing bioreactors. Gibbs free energy changes were calculated from Thauer *et al.* (1977).

Reaction		ΔG°'
	Sulfate reduction	
1	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-48 kJ.mol^{-1} SO_4^{2-}
2	$^4/_3\ CH_3OH + SO_4^{2-} \rightarrow 4/3\ HCO_3^- + HS^- + 4/3H_2O + 1/3H^+$	-121 kJ.mol^{-1} SO_4^{2-}
3	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-152 kJ.mol^{-1} SO_4^{2-}
	Methanogenesis	
4	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31 kJ.mol^{-1} CH_4
5	$^4/_3CH_3OH \rightarrow CH_4 + {}^1/_3HCO_3^- + {}^1/_3H_2O + {}^1/_3H^+$	-104 kJ.mol^{-1} CH_4
6	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-136 kJ.mol^{-1} CH_4

2.4.2 Trace methane oxidation

The results presented in this chapter show that the CH_4 oxidation by anaerobic granular sludge is a side effect of methanogenesis and is not coupled to SR, like Zehnder and Brock (1980), Harder (1997) and Moran (2004) reported for pure cultures of methanogens. This is in agreement with the finding that Eerbeek sludge contains a large population of methanogens, but no ANME (Table 2.2).

During the oxidation of $^{13}CH_4$ to $^{13}CO_2$ a product should be produced in which the reduction equivalents of CH_4 are conserved. Sulfate reducers are known to be able to use a large range of substrates (Muyzer and Stams, 2008). Despite this, SRB were not able to utilize the reduced products of TMO, even when a mix of methanogenic and sulfate-reducing sludge was used (Figure 3D). Possibly, these products are not excreted by the archaea and therefore not accessible for the SRB. Alternatively, the distance between the archaea and the SRB may have been too large. Sørensen *et al*. (2001) calculated that the syntrophic partners involved in AOM in marine sediments at *in situ* conditions should be less than 66 nm apart with formate as interspecies electron carrier. With acetate, hydrogen or methanol as interspecies electron carrier, this distance should be even smaller (13, 8.4 or 0.000004 nm, respectively).

Since methane oxidation by granular sludge was not coupled to SR and other inorganic electron accepters than sulfate and CO_2 were not present, the formation of $^{13}CO_2$ from $^{13}CH_4$ by granular sludge must have been coupled with the formation of $^{12}CH_4$. In this way, the

reduction equivalents of $^{13}CH_4$ would be preserved. This phenomenon could be the result of the reversibility of the enzymes involved in methanogenesis. Because the initial $^{13}CH_4/^{12}CH_4$ ratio was much higher than the initial $^{13}CO_2/^{12}CO_2$ ratio, a net $^{13}CH_4$ oxidation was observed. Much higher methane oxidation to methane production ratios were obtained with sludge than with pure cultures. The reason for this could be that the hydrogen pressure and acetate concentration were kept low in the incubations with sludge, making the reversed conversion of methanogenesis (Table 2.3) less unfavorable. In the experiments presented here no external substrate, other than methane and sulfate, were added. It is likely that any hydrogen, formate or acetate released from endogenous source, was immediately consumed again. In experiments where TMO by hydrogenotrophic methanogens was assessed, hydrogen or formate was added (Zehnder and Brock, 1979; Harder, 1997; Moran *et al.*, 2004).

2.4.3 Marine sediments

At ambient pressure, TMO rates by anaerobic sludge are in the same order of magnitude as the highest AOM rates found in marine sediments. Eerbeek granular sludge oxidizes CH_4 at 12.5 μmol.g_{VSS}^{-1}.day^{-1} or 11.4 μmol $g_{dry\ weight}^{-1}$ day^{-1}, whereas the thus far highest reported AOM rates are 2-8 and 8-21 μmol $g_{dry\ weight}^{-1}$ day^{-1} for Hydrate Ridge sediment (Krüger *et al*., 2005) and Black Sea microbial mats (Treude *et al*., 2007), respectively. All of eight tested AOM mediating sediments also endogenously produced methane, at rates between 0.005 and 0.4 μmol $g_{dry\ weight}^{-1}$ day^{-1} (Krüger *et al*., 2005). Hoehler (1994) and Orcutt (2005) reported the decoupling of SR from AOM and the contemporaneous occurrence of CH_4 production in marine sediments. These findings suggests that TMO can also contribute to methane oxidation in AOM sediments.

2.5 Conclusions

The main conclusions of this work are:

- Anaerobic granular sludge oxidizes methane during net methane production. This process is not coupled to SR. The ratio between methane oxidation and production is positively influenced by the methane partial pressure.
- The oxidation of labeled methane is not an exact measure for net methane oxidation in biological samples in which methanogenesis occurs.

- The methane partial pressure has a stimulating effect on endogenous SR by anaerobic granular sludge and has an inhibitory effect on endogenous methanogenesis. This can be explained by the inhibition of methanogens competing (possibly in syntrophic consortia with acetogenic bacteria) with SRB for the same endogenous substrates.
- In several reported studies the methane dependent occurrence of SR is taken as measure for AOM. As we show here thus may not be correct in sediments in which methanogenesis occurs as well.

Chapter 3

Enrichment of anaerobic methanotrophs in a sulfate-reducing membrane bioreactor

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 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} (196 mmol L^{-1} day^{-1}) and 3.0 mmol L^{-1} day^{-1}, respectively. 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 $g_{volatile\ suspended\ solids}^{-1}$ 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 community 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.

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3.1 Introduction

3.1.1 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 reaction 1 (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).

(1) $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ $\Delta G°' = -16.6\ kJ.mol^{-1}$

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 analogues 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 archaeon 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 was supported by the finding that in Hydrate Ridge sediment, ANME live in consortia with sulfate-reducing bacteria (SRB) (Boetius *et al*. 2000, Hinrichs *et al*., 2000). These archaea/SRB aggregates are not dominant in all AOM sites though. In Black sea microbial mats, SRB occur in microcolonies surrounded by bulk ANME-1 cells clusters (Michaelis *et al.*, 2002; Knittel *et al.*, 2005). In samples from Eel River Basin ANME-1 archaeal group frequently existed in monospecific aggregates or as single filaments, apparently without a bacterial partner (Orphan *et al.*, 2002). And in Eckernförde Bay sediment, clusters of ANME-2a cells were found without sulfate-reducing partners (Treude *et al.*, 2005).

3.1.2 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. 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. Excess sulfide can subsequently 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 will allow the physiological aspects of AOM coupled to SR to be studied.

3.1.3 Current research

In the present study, well-mixed ambient-pressure submerged-membrane bioreactors (MBRs) were used to enrich anaerobic methanotrophs. In these bioreactor systems, the washout of cells and growth limitation, due to substrate 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 of the responsible microorganisms. The microorganisms were identified by constructing a clone library of the enrichment and by monitoring the changes in microbial composition by Denaturing Gradient Gel Electrophoresis (DGGE).

3.2 Material and methods

3.2.1 Origin and storage of the inocula

Sediment samples were taken in Eckernförde Bay (Baltic Sea) at station B (water depth 28 m; position 54º31'15N, 10º01'28E) 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. 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 1 L bottles were closed with butyl rubber stoppers and the headspace was replaced by CH_4 (0.15 MPa). The bottles were stored at 4ºC 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. The sludge was stored anaerobically at 4ºC in the dark.

3.2.2 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 4ºC and connected to the influent pumps of the bioreactors.

3.2.3 Experimental set-up of membrane bioreactors

To enrich for anaerobic methanotrophs, 4 submerged-membrane bioreactors were built (Figure 3.1). The reactor system consisted of a cylindrical glass vessel (height: 520 mm, internal diameter: 70 mm, total volume: 2.0L), 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 3.0 mmol 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 pumps were controlled by level switches (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 bioreactor. The temperature was measured with a PT-100 electrode. The pH in each bioreactor 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 7.4 (±0.2) by 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 each bioreactor. This was done to supply methane to the microorganism, 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} (196 mmol 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_2S) and carbon dioxide (CO_2) stripped from the liquid, left each 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_2S, and was placed on a magnetic stirrer. The sulfide concentration (including the precipitated zinc sulfide) in the bottle was measured once every two weeks. The zinc chloride solution was replaced when the sulfide concentration 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}.

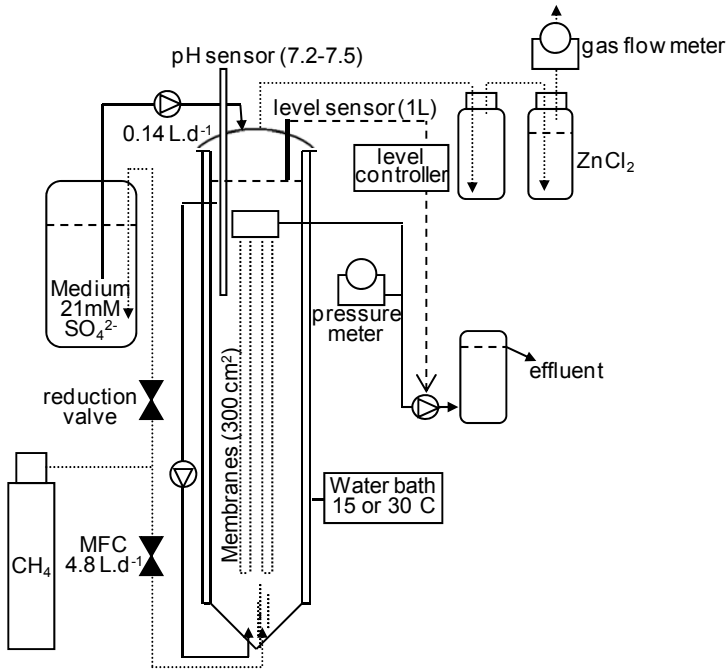


Figure 3.1. Schematic overview of a submerged-membrane bioreactor used for the enrichment experiments.

3.2.4 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. 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.

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

Reactor	Inoculum	Inoculation date	Duration run (days)	Temp. (°C)	Addition of 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 and 2 $g_{dry\ weight}$ sludge	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

3.2.5 Activity assays

CH_4 oxidation rates were estimated from the ^{13}C-labeled CO_2 ($^{13}CO_2$) production rate during batch incubations with sampled reactor suspension and ^{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 sampled 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 sample withdrawn from 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 sampled from R3 diluted with medium with a reduced sulfate concentration (2 mM). The headspace contained not-labeled CH_4 or N_2. Liquid samples were taken and used for sulfide and sulfate analyses, after filtering over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany).

3.2.6 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 (0-0.4 MPa absolute, WAL Mess- 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). The VSS is obtained from the difference between dry and ash weight of the solids (separated from the liquid by filtration).

3.2.7 DNA extraction and amplification

DNA was extracted from the reactor suspension using the FastDNA SPIN for Soil Kit (MP Biomedicals, Ohio, USA). The extracted DNA was purified with the NucleoSpin PCR purification kit (Macherey-Nagel, Germany) and quantified with a Nanodrop spectrophotometer. 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, CA) 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 Tris EDTA (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 the third nucleotide changed into T) and 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. The DNA fragments were partially sequenced (400-740 bp) by BaseClear (Leiden, The Netherlands).

3.2.8 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 by BaseClear (Leiden, The Netherlands).

3.2.9 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. Corrected sequences from representative clones were deposited in GenBank (www.ncbi.nlm.nih.gov; accession numbers FJ210915 and FJ210925).

3.2.10 Calculation of volumetric activities

The volumetric sulfate removal, sulfide production and acetate removal are calculated according to:

$$\text{Sulfate removal rate} = \frac{\left(\left[SO_4^{2-}{}_{\text{inf luent}}\right] - \left[SO_4^{2-}{}_{effluent}\right]\right)}{HRT}$$

$$\text{Sulfide production rate} = \frac{\left(\left[sulfide_{effluent}\right] - \left[sulfide_{\text{inf luent}}\right]\right)}{HRT} + \frac{\Delta\left[sulfide_{gaswash}\right] \times V_{gaswash}}{\Delta t}$$

$$\text{Acetate removal rate} = \frac{\left(\left[Acetate_{\text{inf luent}}\right] - \left[Acetate_{effluent}\right]\right)}{HRT}$$

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

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

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

Nomenclature:

eff = effluent

f = fraction

HRT = Hydraulic retention time

inf = influent

k = Henry's law constant for CO_2 at sampling temperature(20ºC): 0.0388 mol L^{-1}

K_z = dissociation constant of dissolved CO_2 + H_2O: 4.5 10^{-7}

P = pressure

t = time

TSS = total suspended solids

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

VSS = volatile suspended solids

[X] = molar concentration of compound X

3.3 Results

3.3.1 Reactor operation

The MBRs were kept anaerobic during operation and the biomass was retained. To check if biomass was washed-out, 100 ml effluent was monthly collected and centrifuged at 32G. However, no pellet could visually be detected. Moreover the transmembrane pressure in all four bioreactors remained between 15 and 20 kPa, which confirmed that the membranes were not leaking. During the 884 days of operation, the feeding, mixing, heating and/or cooling were five times shortly interrupted (maximum 48h). This was because of power failure, equipment failure or the depletion of CH_4 gas or medium. After these interruptions, the redox potential of the reactor suspension was still below -51 mV (at which the liquid would become pink because of the present resazurin). 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. In all four reactors, the CH_4 gas sparging and the recirculation of the reactor suspension were sufficient to keep all solids 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 between 7.2 and 7.5. In the other reactors the pH stayed between 7.2 and 7.5 without the addition of acid.

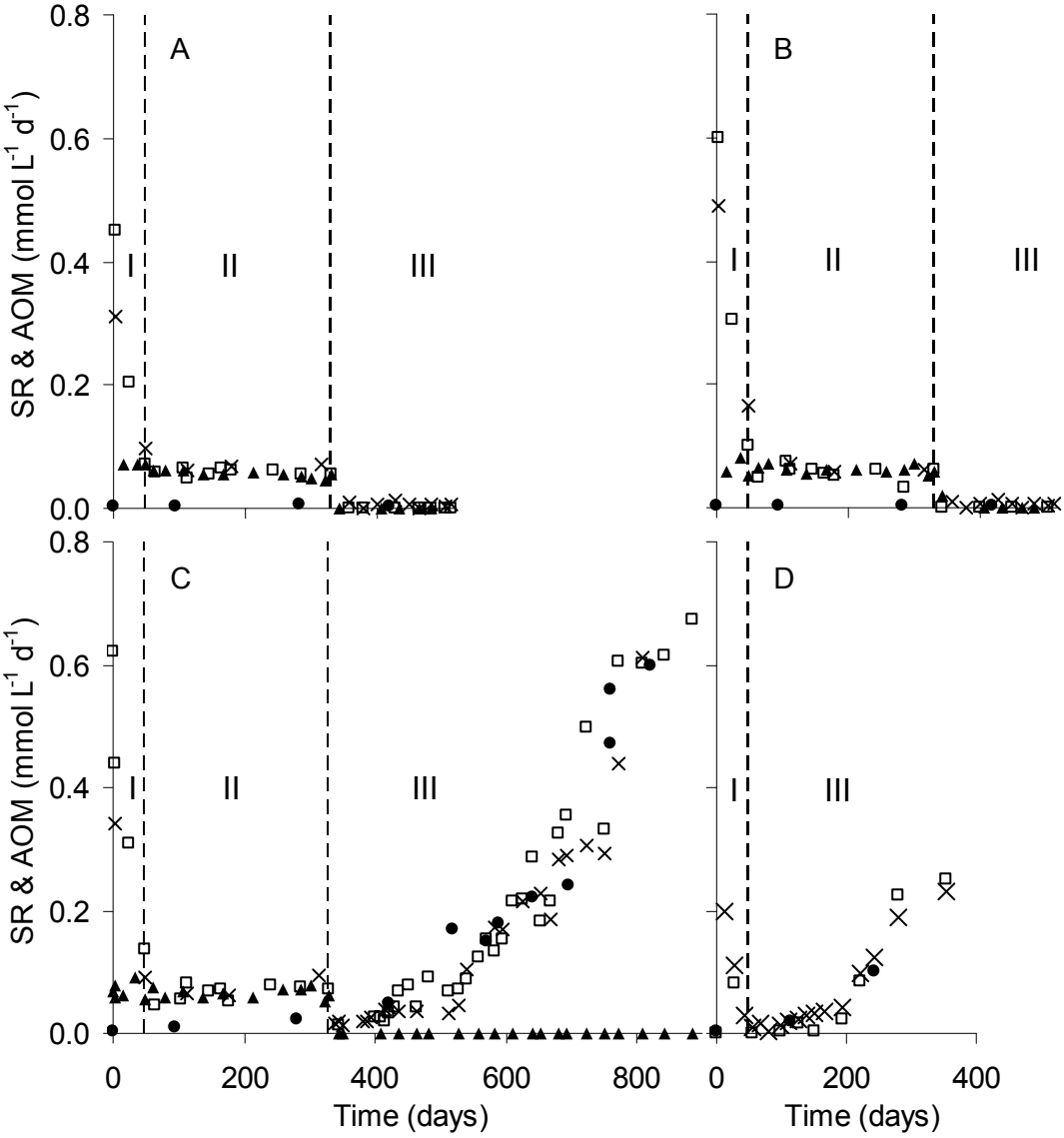


Figure 3.2. Volumetric conversion rates over time of four reactors inoculated with Eckernförde Bay sediment, R1 (A) and R2 (B) both operated at 30ºC, and R3 (C) and R4 (D), operated at 15ºC. R2 was additionally inoculated with anaerobic granular sludge. Symbols indicate: sulfide production rate (X), sulfate removal rate (□), AOM rate (●) and acetate consumption (▲). 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.

3.3.2 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 between 0.3 and 0.7 mmol L^{-1} day^{-1}, 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.

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 sulfate reduction with acetate, according to reaction 2.

(2) $CH_3COOH + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$ $\Delta G°' = -47$ 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). To R4, no acetate has been added to the feed at all, therefore phase I is followed by phase III. In the reactors operated at 15ºC (R3 and R4), the sulfate removal, sulfide production and AOM rates are coupled during phase III, according to reaction 1. Also in the activity assays done with reactor suspension from R3, taken during phase III, simultaneous CH_4 and sulfate consumption was accompanied by $\sum CO_2$ and sulfide production, according to reaction 1 (Figure 3.3A). In control incubations with nitrogen gas instead of CH_4 in the headspace, no conversion was observed (Figure 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 (Figure 3.2C and 3.2D). Figure 3.4A shows that the sulfate concentration in the effluent of R3 decreased over time and the dissolved sulfide concentration increased. A minimum sulfate concentration of 15.7 mM and a maximum sulfide concentration of 1.9 mM was reached.

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} (Figure 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.

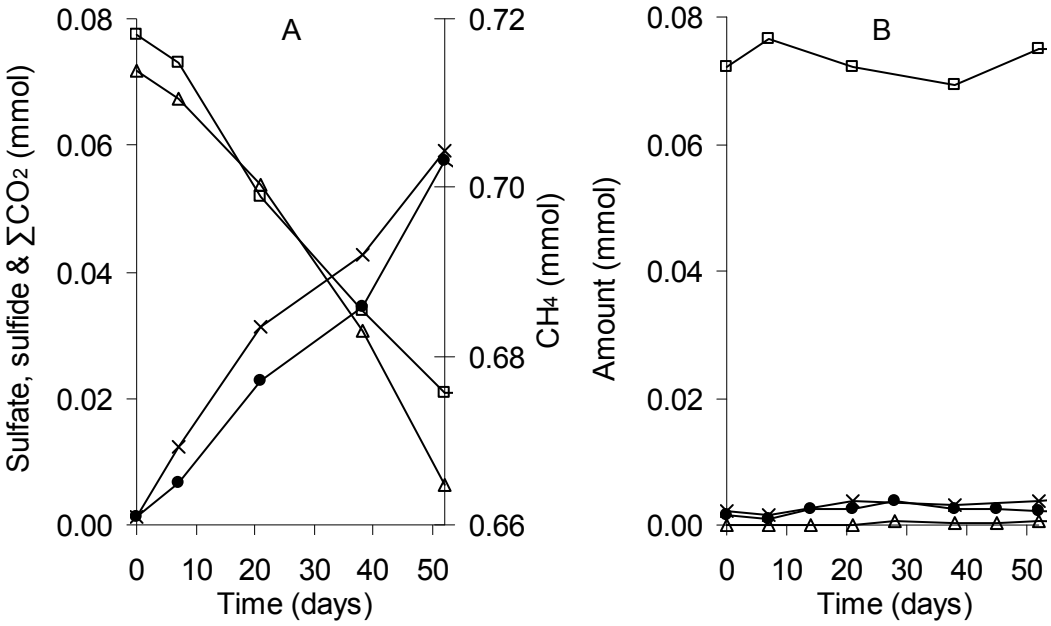


Figure 3.3. Sulfide production (X), sulfate consumption (□), ∑CO_2 production (●) and CH_4 consumption (Δ) in 35-ml batch bottles. The bottles contained 20 ml medium and 0.13 (±0.01) MPa CH_4 (A) or nitrogen (B). The bottles were inoculated with 2.5 ml reactor suspension, taken 760 days after start-up from R3, and incubated shaken at 15°C.

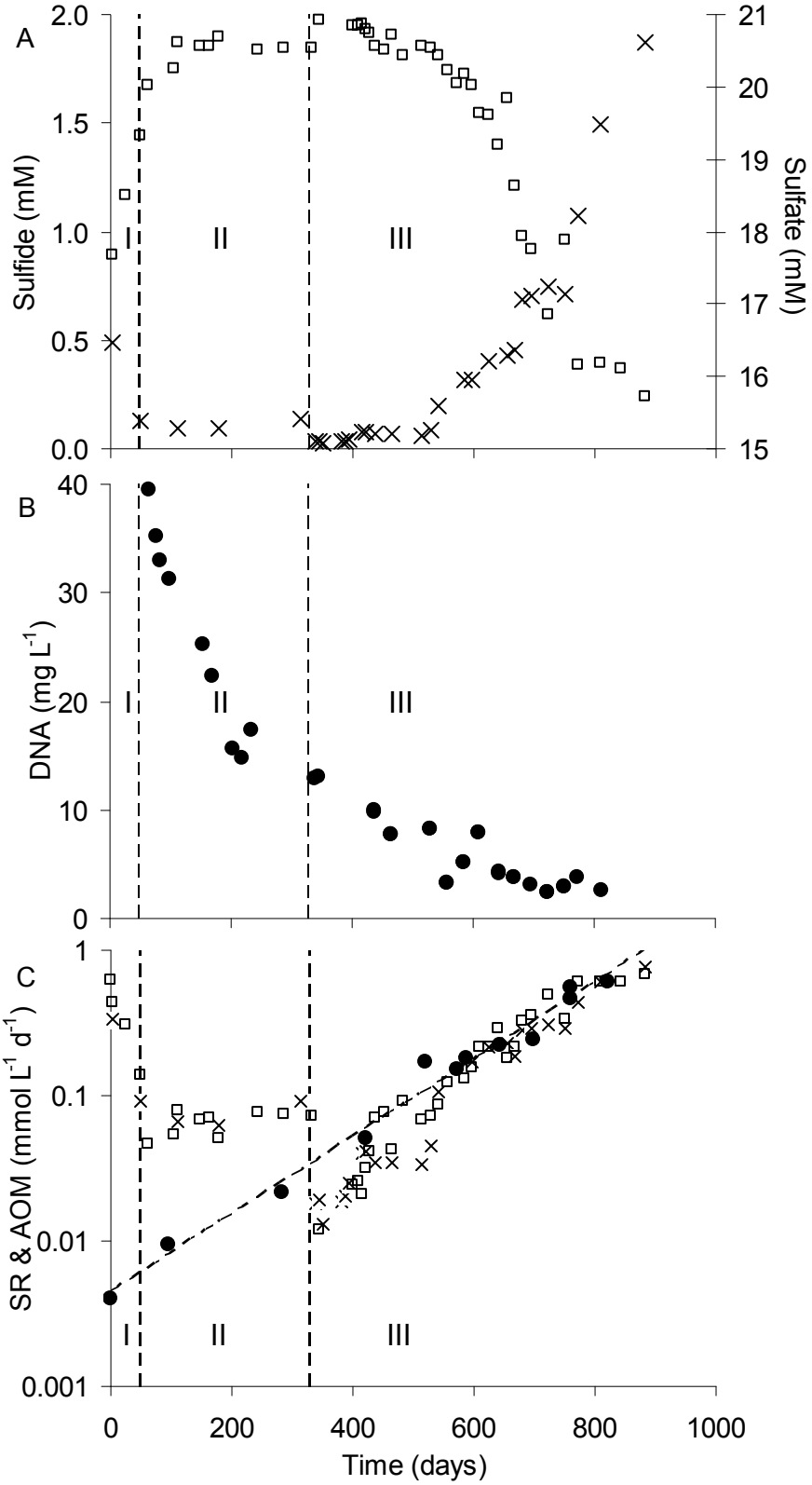
3.3.3 Biomass concentration and composition of R3

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 (Figure 3.4B), despite the 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 mater decomposition can be used as electron donor for SR, this resulted in the relative high SR rate during phase I (Figure 3.2). Maximum 8 mmol sulfate can be reduced from the 0.5 g VSS that was lost during the experiment, when a COD content of 1.07 g g_{VSS}^{-1} is assumed. 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.

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. The sequences of 91% of the obtained archaeal clones were most similar to ANME-2a sequences found by BLASTN analyses (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 (Figure 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).


A
Sulfide (mM)
Sulfate (mM)
2.0
1.5
1.0
0.5
0.0
21
20
19
18
17
16
15
I
II
III
B
DNA (mg L-1)
40
30
20
10
0
C
SR & AOM (mmol L-1 d-1)
1
0.1
0.01
0.001
0
200
400
600
800
1000
Time (days)

Previous page: 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). Three phases can be distinguished: 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.

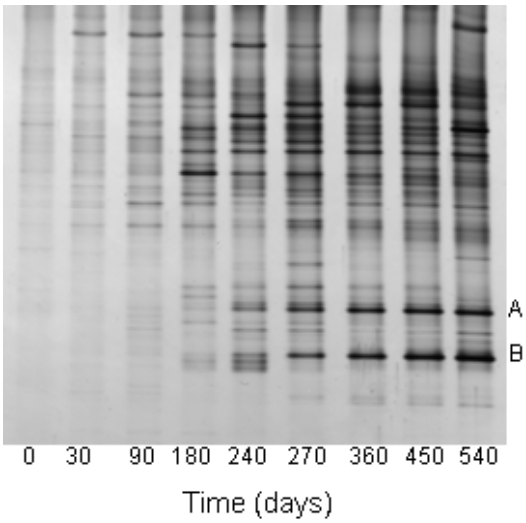


Figure 3.5. Analysis of changes in archaeal community over time, by 16S rRNA gene-targeted PCR-denaturing gradient gel electrophoresis, in a MBR inoculated with Eckernförde Bay sediment, fed with CH_4 and sulfate and operated at 15°C (R3). The sequences obtained from bands A and B can be affiliated with clones from the ANME-2a cluster.

Table 3.2. Phylogenetic summary based on clone library analysis of partial archaeal sequences from the MBR inoculated with Eckernförde Bay sediment, fed with CH_4 and sulfate and operated at 15°C (R3) after 809 days of operation (R3).

Repre-sentative 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%	ANME2a
R3-1A2	FJ210915	23	Clone Hyd24-Arch25 (AJ578107)	99%	ANME2a
R3-1A11	FJ210917	22	Clone GoM_GC232_4463_Arch65 (AM745238)	99%	ANME2a
R3-1E5	FJ210918	8	Clone IV.4.Ar15 (AY367329)	99%	ANME2a
R3-1D10	FJ210919	2	clone SBAK-mid-74 (DQ640234)	99%	ANME2a
R3-1B6	FJ210920	2	Clone WHA34-14 (AB426391)	95%	Methano-coccoides
R3-1G4	FJ210921	2	Methanomicrobiales archaeon 'SBAK-CO2-reducing Enrichment-4' (DQ280485)	99%	Methano-microbiales
R3-1A6	FJ210922	1	clone MOB7-2 (DQ841237)	98%	Methano-sarcinales
R3-1E8	FJ210923	1	Uncultured euryarchaeote EHB95 (AF374283)	97%	Methano-sarcinales
R3-1F5	FJ210924	1	clone SBAK-mid-25 (DQ522923)	96%	Marine Benthic Group –D
R3-1H9	FJ210925	1	clone ss017b (AJ969786)	91%	Thermoplasmat ales - 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.

3.4 Discussion

3.4.1 Bioreactor system

In Figure 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 enriching 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. Moreover, the biomass was 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 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 break up 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, MBRs can operated at suspended solid concentrations up to 31 $g_{dry\ weight}^{-1}$ L^{-1} (Stephenson *et al*, 2000).

3.4.2 Responsible microorganisms

The exponential increase in activity in R3 (Figure 4c) indicates growth. However, VSS content and DNA concentration (Figure 4B) decreased over time. This decrease indicates that the original sediment contained many microorganisms not involved in the exponential increase in AOM coupled to SR and that these microorganisms 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 (Figure 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 from R3 cluster in the ANME-2a subgroup. The dominance of ANME-2a clones in the archaeal clone library of R3 after 809 days of operation and the DGGE bands associated with ANME-2, indicate that ANME-2a were involved in the exponential increase in AOM coupled to SR. ANME-2 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.

3.4.3 Doubling time

Girguis *et al.* (2003; 2005), Nauhaus *et al.* (2007) and Krüger *et al.* (2008) also showed *in vitro* enrichment of anaerobic methanotrophs (Table 3.3). The difference in reported doubling times can be related to the inocula 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 previous reported values, but increase 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).

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	Golf of Mexico	Eckernförde Bay
Incubation technique	Continuous, prop flow	Fed-batch, not mixed	Batch, shaken once a week	Continuous, 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, dominated
Estimated doubling time (months)	1.1 (ANME-2) 1.4 (ANME-1)	7.5	2	3.8
Maximum AOM rate (µmol $g_{dry\ weight}^{-1}$.day^{-1})	0.1	230	13.5	286
Maximum AOM rate (mmol g_{VSS}^{-1} day^{-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

3.4.4 Temperature

The AOM and SR rates increased in both reactors operated at 15°C (Figure 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 (Figure 3.2A and 3.2B). However, there was some AOM activity at 30°C (Figure 3.2A and 3.2B). AOM by the original Eckernförde Bay sediment was only slightly lower at 28ºC than at 20ºC (at which the highest rates were obtained) (Treude *et al*., 2005). Possibly AOM at 30°C was not coupled to net growth because the energy required for maintenance at 30ºC was too high to conserve energy for growth. The maintenance

Gibbs free energy is positively related with the temperature; it doubles for every 10ºC increase (Tijshuis *et al*. 1993).

3.4.5 AOM activity assays

The AOM rates are estimated from the $\sum^{13}CO_2$ production in batch bottles to which only pure $^{13}CH_4$ and reactor liquid were added. Initially also $\sum^{12}CO_2$ was produced due to decomposition of particulate organic matter. As the endogenous activity dropped, the fraction $^{13}CO_2$ became higher. The natural isotopic signature of particulate organic matter is approximately 1.07% ^{13}C, therefore the decomposition contributed to the $\sum^{13}CO_2$ formation. However, because the fraction $\sum^{13}CO_2$ of the total CO_2 production was always at least 10 times higher than the natural isotopic signature, 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). CO_2 reduction during AOM might be similar to the 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 was probably 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). In this study, the activity assays were performed with 100% $^{13}CH_4$ 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 (Figure 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 (Figure 3.4C). This was related with a less accurate quantification of the sulfide production and sulfate removal, due to the small differences between influent and effluent concentrations in this phase.

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.

3.4.6 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 was to supply a carbon source, in case methane could not be used. However, this was not necessary, given the exponential increase in AOM and SR in R4 (to which no acetate was fed; Figure 3.2D) and in R3 after acetate was omitted (Figure 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 main 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.

3.5 Conclusions

The main conclusions of this work are:

- The doubling time of the microorganisms mediating AOM coupled to SR in a bioreactor inoculated with Eckernförde Bay sediment and operated at 15°C is 3.8 months. These microorganisms do not grow at 30°C.
- Sulfate reduction with methane as electron donor is possible in well-mixed bioreactors.
- A submerged-membrane bioreactor system is an excellent system to enrich slow-growing microorganisms, like ANME.

Acknowledgements

Anna Lichtschlag and Tina Treude from the MPI-Bremen are acknowledged for providing access to the Eckernförde Bay sediment. We thank the crew of the LITTORINA from the Leibniz-Institut für Meereswissenschaften for their excellent support with the sediment sampling.

Chapter 4

Microbial characterization of a methane-oxidizing sulfate-reducing enrichment

Abstract

Anaerobic oxidation of methane (AOM) is an important methane sink in the ocean, but the microbes responsible for AOM are resilient to cultivation. Here we describe the microbial characterization of an Eckernförde Bay enrichment capable of high rate AOM (286 μmol.$g_{dry\ weight}^{-1}$.day^{-1}) coupled to sulfate reduction (SR). By constructing a clone library 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 (20, 44 and 49%) and in archaeal lipids, archaeol and hydroxyl-archaeol (21 and 20%, respectively). Confirming that both archaea and bacteria are involved in AOM by the Eckernförde Bay enrichment.

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Christian G. Jagersma, Roel J.W. Meulepas, Ineke Heikamp-de Jong, Jarno Gieteling, Adam Klimiuk, Stefan Schouten, Jaap S. Sinninghe Damsté, Piet N.L. Lens, Alfons J.M. Stams. Microbial diversity and community structure of a highly active anaerobic methane oxidizing sulfate-reducing enrichment.

4.1 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). AOM coupled to SR and mediated by microorganisms has 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-1 and -2 clusters are closely affiliated with methanogenic archaea of the order of *Methanosarcinales* (Hinrichs *et al*., 1999; Orphan *et al*., 2001), whereas those of the ANME-3 cluster are related to *Methanococcoides* and *Methanolobus* sp (Niemann *et al*., 2006). 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 (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). 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 1 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 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 marine sediments. In these reactors, the number of ANME archaea increased, and the rate of AOM increased but did not exceed 140 nmol $g_{dry\ weight}^{-1}$ day^{-1}. 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 its performance are described elsewhere (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.

4.2 Materials and Methods

4.2.1 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). To enrich for anaerobic methanotrophs, a novel submerged-membrane bioreactor was developed (Chapter 3). The hydraulic retention time (HRT) was 7 days. The reactor was equipped with 4 polysulfone membranes. The mean pore size of 0.2 µm guaranteed complete cell retention. The bioreactor was kept anaerobic for the duration of the 884-day run. After 809 days of operation, samples of the reactor suspension (mixture of liquid and suspended solids in the bioreactor) were anaerobically taken and used for FISH analysis and to construct a clone library. In addition, batch incubations with ^{13}C-labeled methane and reactor suspension, taken after 570 days of reactor operation, were done.

4.2.2 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 (Table 1). 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 denaturing gradient gel electrophoresis (DGGE), 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 plus Uni515r-GC clamp (Table 1). The DNA clean and concentrator-5 kit (Zymo research) was used for the purification of almost full-length 16S rRNA gene fragments.

4.2.3 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 using the primers BACT-27f, Uni-515r, Uni-519f, BACT-1100r and Uni-1492r for eubacterial sequences and ARCH-4f, Uni-515r, ARCH-340f, ARCH-915r and Uni-1492r for Archaeal sequences (Table 4.1). 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 analyzed 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.

Table 4.1: 16S rRNA gene primers and probes used in this study

Primer /Probe	Name label	Sequence (5' to 3')[e]	Position[a]	Specificity	Formamide concentration (% vol/vol)[b]	Ref.
PCR primer	ARCH-4f	TCC GGT TGA TCC TGC CRG	4-21	Archaea	n.a.	Hales *et al*., 1996
PCR primer	BACT-27f	GTT TGA TCC TGG CTC AG	27-43	Eubacteria	n.a.	Lane, 1991
PCR primer	ARCH-340f[c]	CCC TAC GGG G(C/T)G CA(G/C) CAG	340–357	Archaea, V3 region	n.a.	Øvreås *et al*, 1997
PCR primer	Uni-515r	ATC GTA TTA CCG CGG CTG CTG GCA	515-538	Universal 16S rRNA gene	n.a.	Lane, 1991
PCR primer	Uni-519f	CAGC(A/C)GCCGCGG TAA(G/A/T/C)(A/T)C	519–533	Eubacteria	n.a.	Lane, 1991
PCR primer	ARCH-915f	GTG CTC CCC CGC CAA TTC CT	915–934	Archaea	n.a.	Stahl and Amann, 1991
PCR primer	BACT-1100r	GGG TTG CGC TCG TTG	1100–1117	Eubacteria	n.a.	Lane, 1991
PCR primer	Uni-1492r	CGG CTA CCT TGT TAC GAC	1492-1509	Universal 16S rRNA gene	n.a.	Lane, 1991
FISH Probe	ANME-IIa-647 (Cy3)	TCT TCC GGT CCC AAG CCT	647-664	ANME-IIa Euryarchaeota	50	Knittel *et al*, 2005
FISH Probe	ANME-I-350 (Cy3)	AGT TTT CGC GCC TGA TGC	350-367	ANME-I	40	Boetius *et al*, 2000
FISH Probe	DSS658 (Fluos[c])	TCC ACT TCC CTC TCC CAT	658-685	*Desulfosarcina* spp. *Desulfofaba* sp. *Desulfococcus* sp. *Desulfofrigus* sp.	60	Manz *et al*, 1998
FISH Probe	EELMS932 (Cy3/Fluos)	AGC TCC ACC CGT TGT AGT	932-949	ANME-II	40	Boetius *et al*, 2000
DGGE Primer	515r-GC[d]	GAT CGT ATT ACC GCG GCT GCT GGC A	-	-	n.a.	Lane, 1991
DGGE Primer	ARCH-109f	ACK GCT CAG TAA CAC GT	-	-	n.a.	Großkopf *et al*, 1998

[a] *Escherichia coli* 16S rRNA positions
[b] Concentration of formamide in *in situ* hybridization buffer
[c] Carboxyfluorescein-N-hydroxysuccinimide ester
[d] GC-Clamp sequence: CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG attached to 5' end of the primer (Øvreås, 1997)
[e] R = A or G, M = A or C, S = C or G, K = G or T
n.a. not availible

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 4.2 and 4.3). Table 4.2 shows the phylogenetic affiliation of the clones. All sequences described in this chapter have been deposited in the databases of Genbank, under accession numbers FJ555674-FJ555687 (archaeal sequences) and FJ615406-FJ615417 (eubacterial sequences).

4.2.4 ^{13}C-CH_4 incubation

Reactor suspension, sampled after 570 days of reactor operation, was incubated in 120 ml bottles under a 0.13 MPa ^{13}C-CH_4 headspace. To remove the accumulating 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 again filled with ^{13}C-CH_4. After three months, the biomass was sampled for lipid analyses.

Table 4.2: 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	*Delta proteobacteria*
23	34	*Bacteroidetes*
6	9	*Planctomycetes*
2	3	*Alpha proteobacteria*
2	3	Uncultured Chloroflexi
1	1	Uncultured *Spirochaetes*
1	1	*Gamma Proteobacteria*

4.2.5 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 hydrolyzed 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. Derivatized compounds were corrected for added methyl and trimethylsilylgroups.

4.2.6 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)

4.3 Results

In 884 days of reaction operation, the AOM rate in the bioreactor had increased exponentially from 0.004 to 0.6 mmol L^{-1} d^{-1}. The final rate was 286 µmol $g_{dry\ weight}^{-1}$ day^{-1} or 1.0 µmol g_{VSS}^{-1} day^{-1} (Chapter 3). Microscopic observations revealed that the biomass in the reactor, after 842 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 4.1).

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, Figure 4.2). The second most dominant group clustered in the *Thermoplasmatales* group of the *Euryarchaeota* (8%, N=172). 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 microorganisms belonging to the *Delta-proteobacteria* (50%, N=68) and the *Flavobacteriales* (34%, N=68; Figure 4.3). 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 4.1) 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. 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 reactor run, was incubated for three months under a pure 0.13 MPa ^{13}C-CH_4 headspace. Subsequently, 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 4.3). 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 4.3). These results confirm that both bacteria and archaea have incorporated label derived from methane into their biomass. The degree of labelling in some 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).

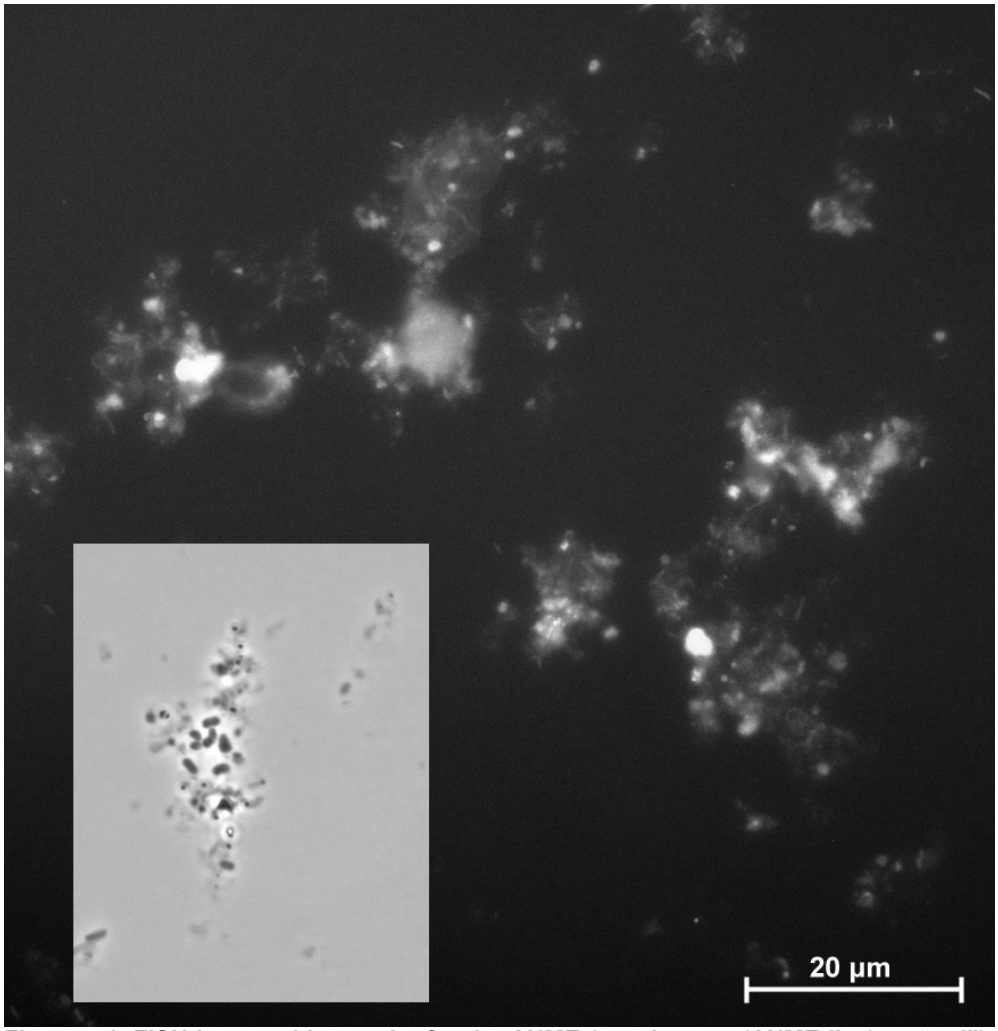


Figure 4.1. FISH image with a probe for the ANME-2a subgroup (ANME-IIa-647, "red") and probe for the DSS subgroup of the sulfate-reducing bacteria (DSS658, "green"), on the backside of this thesis part of the image is presented in color. Insert is a bright field microscopy image of a typical loose aggregate found in the bioreactor enrichment.

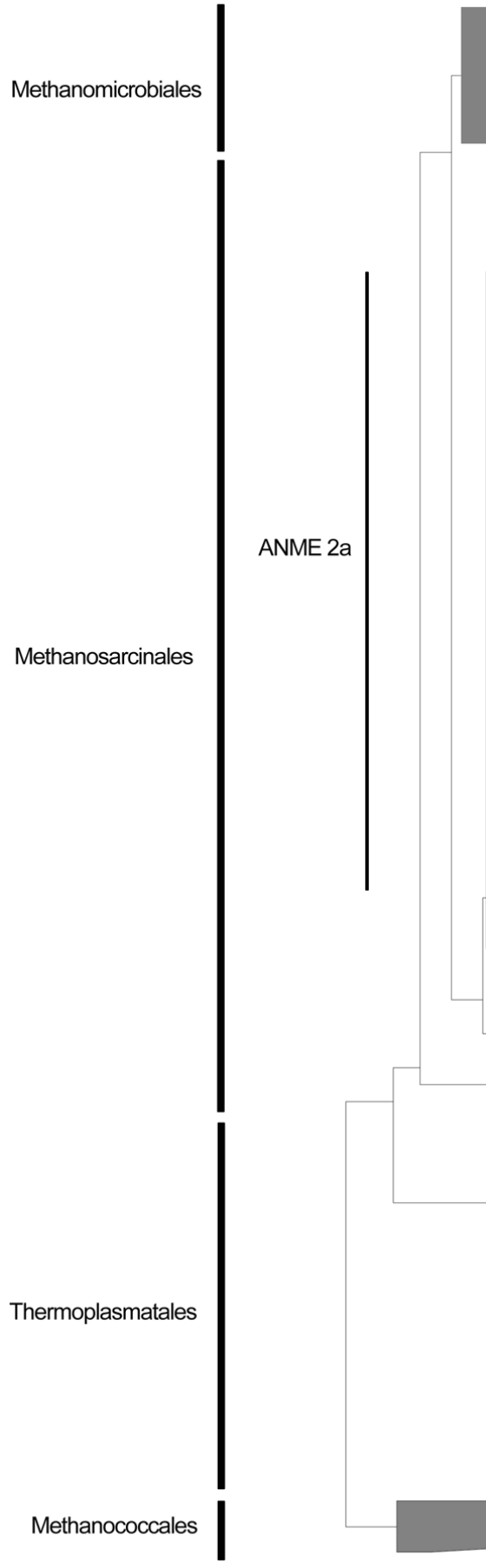
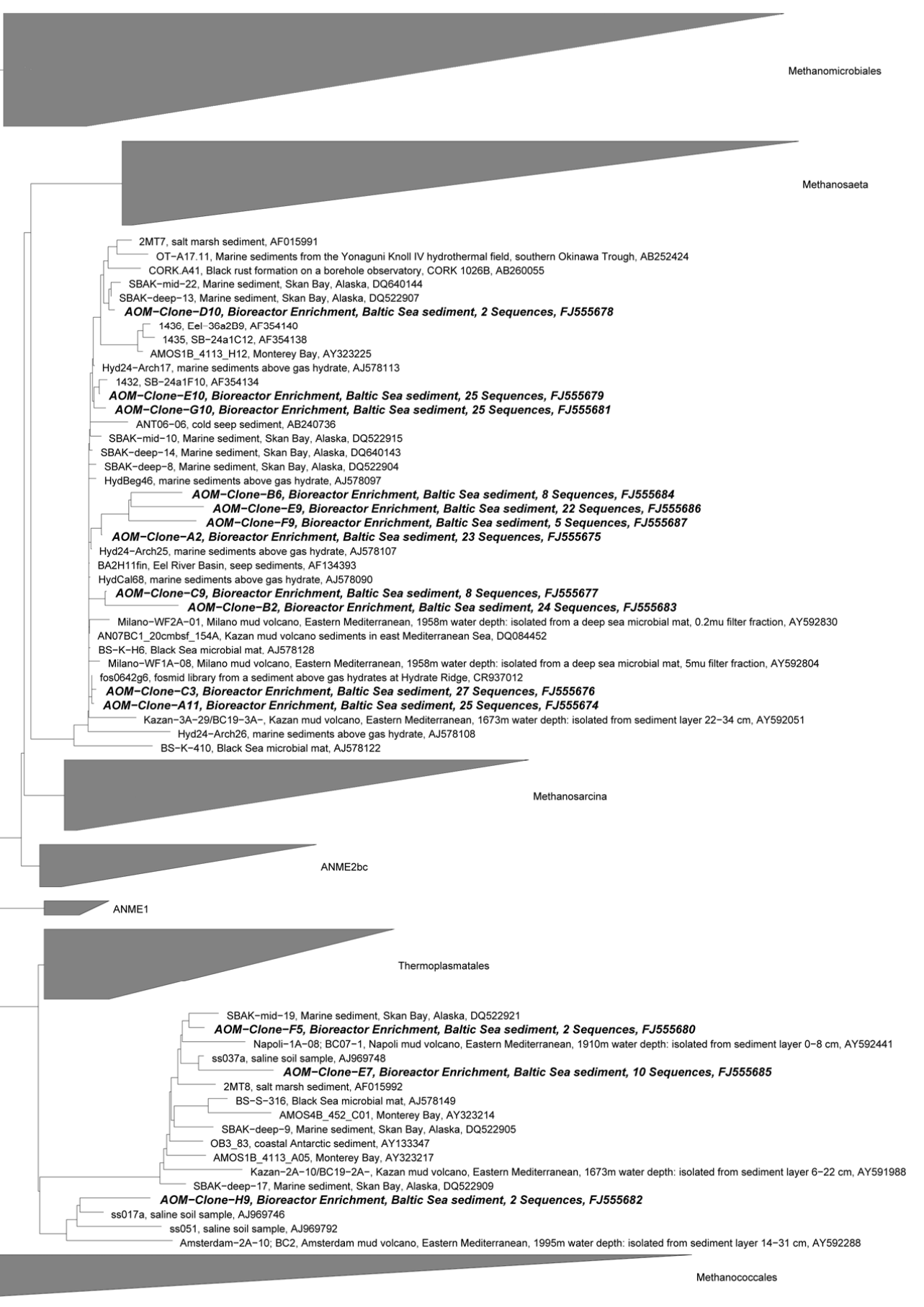


Figure 4.2. Phylogenetic tree showing the affiliation of archaeal 16S rRNA gene sequences (N=172 clones) retrieved from the submerged membrane bioreactor library (printed in boldfaced type) to selected reference sequences.


Methanomicrobiales
Methanosaeta
2MT7, salt marsh sediment, AF015991
OT−A17.11, Marine sediments from the Yonaguni Knoll IV hydrothermal field, southern Okinawa Trough, AB252424
CORK.A41, Black rust formation on a borehole observatory, CORK 1026B, AB260055
SBAK−mid−22, Marine sediment, Skan Bay, Alaska, DQ640144
SBAK−deep−13, Marine sediment, Skan Bay, Alaska, DQ522907
AOM−Clone−D10, Bioreactor Enrichment, Baltic Sea sediment, 2 Sequences, FJ555678
1436, Eel−36a2D9, AF354140
1435, SB−24a1C12, AF354138
AMOS1B_4113_H12, Monterey Bay, AY323225
Hyd24−Arch17, marine sediments above gas hydrate, AJ578113
1432, SB−24a1F10, AF354134
AOM−Clone−E10, Bioreactor Enrichment, Baltic Sea sediment, 25 Sequences, FJ555679
AOM−Clone−G10, Bioreactor Enrichment, Baltic Sea sediment, 25 Sequences, FJ555681
ANT06−06, cold seep sediment, AB240736
SBAK−mid−10, Marine sediment, Skan Bay, Alaska, DQ522915
SBAK−deep−14, Marine sediment, Skan Bay, Alaska, DQ640143
SBAK−deep−8, Marine sediment, Skan Bay, Alaska, DQ522904
HydBeg46, marine sediments above gas hydrate, AJ578097
AOM−Clone−B6, Bioreactor Enrichment, Baltic Sea sediment, 8 Sequences, FJ555684
AOM−Clone−E9, Bioreactor Enrichment, Baltic Sea sediment, 22 Sequences, FJ555686
AOM−Clone−F9, Bioreactor Enrichment, Baltic Sea sediment, 5 Sequences, FJ555687
AOM−Clone−A2, Bioreactor Enrichment, Baltic Sea sediment, 23 Sequences, FJ555675
Hyd24−Arch25, marine sediments above gas hydrate, AJ578107
BA2H11fin, Eel River Basin, seep sediments, AF134393
HydCal68, marine sediments above gas hydrate, AJ578090
AOM−Clone−C9, Bioreactor Enrichment, Baltic Sea sediment, 8 Sequences, FJ555677
AOM−Clone−B2, Bioreactor Enrichment, Baltic Sea sediment, 24 Sequences, FJ555683
Milano−WF2A−01, Milano mud volcano, Eastern Mediterranean, 1958m water depth: isolated from a deep sea microbial mat, 0.2mu filter fraction, AY592830
AN07BC1_20cmbsf_154A, Kazan mud volcano sediments in east Mediterranean Sea, DQ084452
BS−K−H6, Black Sea microbial mat, AJ578128
Milano−WF1A−08, Milano mud volcano, Eastern Mediterranean, 1958m water depth: isolated from a deep sea microbial mat, 5mu filter fraction, AY592804
fos0642g6, fosmid library from a sediment above gas hydrates at Hydrate Ridge, CR937012
AOM−Clone−C3, Bioreactor Enrichment, Baltic Sea sediment, 27 Sequences, FJ555676
AOM−Clone−A11, Bioreactor Enrichment, Baltic Sea sediment, 25 Sequences, FJ555674
Kazan−3A−29/BC19−3A−, Kazan mud volcano, Eastern Mediterranean, 1673m water depth: isolated from sediment layer 22−34 cm, AY592051
Hyd24−Arch26, marine sediments above gas hydrate, AJ578108
BS−K−410, Black Sea microbial mat, AJ578122
Methanosarcina
ANME2bc
ANME1
Thermoplasmatales
SBAK−mid−19, Marine sediment, Skan Bay, Alaska, DQ522921
AOM−Clone−F5, Bioreactor Enrichment, Baltic Sea sediment, 2 Sequences, FJ555680
Napoli−1A−08; BC07−1, Napoli mud volcano, Eastern Mediterranean, 1910m water depth: isolated from sediment layer 0−8 cm, AY592441
ss037a, saline soil sample, AJ969748
AOM−Clone−E7, Bioreactor Enrichment, Baltic Sea sediment, 10 Sequences, FJ555685
2MT8, salt marsh sediment, AF015992
BS−S−316, Black Sea microbial mat, AJ578149
AMOS4B_452_C01, Monterey Bay, AY323214
SBAK−deep−9, Marine sediment, Skan Bay, Alaska, DQ522905
OB3_83, coastal Antarctic sediment, AY133347
AMOS1B_4113_A05, Monterey Bay, AY323217
Kazan−2A−10/BC19−2A−, Kazan mud volcano, Eastern Mediterranean, 1673m water depth: isolated from sediment layer 6−22 cm, AY591988
SBAK−deep−17, Marine sediment, Skan Bay, Alaska, DQ522909
AOM−Clone−H9, Bioreactor Enrichment, Baltic Sea sediment, 2 Sequences, FJ555682
ss017a, saline soil sample, AJ969746
ss051, saline soil sample, AJ969792
Amsterdam−2A−10; BC2, Amsterdam mud volcano, Eastern Mediterranean, 1995m water depth: isolated from sediment layer 14−31 cm, AY592288
Methanococcales

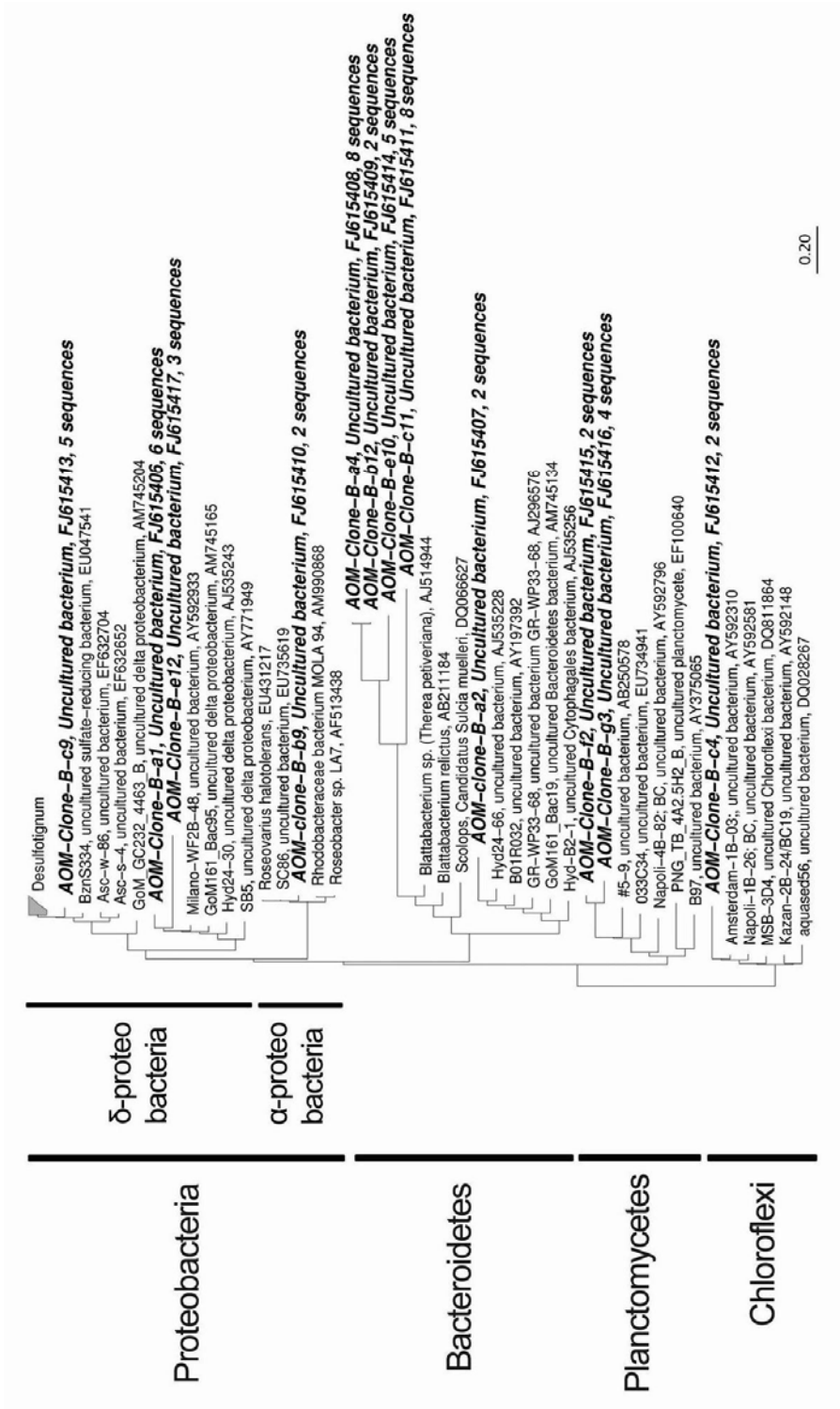


Figure 4.3. Phylogenetic tree showing the affiliation of eubacterial 16S rRNA gene sequences (N=68 clones) retrieved from the submerged membrane bioreactor library (Printed in boldfaced type) to selected reference sequences.

Table 4.3. Relative abundance and degree of ^{13}C labeling of bacterial and archaeal lipids in the lipid extract of the bioreactor enrichment.

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
Archaeal	1.3	21.3
SN2-hydroxyarchaeol	0.9	20.1

FA = fatty acid

4.4 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 the clone library analysis. The presence of *Delta-proteobacteria* can be explained by the fact that the known SRB capable of growth under mesophilic conditions belong to the *Delta-proteobacteria*. 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. 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 *Beta-proteobacteria*, most similar to members of the *Burkholderiaceae*, and *Alpha-proteobacteria*, related to *Sphingomonas*, (Pernthaler *et al*., 2008). Other clones from the bioreactor enrichment can be linked to known marine micro-microorganisms and because of their low abundance after more than 800 days of continuous incubation, they are most probably residual microorganisms 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 microorganisms 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 CO_2 or organic compounds produced by ANME-2a from $^{13}CH_4$. 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 coupled to denitrification occurred, although later it was found that archaea were not involved (Ettwig *et al*., 2008).

4.5 Conclusions

The main conclusions of this work are:

- Both archaea and bacteria are involved in AOM coupled to SR by the Eckernförde Bay enrichment.
- The archaea in the Eckernförde Bay enrichment belong predominately to the ANME-2a subgroup of anaerobic methanotrophs.
- ANME-2a cells occur as single cells without direct contact to a bacterial partner.

Chapter 5

Effect of environmental conditions on sulfate reduction with methane as electron donor

Abstract

Sulfate reduction (SR) coupled to anaerobic oxidation of methane (AOM) is meditated by marine microorganisms and forms an important process in the global sulfur and carbon cycle. In this research, the possibility to use this process for the removal and recovery of sulfur and metal compounds from waste streams was investigated. A membrane bioreactor was used to enrich methane-oxidizing sulfate-reducing microorganisms from Eckernförde Bay sediment. The SR rate of the obtained enrichment was 1.0 mmol g_{VSS}^{-1} day^{-1}. The operational window and optimal environmental conditions for SR with methane as electron donor were assessed. The optimum pH, salinity and temperature were 7.5, 30‰ and 20°C, respectively. The enrichment had a good affinity for sulfate ($K_m \leq 1.0$ mM) and a low affinity for methane ($K_m > 75$ kPa). AOM coupled to SR was completely inhibited at 2.4 (±0.1) mM sulfide. AOM occurred with sulfate, thiosulfate and sulfite as electron acceptors. Sulfate reduction with methane as electron donor can be applied for the removal of sulfate or for the production of sulfide, for metal precipitation. However, the low optimal temperature and the high salt requirement limit the operational window of the process.

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Roel J.W. Meulepas, Christian G. Jagersma, Ahmad F. Khadem, Cees J.N. Buisman, Alfons J.M. Stams en Piet N.L. Lens. Effect of environmental conditions on sulfate reduction with methane as eectron donor by an Eckernförde Bay enrichment

5.1 Introduction

5.1.1 Sulfate reduction with methane as electron donor in marine sediments

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) in marine sediments contributes for a large part to the removal of the greenhouse gas methane (CH_4) (Reeburgh, 1996; Hinrichs and Boetius, 2002). Thus far, the highest AOM rates have been reported for Hydrate Ridge and Black Sea sediments, 2-8 µmol $g_{dry\ weight}^{-1}$ day^{-1} (Krüger *et al*., 2005) and 8-21 µmol $g_{dry\ weight}^{-1}$ day^{-1} (Treude *et al*., 2007), respectively. Uncultured Archaea, called anaerobic methanotrophs (ANME), often living in consortia with sulfate-reducing bacteria (SRB) were found to be involved in AOM coupled to SR (Hinrichs *et al.*, 1999; Boetius *et al*., 2000; Orphan *et al*., 2002; Knittel *et al*., 2005).

5.1.2 *In vitro* studies

In vitro studies have demonstrated the coupling between AOM and SR according to conversion 1 (Table 1) (Nauhaus *et al.*, 2002) and have shown that the exponential increase in AOM activity is coupled to growth of ANME/SRB consortia (Nauhaus *et al.*, 2007). Reported doubling times vary from 1 to 7 months (Girguis *et al*., 2005; Nauhaus *et al*., 2007; Krüger *et al*., 2008; Chapter 3). Nauhaus *et al*. (2005a) and Treude *et al*. (2005) investigated the effect of temperature on SR by Hydrate Ridge, Black Sea and Eckernförde Bay sediment, the optimal temperatures are respectively: 16°C or lower, around 20°C, and between 20 and 28°C. AOM is enhanced by applying higher CH_4 partial pressures (Nauhaus *et al*., 2005a). Mn (IV), Fe (III), S° and fumarate are not used as electron acceptor for AOM by Hydrate Ridge sediment (Nauhaus *et al*., 2005a).

5.1.3 Application of sulfate reduction with methane as electron donor

Sulfate reduction with other electron donors than CH_4, like hydrogen, ethanol and acetate, is well understood (Widdel, 2007; Muyzer and Stams, 2008) and is an established biotechnological process (Weijma *et al.*, 2002; Lens *et al*., 2002; Liamleam and Annachhatre, 2007; Kaksonen. and Puhakka, 2007). Biological sulfate reduction can prevent the emission of toxic metals and oxidized sulfur compounds that would acidify the environment. Dissolved metals can be removed by precipitation with biologically produced sulfide. The formed insoluble metal sulfides can be separated from the water and reused in the metallurgical industry (Weijma *et al.*, 2002). Oxidized sulfur compounds can be converted to the insoluble and reusable elemental sulfur by subsequently applying biological

sulfate reduction and partial sulfide oxidation (Janssen *et al.*, 2001). Wastewaters rich in oxidized sulfur compounds are produced in flue gas desulfurization and in industries that use sulfuric acid or sulfate-rich feedstock, e.g. fermentation or sea food processing industry (Lens *et al*., 1998), and wastewater containing both sulfur and metal compounds are produced in the mining and the metallurgical industry.

However, the costs of the electron donor limit the application of biological sulfate reduction. Of the conventional electron donors, hydrogen is the most attractive for large-scale applications (van Houten, 1996). Hydrogen is commonly derived from synthesis gas, produced by steam reforming CH_4 (natural gas). Natural gas is 2 to 4 times cheaper than hydrogen per amount needed for sulfate reduction (Mueller-Langer et al., 2007). The operation costs of the treatment plant will thus be significantly reduced if CH_4 could be fed directly to sulfate-reducing bioreactors.

5.1.4 Current research

To assess the potential of CH_4 as electron donor for biological sulfate reduction in industrial applications, ANME were enriched in a membrane bioreactor (Chapter 3), the obtained CH_4-oxidizing sulfate-reducing enrichment had an activity of 1.0 mmol g_{VSS}^{-1} day^{-1}. In this research, the operational window for AOM coupled to SR by this enrichment was assessed. The effect of the temperature, pH, salinity, CH_4 partial pressure, sulfate concentration, dissolved inorganic carbon concentration and sulfide concentration, on the AOM and SR rate of the CH_4-oxidizing sulfate-reducing enrichment was investigated. Additionally, alternative electron acceptors for sulfate were tested. AOM was quantified from the production of $\sum^{13}CO_2$ (=^{13}C-labeled CO_2 + ^{13}C-labeled HCO_3^-) during incubation with ^{13}C-labeled CH_4 ($^{13}CH_4$). Labeled CH_4 was used to prevent an overestimation of the AOM rate due endogenous carbon dioxide production.

5.2 Materials and methods

5.2.1 Eckernförde Bay enrichment

The biomass used for this research was taken from a 1-L submerged-membrane bioreactor, used to enrich anaerobic methanotrophs. The reactor was inoculated with 10 $g_{dry\ weight}$ sediment from Eckernförde Bay (Baltic Sea) and fed with sulfate as electron acceptor and CH_4 as electron donor and carbon source. During these 884 days, the volumetric CH_4 oxidation rate increased exponentially from 0.002 to 0.6 mmol L^{-1} day^{-1} (Chapter 3). The

AOM and SR rate of the obtained enrichment were both 1.0 mmol g_{VSS}^{-1} day^{-1}. Molecular analyses of the reactor suspension (mixture of liquid and suspended solids in the bioreactor) revealed that ANME became the dominant archaea (Chapters 3 and 4). Samples of the reactor suspension used for this research were taken between day 420 and day 884 of the bioreactor run. 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. A biomass stock was made by collecting the reactor suspension in a bottle filled with nitrogen gas. After the solids were allowed to settle, the liquid could be removed with syringe and needle. In this way, the biomass was concentrated 15 times and washed 2 times with fresh medium.

5.2.2 Standard incubations procedure

The preparation procedure and the composition of the basal medium are described in Chapter 3. Unless stated otherwise, the medium was buffered at pH 7.4 and the salinity and sulfate concentration of the medium were 30‰ and 21 mM, respectively.

Experiments were done in serum bottles of 35 ml closed with butyl rubber stoppers and caps. After determination of the exact weight and volume, the bottles were flushed for eight times with nitrogen gas and made vacuum. Subsequently, 28 mL medium and 2 ml from the biomass stock were transferred to the bottles by syringe. The headspaces were made vacuum again and filled with 0.13 MPa ^{13}C-labeled CH_4 ($^{13}CH_4$) with a purity of 99% from Campro (Veenendaal, the Netherlands). The bottles were incubated shaken at 15°C, unless stated otherwise. The gas composition, the pH, the pressure and the liquid and gas volume were measured once a week. Liquid samples were taken once every two weeks and used for sulfide and sulfate analyses, after filtering over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany).

5.2.3 Experimental set-up

To assess the effect of temperature, pH and salinity on the conversion rate of the CH_4-oxidizing sulfate-reducing enrichment, incubations were done at 15, 20, 25 and 30°C; a pH of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0; and a salinity of 4, 10, 20, 30, 40 and 50‰. To obtain these conditions, the following modifications were done to the basal medium and the standard incubation procedure. The pH was altered by adding NaCl or HCl solution (1.0 M) to the individual bottles till the final pH was reached. The salinity was varied by mixing medium from which all salts, other than magnesium sulfate, were omitted with medium with a salinity of 50‰.

To assess the effect of CH_4, sulfate and total dissolved inorganic carbon ($\sum CO_2$) concentrations, incubations with different CH_4 partial pressures (from 0.00 to 0.15 MPa), sulfate concentrations (0.5, 2, 5, 10 and 20 mM) and $\sum CO_2$ concentrations (0.5, 2, 10 and 25 mM) were done. To obtain the different CH_4 partial pressures, nitrogen and $^{13}CH_4$ gas were sequentially added from pressurized bottles. The sulfate concentration was adjusted by adding sulfate from a stock solution (1.0 M) to sulfate-free medium. To obtain the different $\sum CO_2$ concentrations, HCO_3^- (from a 1 M stock) and CO_2 (from a pressurized bottle) were added. The amount of gas added could be controlled by a reduction valve.

To assess the effect of sulfide, sulfide was allowed to accumulate in two bottles with standard medium and 0.15 MPa $^{13}CH_4$. After the accumulation stopped, the sulfide was removed by flushing the bottle with CH_4 gas. Subsequently, the bottles were incubated again to test whether the inhibition was reversible.

Nitrate (10 mM), sulfur (0.5 mg L^{-1}), sulfite (10 mM) and thiosulfate (10 mM) were tested as potential alternative electron acceptors for sulfate. For these experiments, sulfate-free medium was used. Elemental sulfur was added before closing the bottles and other electron acceptors were added from stock solutions (1.0 M).

5.2.4 Calculations and estimation conversion rates

The volumetric AOM rate, sulfate removal rate and sulfide production rate are estimated from: the increase in $\sum^{13}CO_2$, the decrease in sulfate concentration and the increase in sulfide concentration over time, respectively. A line was plotted over the period were the increase or decrease was linear; at least four successive measurements were used. The analytical procedures and the $\sum^{13}CO_2$ calculation are described in Chapter 3.

5.3 Results

5.3.1 Effect of temperature, pH and salinity

Simultaneous and stoichiometric AOM and SR by the Eckernförde Bay enrichment were observed at all tested temperatures (15-30ºC), at a pH between 6.5 and 9.0 and at a salinity between 10‰ and 50‰ (Figure 5.1). At a pH of 6.0 and a salinity of 4‰, no AOM or SR was observed. The conversion was highest at a temperature of 20°C, a pH of 7.5 and a salinity of 30‰. The incubations to determine the effect of temperature were done at optimal pH and salinity, but the incubations to determine the effect of pH and salinity were done at

suboptimal temperature (15°C). Therefore, the maximum rates in Figure 5.1B and 5.1C are lower than in Figure 5.1A.

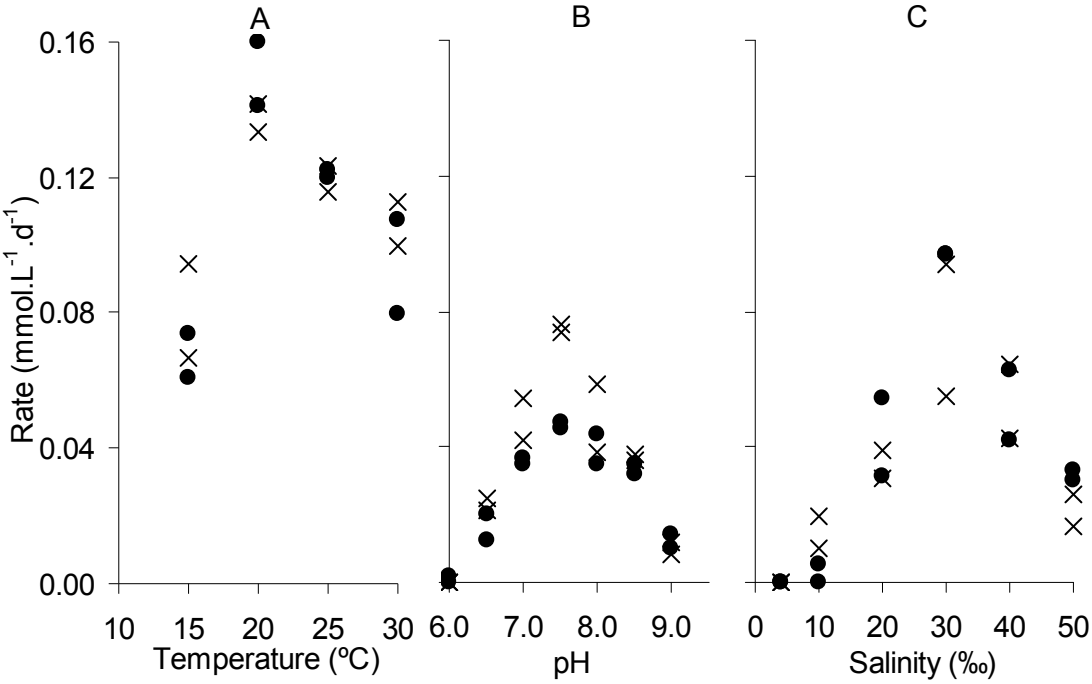

Figure 5.1. Effect of temperature (A), pH (B) and salinity (C) on the AOM (●) and sulfide production (X) rates, over a period of 28 days, by the Eckernförde Bay enrichment. The bottles contained initially 0.13 (±01) MPa $^{13}CH_4$ as sole energy and carbon source, 19 (±1) mM sulfate as sole electron acceptor and less than 0.2 mM $\sum CO_2$ and sulfide. The standard temperature, pH and salinity were 15°C, 7.4 and 30‰, respectively.

5.3.2 Effect of the methane, sulfate, $\sum CO_2$ and sulfide

Figure 5.2 presents the AOM and SR rates, of the Eckernförde Bay enrichment, for different initial CH_4 partial pressures, sulfate concentrations and $\sum CO_2$ concentrations. A more or less linear correlation between the CH_4 partial pressure and the AOM and SR rates, over the tested range, was observed. This indicates that the maximum conversion rate can be found at much higher CH_4 partial pressures. The Michaelis constant (K_m) for CH_4 is therefore more than half of the maximum CH_4 partial pressure tested, which is 75 kPa.

Above a sulfate concentration of 2.0 mM, the AOM and SR rates were independent on the sulfate concentration, indicating that the K_m for sulfate is 1 mM or less. Only at the lowest sulfate concentration tested (0.5 mM), the AOM and SR rates were affected. In those incubations, sulfate was almost completely converted, the final concentrations were 0.045 and 0.052 mM.

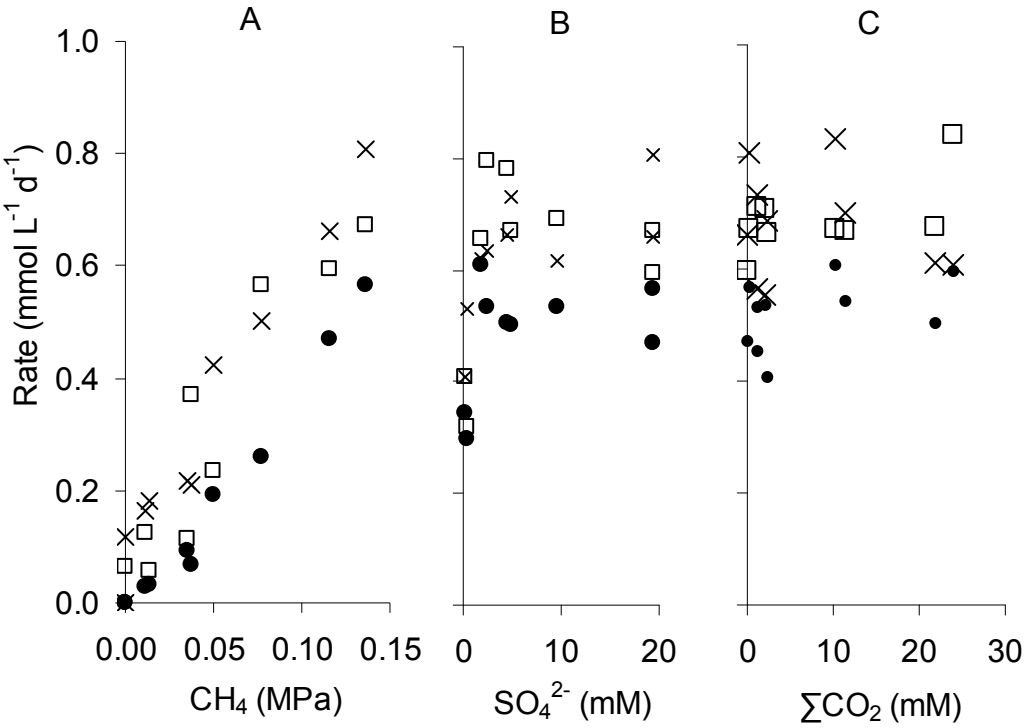

Figure 5.2. The effect of the CH_4 partial pressure (A), sulfate concentration (B) and dissolved $\sum CO_2$ concentration (C) on the AOM (●), sulfide production (X) and sulfate removal (□) rates over a period of 28 days by the Eckernförde Bay enrichment with $^{13}CH_4$ as sole energy and carbon source and sulfate as sole electron acceptor. If not varied, the CH_4 partial pressure and sulfate concentration were initially 0.13 (±0.01) MPa and 19 (±1) mM, respectively. Both the initial $\sum CO_2$ and sulfide concentration were less than 0.2 mM.

In the incubations where sulfide was allowed to accumulate, maximum dissolved sulfide concentrations of 2.4 (±0.1) mM (N=4) were reached, after which both AOM and SR stopped

(Figure 5.3). This inhibition was reversible, as the conversion started again after removing the sulfide on day 57 by flushing the liquid with CH_4 gas. As a result of the stripping of H_2S and CO_2 from the liquid, the alkalinity increased from 7.5 to 7.9, which was compensated by adding HCl. The inhibitory effect must have been caused by sulfide, since incubations with $\sum CO_2$ concentrations up to 23 mM did not show an inhibitory effect (Figure 5.2C).

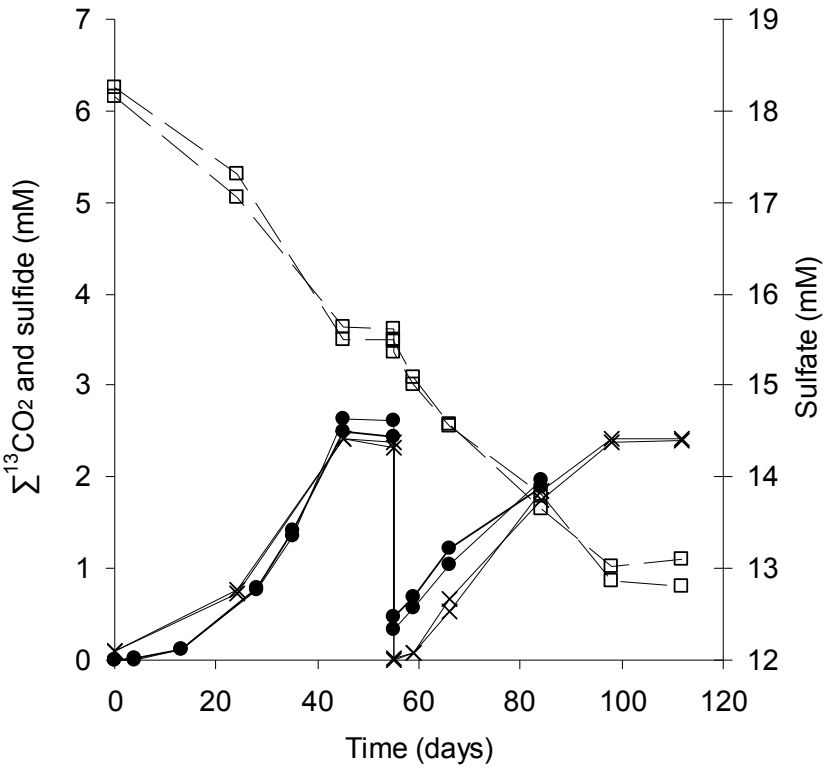

Figure 5.3. $\sum^{13}CO_2$ (●), sulfide (X) and sulfate (□) concentrations over time in two duplicate batch bottles inoculated with the Eckernförde Bay enrichment and with initially 0.13 (±01) MPa $^{13}CH_4$ in the headspace as sole energy and carbon source. On day 57 the liquid was flushed with CH_4 gas.

5.3.3 Alternative electron acceptors for AOM

CH_4 was oxidized by the Eckernförde Bay enrichment in the presence of sulfate, thiosulfate, sulfite and sulfur as sole available electron acceptors (Figure 5.4). AOM with sulfite proceeded approximately 5 times slower than with thiosulfate or sulfate. Even smaller amounts of CH_4 were oxidized in the incubations with sulfur. CH_4 was not oxidized by the Eckernförde Bay enrichment in the presence of nitrate as sole electron acceptor, nor was nitrate removed (Figure 5.4).

The utilized sulfite and sulfur were not completely reduced, but also partly oxidized, as sulfate was produced (Figure 5.5). In addition, in the bottles without CH_4, thiosulfate and sulfite are removed as well, resulting in both sulfide and sulfate production (Figure 5.5). This can be explained by disproportionation, according to conversions 6, 7 and 8 (Table 5.1). In the presence of CH_4 and, sulfate, thiosulfate, sulfite or sulfur, the sulfide production is higher than the sulfide production based on disproportionation alone (Figure 5.5), there is net reduction (Figure 5.4). For sulfate, thiosulfate and sulfite, the net reduction can be coupled to CH_4 oxidation, according to conversions 2 and 3 (Table 5.1). In the incubations with sulfur, the net reduction was about half of what can be expected based on the CH_4 oxidation rate.

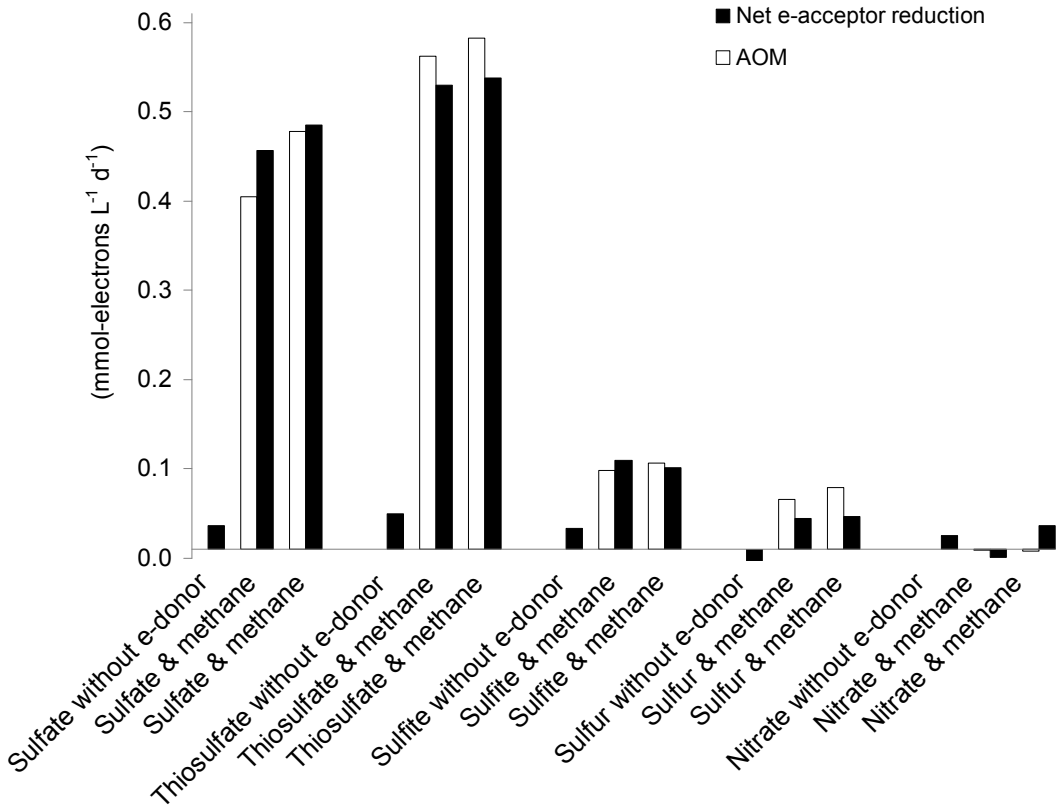

Figure 5.4. Consumption and production of reducing equivalents in bottles with different electron acceptors and with or without CH_4.

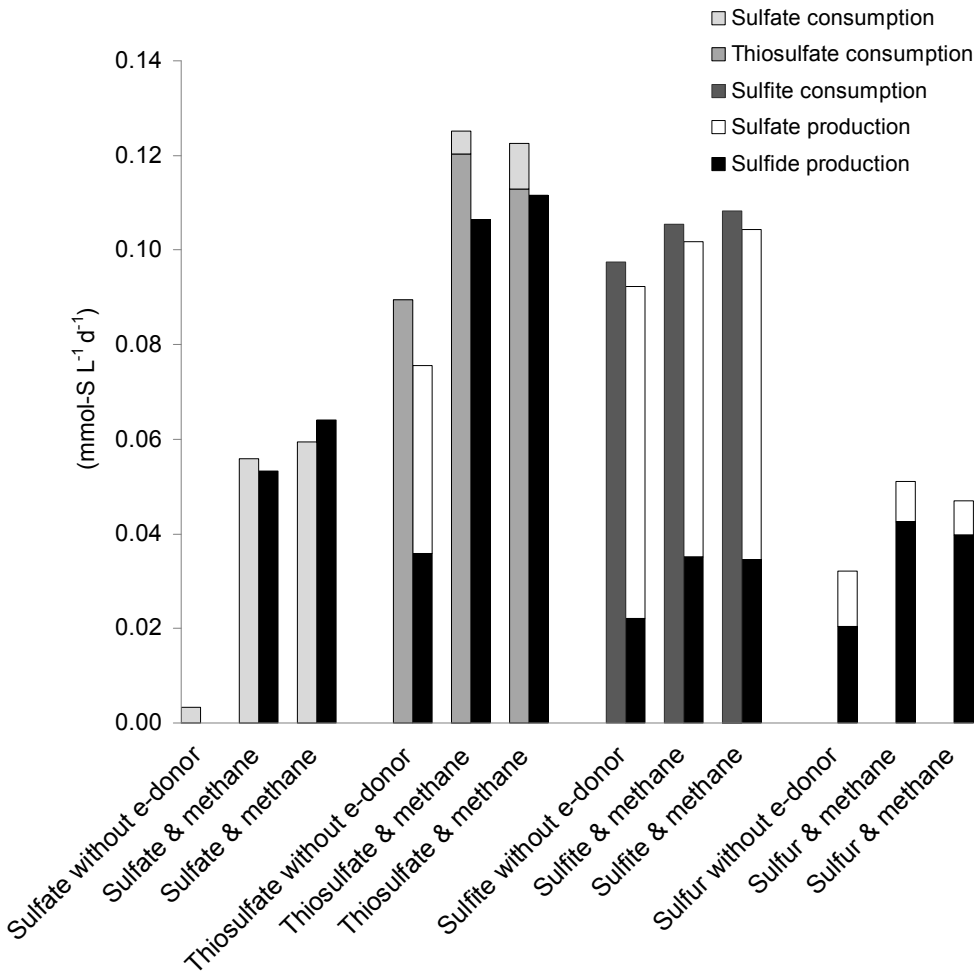

Figure 5.5. Consumption and production of sulfur-compounds in bottles with different electron acceptors and with or without CH_4.

Table 5.1. Reduction and disproportionation conversions of oxidized sulfur compounds, and their standard Gibbs free energy changes at pH 7.0 (∆G°'). Gibbs free energy changes were obtained from Thauer *et al*. (1977).

Conversion	Stoichiometry	∆G°'
1	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	-16.6 kJ mol^{-1} CH_4
2	$CH_4 + S_2O_3^{2-} \rightarrow HCO_3^- + 2\ HS^- + H^+$	-39 kJ mol^{-1} CH_4
3	$CH_4 + 4/3\ SO_3^{2-} + 1/3\ H^+ \rightarrow HCO_3^- + 4/3\ HS^- + H_2O$	-95 kJ mol^{-1} CH_4
4	$CH_4 + 4\ S° + 3\ H_2O \rightarrow HCO_3^- + 4\ HS^- + 5\ H^+$	+24 kJ mol^{-1} CH_4
5	$CH_4 + 8/5\ NO_3^- + 8/5\ H^+ \rightarrow HCO_3^- + 4/5\ N_2 + H_2O$	-765 kJ mol^{-1} CH_4
6	$4\ SO_3^{2-} + H^+ \rightarrow\ 3\ SO_4^{2-} + HS^-$	-236 kJ mol^{-1} SO_3^{2-}
7	$S_2O_3^{2-} + H_2O \rightarrow\ SO_4^{2-} + HS^- + H^+$	-22 kJ mol^{-1} $S_2O_3^{2-}$
8	$4\ S° + 4\ H_2O \rightarrow\ SO_4^{2-} + 3\ HS^- + 5\ H^+$	+40 kJ mol^{-1} S°

5.4 Discussion

5.4.1 Effect of temperature, pH and salinity on AOM and SR

The AOM and SR rate of the Eckernförde Bay enrichment were optimal at a temperature around 20°C. Treude *et al*. (2005a) found a similar temperature optimum and range for Eckernförde Bay sediment. Although the AOM mediating microorganisms are still active at 30°C, attempts to grow CH_4-oxidizing sulfate-reducing microorganisms at 30°C in membrane bioreactors inoculated with Eckernförde Bay sediment were not successful (Chapter 3). ANME-2 were shown to be involved in AOM in Eckernförde Bay sediment (Treude *et al*., 2005a) and by the Eckernförde Bay enrichment (Chapter 3 and 4). ANME-2 archaea are more adapted to low temperatures than ANME-I (Nauhaus *et al.*, 2005). For biotechnological application, the low temperature optimum forms a limitation, as many industrial wastewaters are warmer than 20°C. However, in many countries legislation requires treated wastewater to be cooled before discharge. Moreover, if the wastewater is cooled in a heat exchanger the energy loss can be minimized.

The pH and salinity optima found in this study (7.5 and 30‰, respectively) are what can be expected for marine microorganisms, although at the sampling site the salinity in the top 30 cm of the sediment column was a bit lower, between 15 and 20‰ (Treude *et al*., 2005a). Due to the high salinity requirement, wastewaters low in salts (other than sulfate) can not be treated with the biomass investigated in this study. However, for applications in which the

liquid is recirculated (e.g. flue gas desulfurization), a high salinity optimum is an advantage, since salts accumulate in such systems.

Many sulfate and metal containing wastewaters are acid (Weijma *et al.*, 2002; Kaksonen. and Puhakka, 2007). However, below a pH of 6.5, H_2S and CO_2 will be the main products of sulfate reduction, instead of HS^- and HCO_3^-. This will result in the generating of alkalinity. Therefore, a sulfate-reducing bioreactor fed with acidic wastewater, can often be maintained at a neutral pH.

5.4.2 Effect of the methane partial pressure on AOM and SR

The positive relation between AOM rates and the CH_4 partial pressure was also found by Krüger *et al*. (2005), Nauhaus *et al.* (2005) and Kallmeyer and Boetius (2004), even up to pressures up to 45 MPa. This implies that ANME archaea at ambient pressure are always limited by the CH_4 availability. Shima and Thauer (2005; 2008) showed that the activity of the methyl-CoM reductase involved in AOM depends on the CH_4 concentration. In industrial applications, the availability of CH_4 for the microorganisms can be optimized by applying thorough mixing, e.g. by CH_4 gas recirculation, since this improves the contact between the CH_4 gas and the biomass.

5.4.3 Effect of sulfate, $\sum CO_2$ and sulfide concentrations on AOM and SR

The ability of the CH_4-oxidizing sulfate-reducing enrichment to remove sulfate almost completely (down to 0.05 mM), shows that a sulfate removal process with CH_4 as electron donor is possible. For sites with legislation on sulfate emissions, the maximum discharge concentration for sulfate is much higher than 0.05 mM, around 200 mg L^{-1} or 2 mM. Measurements in the Black Sea sediment (Neretin *et al.*, 2004) and Baltic Sea (Knab *et al.*, 2008) showed a residual sulfate concentration of a few hundred µM or less beneath the sulfate to CH_4 transition zone. Compared to many AOM mediating sediments, the Eckernförde Bay enrichment has a good affinity for sulfate, but a low tolerance for sulfide. Sulfide levels in CH_4 seeps can reach up to 10 mM (Joye *et al.*, 2004) or 15 mM (Valentine, 2002). Also in *in vitro* incubations of hydrate ridge sediment, sulfide accumulated to 14 mM (Nauhaus, 2005). During the 3 years of enrichment, in the CH_4 and sulfate fed membrane bioreactor operated at 15°C, the sulfide concentration remained below 2.0 mM due to stripping. Therefore, microorganisms with a low tolerance for sulfide could have become dominant. The low tolerance for sulfide forms no problem in applications in which dissolved sulfide is continuously removed, e.g. by stripping or due to precipitation with metals.

5.4.4 Alternative electron acceptors for AOM

The Eckernförde Bay enrichment was able to utilize sulfate, thiosulfate and sulfite as electron acceptors for CH_4 oxidation. It is possible that thiosulfate or sulfite were not utilized directly for AOM, but that the sulfate produced by disproportionation was utilized by the CH_4-oxidizing sulfate-reducing community. Most sulfate reducers can use thiosulfate and sulfite as substrates (Widdel, 2008) though, as these compounds are intermediates in the sulfate reduction pathway (Widdel and Hansen, 1980). The Gibbs free energy change that can be obtained from CH_4 oxidation coupled to thiosulfate or sulfite reduction is larger than that with sulfate (Table 5.1). Thiosulfate and sulfite do not need to be activated at the cost of one ATP, like sulfate. The methyl-coenzyme M reductase, of which an analogue was shown to be involved in AOM (Hallam *et al*., 2003; Kruger *et al*., 2003) was shown to be inhibited by sulfite (Mahlert *et al*., 2002). Possibly this inhibitory effect of sulfite resulted in lower rates than obtained with thiosulfate. These alternative electron acceptors have application possibilities as well. Thiosulfate containing wastewater is produced at pulp bleaching and for fixing of photographs (Lens *et al*. 1998) and sulfite is the main compound in the liquid of flue gas scrubbing. These sulfur compounds can be recovered as elemental sulfur in a combined anaerobic/aerobic process, as described by Janssen *et al.* (2001).

Not all sulfate reducers can utilize sulfur. The Gibbs free energy change for sulfur reduction with CH_4 as electron donor is positive at standard conditions. Our result show that some disproportionation and CH_4 oxidation did occur though, but there was no clear coupling between net reduction and CH_4 oxidation.

5.5 Conclusions

The main conclusions of this work are:

- The optimum pH, salinity and temperature for AOM coupled to SR by the Eckernförde Bay enrichment were 7.5, 30‰ and 20°C, respectively.
- The Eckernförde Bay enrichment has a low affinity for CH_4 (K_m > 75 kPa) and a good affinity for sulfate ($K_m \leq$ 1.0 mM), sulfate can be almost completely removed (down to 0.05mM).
- AOM coupled to SR by the Eckernförde Bay enrichment was not inhibited at dissolved inorganic carbon concentrations up to 23 mM, but was completely inhibited at a sulfide concentration of 2.4 (±0.1) mM.

- The Eckernförde Bay enrichment can utilize sulfate, thiosulfate and sulfite as electron acceptors for AOM.
- CH_4 can be used as electron donor for biological sulfate reduction for the removal of sulfate or for the production of sulfide, for metal precipitation. However, the low temperature optimum and the high salinity requirement of the Eckernförde Bay enrichment limit the operational window of the process.

Chapter 6.

Long-term effect of elevated methane partial pressure on the anaerobic oxidation of methane

Abstract

The anaerobic oxidation of methane (AOM) rate and the Gibbs free energy change of AOM coupled to sulfate reduction are positively affected by the methane (CH_4) partial pressure. To investigate the effect of the CH_4 partial pressure on the growth of AOM mediating microorganisms, Eckernförde Bay sediment was incubated at a CH_4 partial pressure of 10.1 MPa. In 240 days, the AOM rate of this sediment increased from 0.006 to 0.024 mmol g_{VSS}^{-1}. This increase was not or hardly faster than the increase obtained at a CH_4 partial pressure of 0.10 MPa in a membrane bioreactor. Possibly, Eckernförde Bay sediment is not able to take advantage of a 100 fold increase in CH_4 partial pressure, as it originates from relative shallow waters (28 meter dept). However, also the different incubation technique could have counteracted a positive effect of the CH_4 partial pressure on the exponential increase of AOM.

6.1 Introduction

Anaerobic oxidation of methane (AOM) in marine sediments is coupled to sulfate reduction (SR) and mediated by archaea, called anaerobic methanotrophs (ANME), coexisting with sulfate-reducing bacteria. AOM is sensitive to the CH_4 partial pressure. Between 0 and 0.15 MPa, there is a positive linear correlation between the CH_4 partial pressure and the AOM and SR rates of a CH_4-oxidizing sulfate-reducing Eckernförde Bay enrichment (Chapter 5). The SR rate of Hydrate Ridge sediment was significantly higher at a CH_4 partial pressure of 1.1 MPa than at a CH_4 partial pressure 0.1 MPa (Nauhaus *et al.*, 2002; Krüger *et al*., 2005). In addition, Kallmeyer and Boetius (2004) reported a 40 fold increase of the SR rate of a thermophilic sediment when the pressure was increased from 0.101 to 45 MPa, however, SR was only partly fueled by CH_4 in those experiments.

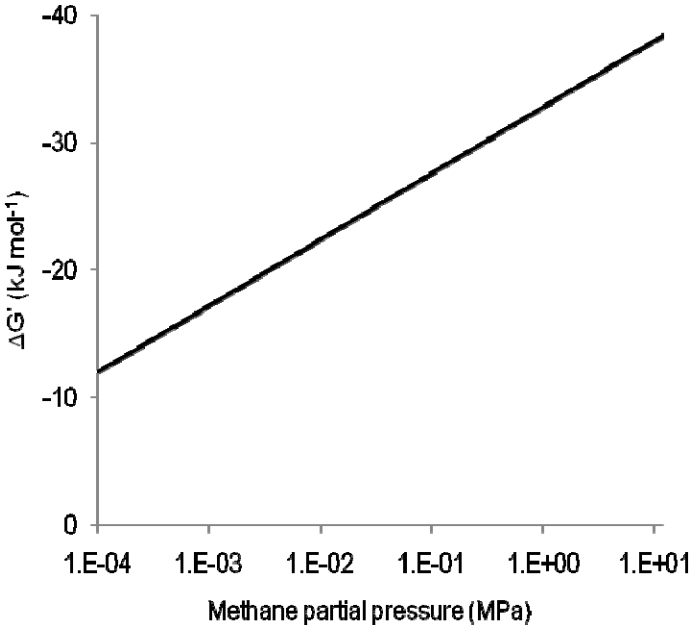

Figure 6.1. The cinfluence of the CH_4 partial pressure on the Gibbs Free Energy yield (kJ mol^{-1}) for AOM coupled to SR. Calculations assume the following conditions: Temperature 4°C; pH 7.2; [HCO_3^-] = 20 mM; [HS^-] = 2 mM; [SO_4^{2-}] = 10 mM (Valentine, 2002).

Because of the relatively low $\Delta G^{\circ\prime}$ of AOM coupled to SR (-16.6 kJ mol^{-1}), the $\Delta G'$ is sensitive to the CH_4 partial pressure. Valentine (2002) calculated this effect for typical *in situ* conditions (Figure 6.1). The growth of the microorganisms responsible for AOM coupled to SR is extremely slow (Nauhaus *et al.*, 2007; Chapter 3). Because of higher AOM rates and a more negative $\Delta G'$ at elevated CH_4 partial pressures, the growth of the AOM mediating microorganisms is expected to be faster at elevated CH_4 partial pressures. This hypothesis was tested by assessing the increase in AOM activity during a long-term incubation of the Eckernförde Bay sediment at 10.1 MPa CH_4, and comparing this with the activity increase at ambient pressure (Chapter 3).

6.2 Material and Methods

High-pressure incubation

For the long-tem incubation, a 2.0 L high-pressure vessel (Parr, Moline, USA) was used, which was placed in a water bath controlled at 20 (±1)°C and equipped with a stirrer controlled at 100 rpm (Figure 6.2). The vessel was filled with 1.8 L marine medium, containing 21 mM sulfate and a 8 mM phosphate buffer (composition given in Chapter 3). The vessel was inoculated with 25 membrane capsules containing the Eckernförde Bay sediment. Each capsule contained 0.038 (±0.003) g_{VSS}. The membrane capsules were cylindrically shaped, 14 mm in diameter, 20 mm long and had a membrane surface of 840 mm^2. The membranes (Triqua BV, Wageningen, the Netherlands) were made of polysulfone and had a pore size of 0.2 µm to retain microorganisms. The filled capsules were slightly lighter than water, which made them float when the stirrer was turned off.

For inoculation of the high pressure vessel, the lit of the vessel needed to be removed, which was done in an anaerobic glove box (containing 90% N_2 and 10% H_2). After the lit was put back, the high-pressure vessel was taken out of the glove box and connected to a bottle with pressurized CH_4 (purity 99.9995%). The vessel was flushed with approximately 10 L CH_4 (the gas entered the vessel at the bottom to remove any dissolved gas) and subsequently slowly (over a period of 2 hours) pressurized to a pressure of 10.1 MPa.

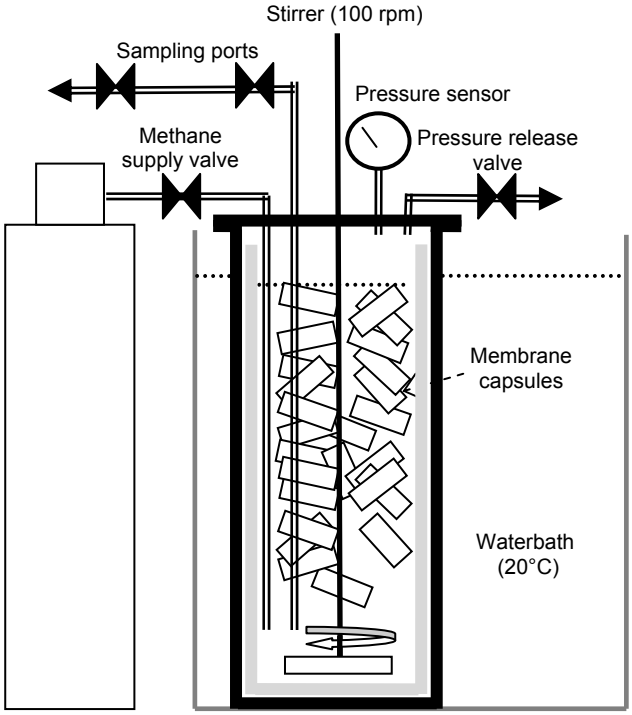

Figure 6.2. Schematic representation of the high-pressure vessel, containing membrane capsules, used in this study.

Every two months, the pressure was gradually (over a period of two hours) released, and the vessel was opened in an anaerobic glove box. This was done to replace the medium by fresh anaerobic medium and to sample three membrane capsules. Immediately after this, the vessel was closed, flushed and pressurized again with CH_4 gas as described above.
The high-pressure vessel was equipped with a sampling port. A sample of the liquid phase was taken just before depressurization and used to measure the dissolved sulfide concentration in the vessel, according to the method described in Chapter 3.

Ambient-pressure activity assays

The membrane capsules were incubated in 25-ml tubes, closed with butyl rubber stoppers and filled with 20 ml marine medium. The 5 ml headspace was filled with pure ^{13}C-labeled CH_4 (0.13 MPa). The tubes were incubated at 20°C in orbital shakers (100 rpm). Weekly the pH, liquid and gas volume, pressure and gas composition in the tubes were measured, as described in Chapter 3. From each activity assay, an AOM rate was obtained from the $^{13}CH_4$ oxidation to $\sum^{13}CO_2$ as described in Chapter 3. In the same way, the $\sum^{12}CO_2$ production rate was obtained.

6.3 Results

Figure 6.3 shows the AOM rate (determined at ambient pressure) of Eckernförde Bay sediment after different periods of incubation at 10.1 MPa CH_4. The AOM rate increased, during 240 days of incubation, from 0.006 mmol g_{VSS}^{-1} day^{-1} to 0.024 mmol g_{VSS}^{-1} day^{-1}, suggesting that the microorganisms responsible for the AOM were growing. The $\sum^{12}CO_2$ production rate on the other hand decreased. Since no other carbon source than $^{13}CH_4$ was added to the activity assays, the $^{12}CO_2$ must have been released from the sediment, likely due to organic matter decomposition. Endogenous $\sum^{12}CO_2$ production also occurs during incubations with anaerobic granular sludge (Chapter 2). Because the organic matter available for decomposition was being depleted during the 240-day incubation, the endogenous activity was decreasing. After 240 days, AOM was faster than the endogenous $^{12}CO_2$ production.

Chapter 3 describes the incubation of the same sediment in a membrane bioreactor (MBR) operated at ambient pressure (0.10 MPa CH_4) and at 15°C. There, the AOM rate doubled every 3.8 months, during this incubation an ANME-2a enrichment was obtained (Chapter 3 and 4). The increase of the AOM rate during the incubation at 10.1 MPa CH_4 was not or hardly faster than the increase obtained during the incubation at ambient pressure (Figure 6.3). Besides the difference in pressure, there was a difference in incubation temperature; 20°C for the high-pressure vessel and 15°C for the MBR. Note that the AOM and SR rates at ambient pressure were higher at 20°C than at 15°C (Chapter 5). The higher temperature in the high-pressure vessel did not result in a faster increase of the AOM rate though.

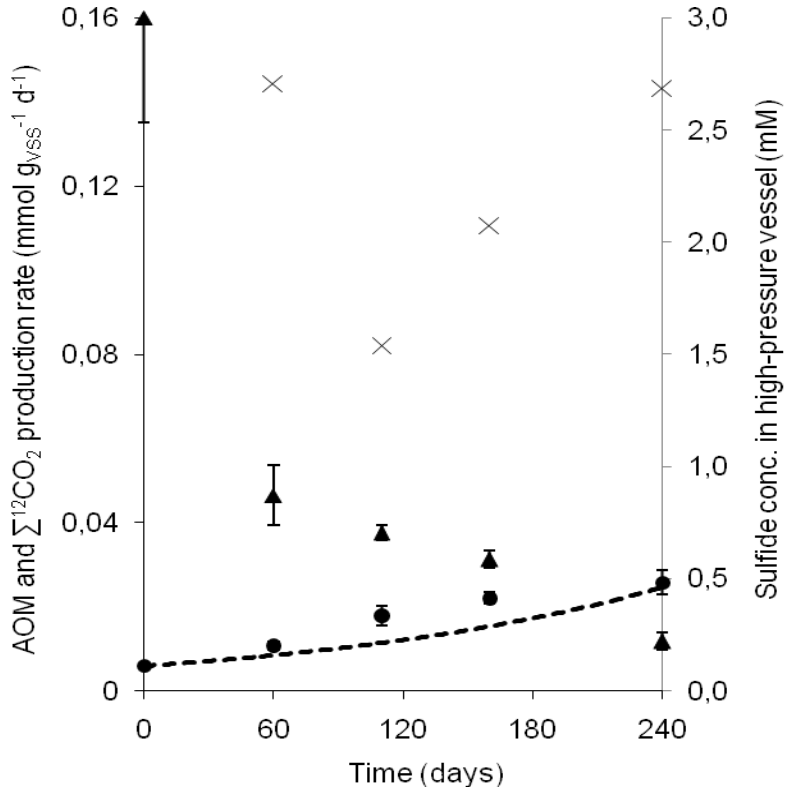

Figure 6.3. The AOM (●) and $\sum^{12}CO_2$ production (▲) rate of Eckernförde Bay sediment after different periods of incubation at 10.1 MPa CH_4 (N=3), and the dissolved sulfide concentration (X) in the high-pressure vessel just before biomass sampling and medium replacement. The dotted line represents the theoretical AOM rate at a doubling time of 3.8 months.

6.4 Discussion

The microorganisms from Eckernförde Bay sediment mediating AOM had no advantage of the 100 fold increase in CH_4 partial pressure. This may be related to the fact that the sediment originates from relative shallow waters (28 m dept; Treude *et al*., 2005a). However, also the different incubation technique could have counteracted a more pronounced positive effect of the CH_4 partial pressure. In the high-pressure vessel, sulfide and bicarbonate accumulated until the vessel was opened and the medium replaced. During the first (between day 0 and 60) and the last (between day 180 and 240) incubation periods,

the sulfide concentration reached 2.7 mM. This could have been limiting for the AOM mediating microorganism, as 2.4 (±0.1) mM sulfide was found to completely inhibit AOM and SR by an Eckernförde Bay enrichment at ambient pressure (Chapter 5). In the MBR, the sulfide concentration never exceeded 2.0 mM (Chapter 3). The dissolved inorganic carbon and sulfate concentration had little effect on the AOM rate up to a concentration of 23 mM and down to a concentration of 2 mM, respectively (Chapter 5). Therefore, the dissolved inorganic carbon and sulfate concentration were probably not limiting during the high-pressure incubation. A third possibility is that, despite the good mixing of the high-pressure vessel, the increase of the AOM rate was limited by the larger diffusion distances, since in the high-pressure vessel the biomass was present in membrane capsules with a diameter of 14 mm, and in the MBR the biomass was present as 0.1-mm flocks directly in contact with the bioreactor medium (Chapter 3).

6.5 Conclusions

During incubation of Eckernförde Bay sediment in a high-pressure vessel at 10.1 MPa CH_4, the AOM rate increases, indicating growth. However, this increase is not or hardly faster than in a MBR operated at ambient-pressure. Since a MBR is a more practical enrichment technique compared to the high-pressure vessel, it is more suited to grow CH_4-oxidizing sulfate-reducing biomass.

Chapter 7

Effect of methanogenic substrates on anaerobic oxidation of methane and sulfate reduction

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-day 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 thermodynamical 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.

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Roel J.W. Meulepas, Christian G. Jagersma, Ahmad F. Khadem, Cees J.N. Buisman, Alfons J.M. Stams en Piet N.L. Lens. Effect of methanogenic substrates on anaerobic oxidation of methane by a methane-oxidisinf sulfate-reducing enrichment

7.1 Introduction

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), methanogenic substrates were therefore 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 7.1). 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.

This study investigates whether methanogenic substrates act as IEC. This is done 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 7.1. If AOM still occurs at IEC concentrations far above the theoretical maximum, the AOM does not proceed via the production of that particular IEC.

Table 7.1. 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		
Acetate	$CH_4 + HCO_3^- \rightarrow CH_3COO^- + H_2O$ $CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+31 kJ mol^{-1} CH_4 -47 kJ mol^{-1} SO_4^{2-}
Formate	$CH_4 + 3HCO_3^- \rightarrow 4HCO_2^- + H^+ + H_2O$ $4HCO_2^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^-$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+128 kJ mol^{-1} CH_4 -144 kJ mol^{-1} SO_4^{2-}
Methanol	$CH_4 + {}^1/_3HCO_3^- + {}^1/_3H^+ + {}^1/_3H_2O \rightarrow {}^4/_3CH_3OH$ ${}^4/_3CH_3OH + SO_4^{2-} \rightarrow HS^- + {}^4/_3HCO_3^- + {}^1/_3H^+ + {}^4/_3H_2O$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+104 kJ mol^{-1} CH_4 -120 kJ mol^{-1} SO_4^{2-}
Carbon monoxide	$CH_4 + 3HCO_3^- + 3H^+ \rightarrow 4CO + 5H_2O$ $4CO + SO_4^{2-} + 4H_2O \rightarrow HS^- + 4HCO_3^- + 3H^+$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+196 kJ mol^{-1} CH_4 -212 kJ mol^{-1} SO_4^{2-}
Methane-thiol	$CH_4 + {}^1/_3HCO_3^- + {}^5/_3H^+ + {}^4/_3HS^- \rightarrow {}^4/_3H_3CSH + H_2O$ ${}^4/_3H_3CSH + SO_4^{2-} \rightarrow {}^7/_3HS^- + {}^4/_3HCO_3^- + {}^5/_3H^+$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+55 kJ mol^{-1} CH_4 -71 kJ mol^{-1} SO_4^{2-}
Hydrogen	$CH_4 + 3H_2O \rightarrow 4H_2 + HCO_3^- + H^+$ $4H_2 + SO_4^- + H^+ \rightarrow HS^- + 4H_2O$	$\Delta G°'_{ANME}$ $\Delta G°'_{SRB}$	+136 kJ mol^{-1} CH_4 -152 kJ mol^{-1} SO_4^{2-}

7.2 Material and methods

7.2.1 Eckernförde Bay enrichment

The biomass used for this research was taken from a 1-L submersed-membrane bioreactor, in which anaerobic methanotrophs were enriched. 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.

7.2.2 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).

7.2.3 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 in Chapter 2.

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 (Simpa *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).

7.2.4 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 in Chapter 2. 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 7.1) was calculated using the following equation:

$$\Delta G'_{anme} = \Delta G^{o}_{\text{anme}} + \text{RTLn}\frac{\prod[products]}{\prod[substrates]}$$

Nomenclature

R = gas constant = 8.314 J mol^{-1} K^{-1}

T = temperature in ºK = 288.15 ºK

7.3 Results

7.3.1 Incubations

Figure 7.1 shows the accumulation of $^{13}CO_2$ for each incubation. The presence of acetate, formate, methanol and hydrogen did not inhibit $^{13}CH_4$ 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.

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 7.2 shows the carbon balance, 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.

Figure 7.3 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. SR and CH_4 oxidation were both inhibited by the presence of methanethiol.

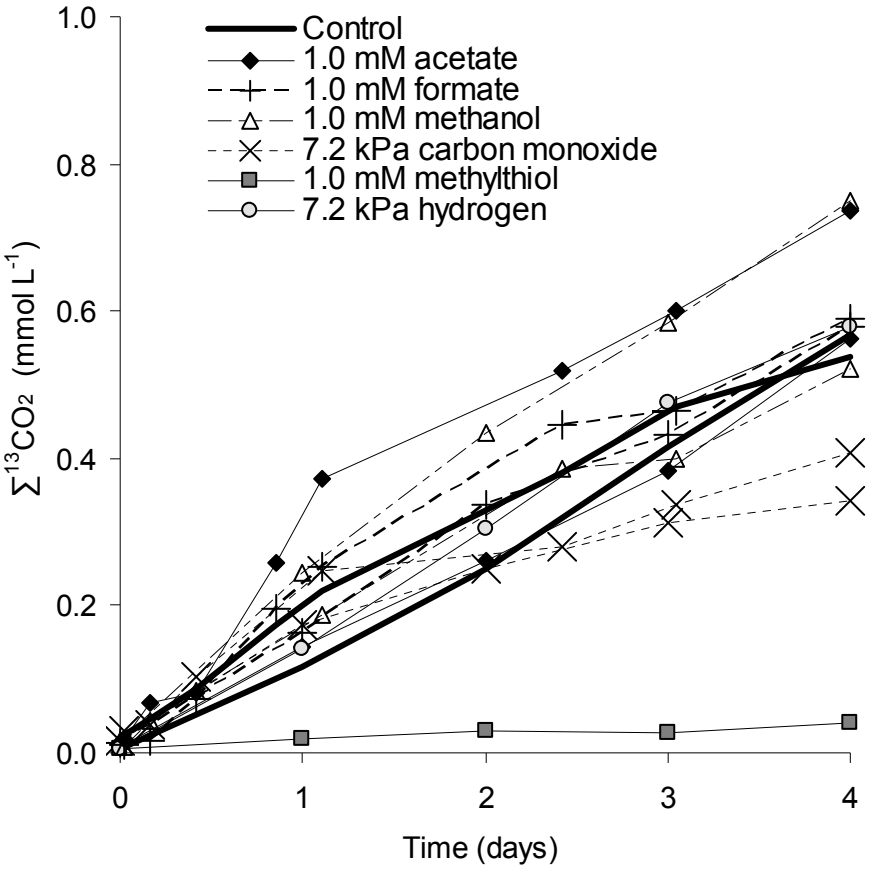

Figure 7.1. $\sum^{13}CO_2$ production in time, during four-day batch incubations, in the absence (control) or in the presence of one candidate IEC. The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa $^{13}CH_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.

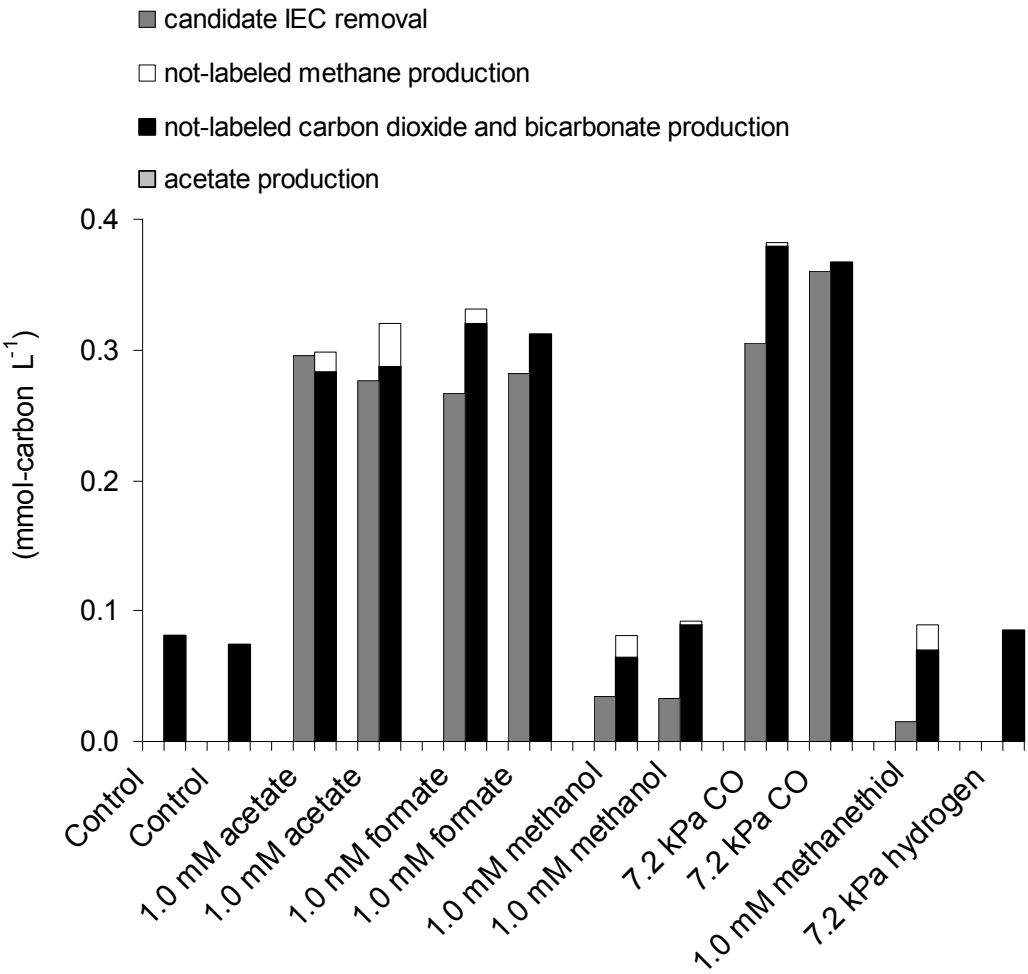

Figure 7.2. Candidate IEC removal compared to the $^{12}CH_4$ production, $\sum^{12}CO_2$ production and acetate production after four days of incubation in batch, in the absence (control) or in the presence of one of the candidate IEC. The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa $^{13}CH_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.

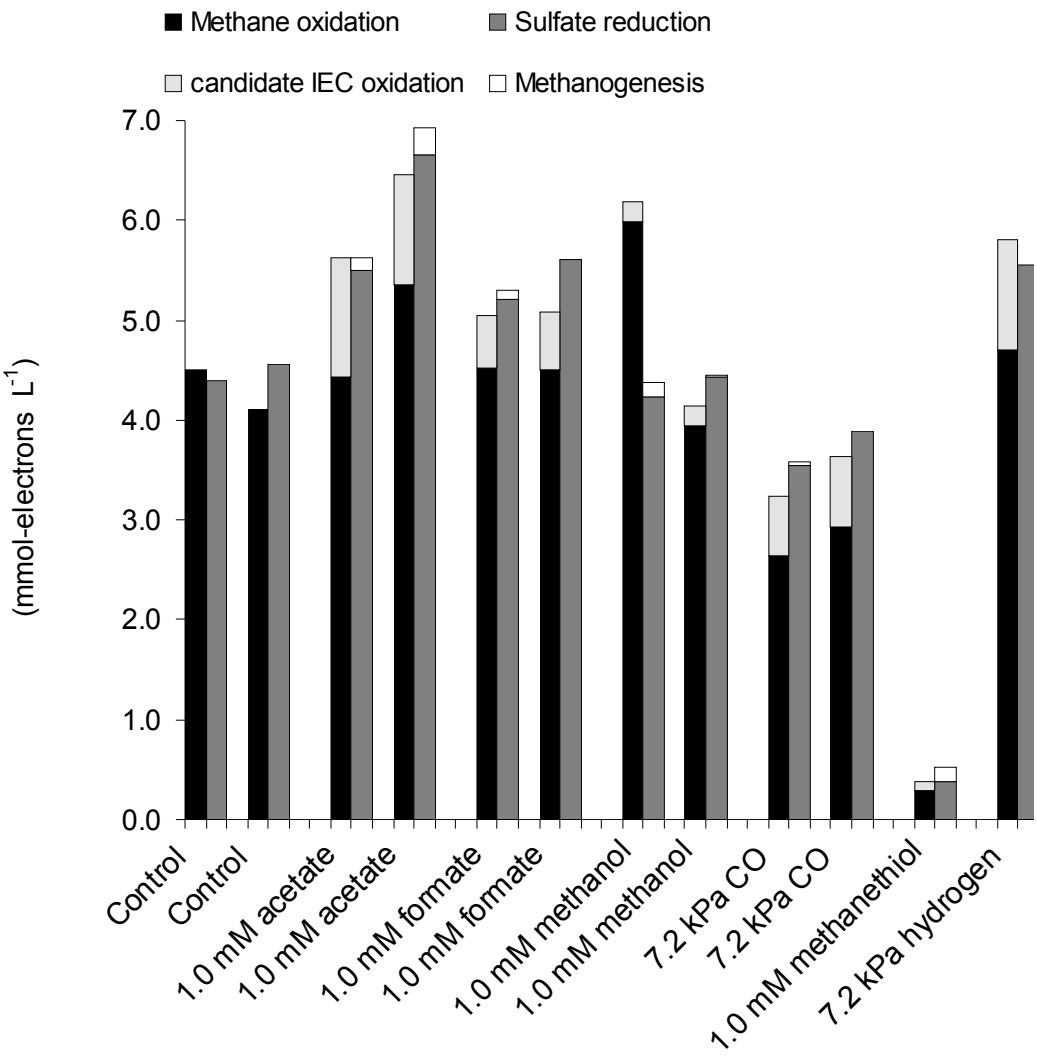

Figure 7.3. $^{13}CH_4$ and candidate IEC oxidation compared to SR and methanogenesis after four days of incubation in batch, in the absence (control) or in the presence of one candidate IEC. The bottles contained undiluted Eckernförde Bay enrichment and initially 0.16 (±0.01) MPa $^{13}CH_4$, 15 (±1) mM sulfate and 0.2 (±0.1) mM sulfide.

7.3.2 Thermodynamic calculations

Table 7.2 presents the concentrations of candidate IECs at which their production, under the applied experimental conditions, is no longer thermodynamically possible. 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.

Table 7.2. The concentration of candidate IECs at which their production from CH_4 is no longer thermodynamically possible ($\Delta G'=0$), at 1.4 atm CH_4, 1 mM HCO_3^-, 1 mM HS^- and a pH of 7.0.

IEC	IEC concentration at which $\Delta G_{ANME} = 0$	IEC concentration at day 4
Acetate	$3.4.10^{-6}$ mM	0.85 and 0.86 mM
Formate	$9.7.10^{-6}$ mM	0.73 and 0.72 mM
Methanol	$1.7.10^{-12}$ mM	0.93 and 0.93 mM
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 mM
Hydrogen	$4.2.10^{-6}$ atm.	3.2 kPa / 0.032 atm.

7.4 Discussion

7.4.1 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 were between 61 and 139% of the rates obtained in the controls. During the 4-day 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 7.2), 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 7.2). Therefore, these compounds can be excluded as sole 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 7.3). However, SR could also have been inhibited by methanethiol.

Many of the candidate IECs tested were consumed (Figure 7.2), 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; Chapter 3) and the IEC consumption rates were low (<0.6 mmol L^{-1} day^{-1}).

7.4.2 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), via membrane bound redox shuttles or via so-called “nanowires” (Reguera *et al.*, 2005; Stams *et al.*, 2006; Thauer and Shima, 2008; Wegener *et al.*, 2008).

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; Chapter 4). Further research should consider the possibility that one microorganism is capable of AOM coupled to SR.

7.4.3 Alternative electron donors

The Eckernförde Bay enrichment was able to utilize acetate, formate, methanol, carbon monoxide and hydrogen as electron donor for SR (Figure 7.3), 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 small amounts of 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-day duration of the experiment, which is a rather short time span.

SR with any of the added candidate IECs would yield more Gibbs free energy change than AOM coupled to SR (Table 7.1). 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, 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.

7.5 Conclusions

The main conclusions of this work are:

- Acetate, formate, methanol, carbon monoxide and hydrogen can be excluded as interspecies electron carriers in AOM coupled to SR by an Eckernförde Bay enrichment, because AOM was not or hardly inhibited by the presence of these compounds at concentrations at which their production from CH_4 is thermodynamically not possible.
- The CH_4-oxidizing sulfate-reducing Eckernförde Bay enrichment is able to utilize acetate, formate, methanol, carbon monoxide and hydrogen as electron donor for SR. The microorganisms capable of utilizing these compounds for SR can survive 512 days in a MBR fed with CH_4 as the sole electron donor and carbon source.

Chapter 8

General Discussion

8.1 Introduction

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) in marine environments has been studied intensively. However, thus far, the responsible microorganisms have not been isolated and the exact metabolic pathway of AOM remains unknown. *In vitro* growth has been demonstrated just a few times and only at extremely low growth rates. Therefore, research on the biotechnological aspects of AOM coupled to SR described in this thesis mainly focused on cultivation, the demonstration of the process in a bioreactor and on environmental factors that may influence AOM coupled to SR. This chapter discusses the progress made within this research with respect to the biotechnological aspects of AOM coupled to SR. In addition, recommendations for further biotechnological research are given.

8.2 Methane-dependent sulfate reduction and methane oxidation and by anaerobic granular sludge

All of the three tested granular sludge samples from anaerobic bioreactors anaerobically oxidized $^{13}CH_4$ to $\sum^{13}CO_2$ during net CH_4 production (Chapter 2). AOM rates followed CH_4 production rates and both AOM and methanogenesis were equally hampered by the presence of sulfate or bromoethanesulfonate, an inhibitor for methanogenesis. Therefore, AOM by granular sludge is a side effect of methanogenesis and not coupled to SR. The implication of this is that granular sludge cannot be used as inoculum for sulfate-reducing bioreactors fed with CH_4 as electron donor.

In the presence of sulfate, methanogens and sulfate reducers in granular sludge compete with each another for substrates released from an endogenous source (Chapter 2). Methanogenesis increased when sulfate was omitted and SR increased when methanogenesis was inhibited. The CH_4 partial pressure had a positive effect on sulfate reduction and a negative effect on methanogenesis (Chapter 2). Given the findings that CH_4 oxidation by Eerbeek sludge was not coupled to SR, the additional SR at elevated CH_4 pressure must have been caused by an increased substrate availability due to the inhibition of methanogens (Figure 8.1). The contribution of this phenomenon to CH_4-dependent sulfate reduction must be accounted for when investigating AOM coupled to SR in reactor sludge or sediments that show endogenous methanogenesis.

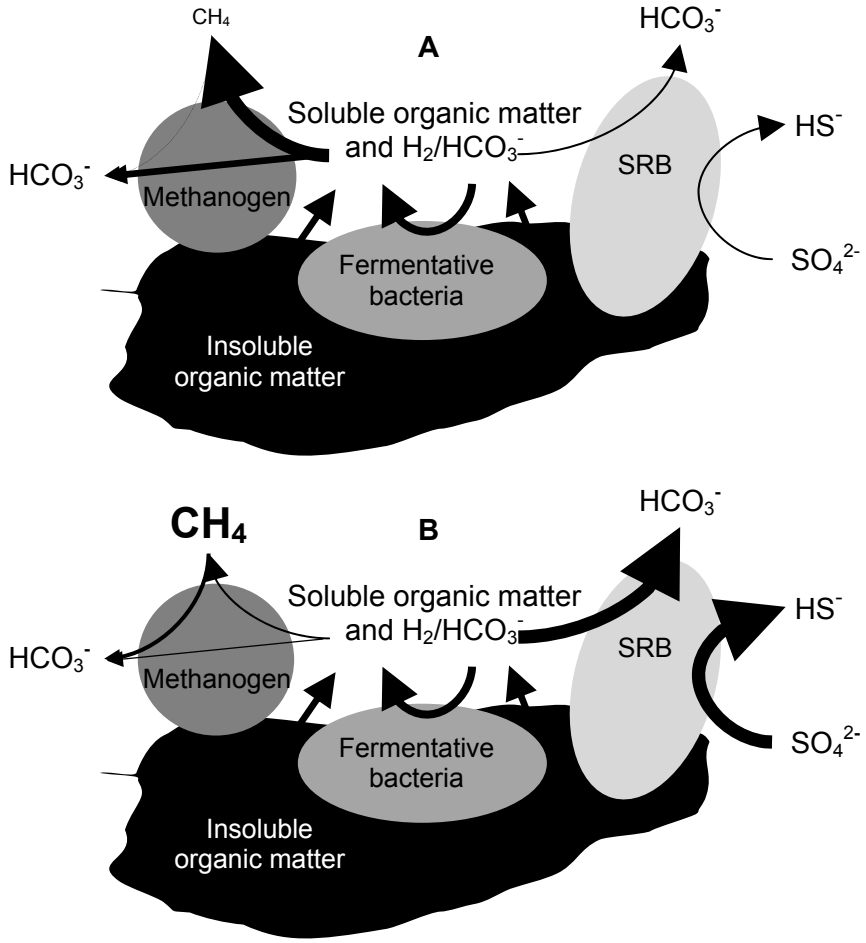

Figure 8.1. Methanogenesis, CH_4 oxidation and sulfate reduction by anaerobic granular sludge at a low CH_4 partial pressure (A) and at a high CH_4 partial pressure (B)

8.3 The Eckernförde Bay enrichment

Well-mixed ambient-pressure submersed-membrane bioreactors (MBR) were inoculated with Eckernförde Bay sediment and fed with CH_4 and sulfate as sole substrates, after an initial phase of 330 days during which acetate was added as co-substrate (Chapter 3). The AOM and SR rates in two reactors operated at 15°C increased over time, the AOM doubled approximately every 3.8 months. At 30ºC there was no increase in activity.

Table 8.1. The physiological and microbial characteristics of a CH_4-oxidizing sulfate-reducing Eckernförde Bay enrichment.

Parameter	Result	Chapter
Doubling time*	3.8 months, in a MBR at 15°C and 0.10 MPa CH_4	3
	no activity increase, in a MBR at 30°C and 0.101 MPa CH_4	3
	±3.8 months, in a fed-batch system at 20°C and 10.1 MPa CH_4	6
K_m P_{CH4}	> 75 kPa	5
K_m $[SO_4^{2-}]$	≤ 1.0 mM	5
$[SO_4^{2-}]_{threshold}$	0.05 mM	5
[Sulfide]	complete inhibition at 2.4 mM	5
$[\sum CO_2]$	no clear effect from 0 to 23 mM	5
Temperature optimum	20°C	5
Temperature range	no limit was reached	5
pH optimum	7.5	5
pH range	from, between 6 and 6.5, to 9 or higher	5
Salinity optimum	30‰	5
Salinity range	from, between 4 and 10‰, to 50‰ or higher	5
Electron acceptors for AOM	sulfate, thiosulfate and sulfite	5
Electron donors for SR	CH_4, acetate, formate, carbon monoxide and hydrogen	7
Dominant archaea	ANME-2a	4
Dominant bacteria	SRB, *Delta-proteobacteria* and the phylum *Bacteroidetes*	4
Incorporation of ^{13}C during a 3 month incubation with $^{13}CH_4$ and sulfate as sole substrates	both archaea and bacteria incorporated significant amounts of ^{13}C and are therefore both involved in AOM coupled to SR	4
Excluded as interspecies electron carrier	formate, acetate, methanol, carbon monoxide and hydrogen	7

***** Obtained from incubations with the original Eckernförde Bay sediment

During a reactor run at 15°C, the anaerobic methanotroph (ANME) of type 2a became the dominant archaea. The AOM and SR rate of the obtained ANME-2a enrichment were 1.0 mmol g_{VSS}^{-1} day^{-1}. Girguis *et al*. (2005), Nauhaus *et al*. (2007) and Krüger *et al*. (2008) also showed *in vitro* enrichment of ANME. An important difference in the approach of this research compared to the other studies is that the aim was not to mimic the natural conditions, but to apply conditions that allowed a maximal conversion rate. The results show that AOM coupled to SR is possible in well-mixed bioreactors, despite severe gas sparging and the continuous wash-out of dissolved compounds. The obtained enrichment was physiological and microbiological characterized, the results are summarized in Table 8.1.

8.4 Implication for biotechnological applications

8.4.1 Growth rate

The extreme low growth rate of the microorganisms mediating AOM coupled to SR (doubling time of 3.8 months, Chapter 3) indicates that biomass retention is crucial for applications of the process. A MBR allows complete cell retention, but requires energy input to overcome the trans-membrane pressure. Thus far, it is unknown whether sufficient CH_4-oxidizing sulfate-reducing biomass can be retained in a bioreactor by settling alone (like in gas-lift bioreactors or UASB systems). The formation of CH_4-oxidizing sulfate-reducing biofilms under turbulent reactor conditions has not yet been described. Naturally AOM mediating biofilms do occur though, in the form of microbial mats in the Black Sea (Michaelis *et al*., 2002).

From the growth rate (μ) and the specific conversion rate (V) the growth yield (Y) can be calculated, according to $Y = \mu.V^{-1}$. Nauhaus *et al.* (2007) calculated a molar yield of 0.6 g cell dry weight (mol CH_4 oxidized)$^{-1}$. This was based the sulfate reduction rate per gram ANME/SRB consortia. From our results (Chapter 3 and 4), it was not clear which fraction of the biomass contributed to AOM. Based on the AOM rate per gram VSS (1.0 mmol g_{VVS}^{-1} day^{-1}) and a growth rate of 0.0086 day^{-1} (1/3.8 months), a yield of 8.6 g dry weight (mol CH_4 oxidized)$^{-1}$ is obtained, the actual yield must be lower then this. The slow growth and low growth yield makes it difficult to combine AOM coupled to SR and metal precipitation in one system, since the metal sulfides need to be harvested without the loss of biomass. However, sulfate reduction with methane as electron donor can be used to remove and recover metals from wastewater if SR and metal precipitation are separated, like illustrated in Figure 8.2.

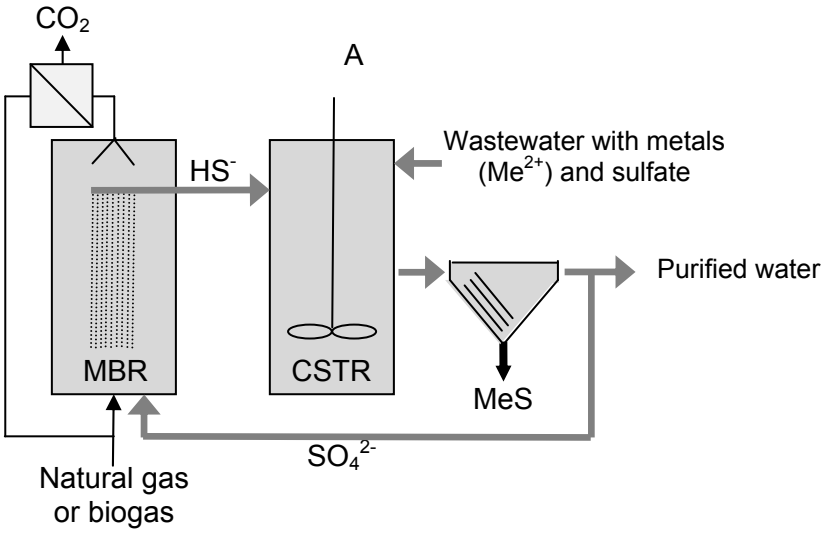

Figure 8.2. Schematic representation of a metal-removal process, in which SR with methane as electron donor and metal precipitation with sulfide are separated.

8.4.2 Temperature, pH and salinity

For biotechnological application, the low temperature optimum forms a limitation, as many industrial wastewaters are warmer than 20°C. However, in many countries legislation requires treated wastewater to be cooled before discharge. Moreover, if the wastewater is cooled in a heat exchanger the energy loss can be minimized.

The pH and salinity optima of the enrichment were 7.5 and 30‰, respectively. Many sulfate and metal containing wastewaters are acid (Weijma *et al.*, 2002; Kaksonen. and Puhakka, 2007), thus far, AOM coupled to SR has not been demonstrated at acid conditions. However, below a pH of 6.5, H_2S and CO_2 will be the main products of sulfate reduction, instead of HS^- and HCO_3^-. This will result in the generating of alkalinity. Therefore, a sulfate-reducing bioreactor fed with acidic wastewater, can often be maintained at a neutral pH.

Due to the high salinity requirement, wastewaters low in salts (other than sulfate) can not be treated with the biomass investigated in this study. However, for applications in which the liquid is recirculated (e.g. flue gas desulfurization), a high salinity optimum is even an advantage, since salts accumulate in such systems.

8.4.3 Methane, sulfate and sulfide concentrations

The affinity of the enrichment for CH_4 is low (K_m > 75 kPa). In industrial applications, the availability of CH_4 for the microorganisms can be optimized by applying thorough mixing, so that the CH_4 concentration near the microorganisms is close to saturation concentration, even at high conversion rates. The use of high-pressure reactors at full-scale, to obtain higher saturation concentrations, is not appealing; mainly because of the energy required to pressurize CH_4 gas.

The ability of the CH_4-oxidizing sulfate-reducing enrichment to remove sulfate almost completely (down to 0.05 mM), makes it possible to use this process for sulfate removal. However, the obtained Eckernförde Bay enrichment has a low tolerance for sulfide (inhibition at 2.4 mM). The low tolerance for sulfide forms no problem in applications in which sulfide is continuously stripped or used to precipitate metals. However, in a two-stage sulfate reduction and metal precipitation system (Figure 8.2), more liquid needs to be transported between the bioreactor and the precipitation reactor when the dissolved sulfide concentration is lower.

8.4.4 Alternative electron acceptors

The Eckernförde Bay enrichment was able to utilize thiosulfate and sulfite as electron acceptors for CH_4 oxidation. These alternative electron acceptors have application possibilities as well. Thiosulfate containing wastewater is produced at pulp bleaching and by the photographs fixing process (Lens *et al*. 1998), and sulfite is the main compound in the liquid from flue gas scrubbing.

8.5 Recommendation for further research

8.5.1 Introduction

The low growth rate of the microorganisms mediating AOM coupled to SR forms a major bottleneck for biotechnological applications. In this research, 0.59 g_{VSS} enrichment was obtained with an AOM and SR activity of 1.0 mmol g_{VSS}^{-1} day^{-1} (Chapter 3). The full-scale sulfate-reducing bioreactor at Nyrstar (Budel, the Netherlands) is capable of reducing 87.5 kmol (8.4 ton) sulfate per day (Weijma *et al*., 2002). At a doubling time of 3.8 months, it would take 8.6 years to grow enough CH_4-oxidizing sulfate-reducing biomass from the obtained enrichment to be able to replace the current process at Nyrstar, in which hydrogen is supplied as electron donor for biological sulfate reduction. Once enough CH_4-oxidizing sulfate-reducing biomass is produced, an operational failure, resulting in biomass wash-out or decay, could set the operation a few years back.

Alternatively, large amounts of AOM biomass could be sampled from the seafloor and used as inoculum for full-scale bioreactors. The highest AOM rate of a natural AOM enrichment is 8-21 µmol g_{dw}^{-1} day^{-1} (Black Sea microbial mats; Treude *et al.*, 2007). At least 4100 ton dry weight sediment would be needed to replace the current sulfate reduction process; this is from a technological, economical and ecological point of view undesirable. For biotechnological applications it is essential that CH_4-oxidizing sulfate-reducing biomass can be grown much faster. Three approaches to obtain faster growth rates are discussed below.

8.5.2 Other inocula

One straight forward approach is to sample and incubate other (more promising) AOM inocula, e.g. Black Sea microbial mats or sediments from thermophilic CH_4 seeps (Lost city; Boetius, 2005). Black Sea microbial mats form the most active natural AOM inocula. Possibly, the relative high conversion rates are related to faster maximum growth rates. Black Sea microbial mats occur above CH_4 seeps in the deep sea and are therefore adapted to higher dissolved CH_4 concentrations than the microorganisms from Eckernförde Bay sediment. Possibly, these microorganisms do grow faster in a high-pressure system compared to an ambient-pressure bioreactor (unlike the microorganisms from Eckernförde Bay, Chapter 6). Thermophilic AOM coupled to SR has hardly been investigated, possibly it would proceed much faster than AOM at cold-seeps. It would be worth to investigate AOM with samples from a thermophilic “Lost city” site (Boetius, 2005).

8.5.3 Other incubation techniques

A second approach is to test other incubation techniques to enrich the CH_4-oxidizing sulfate-reducing microorganisms, e.g. hollow-fiber bioreactors or continuous high-pressure bioreactors. Hollow fibers are semi-permeable tubes, via which for example CH_4 can be supplied to microorganisms growing in a biofilm on the fiber. At the other site of the semi-permeable tube, the sulfate containing liquid phase can be recirculated and refreshed. Transport distances in such system are minimal and the shear forces are relative low compared to gas-lift bioreactors. High shear forces might prevent the formation of CH_4-oxidizing sulfate-reducing biofilms. Sulfide can be removed from the recirculation stream, for example by precipitation with metals.

During the fed-batch high-pressure incubation (Chapter 6), sulfide accumulation and mass transfer limitation potentially limited the growth. In continuous well-mixed high-pressure bioreactors equipped with sulfide scrubbers this could be prevented.

8.5.3 Growth on alternative substrates

A third approach is to grow anaerobic methanotrophs on alternative substrates. The enrichment obtained in this study was able to utilize thiosulfate and sulfite as alternative electron acceptor for sulfate (Chapter 5), and acetate, formate carbon monoxide and hydrogen as alternative electron donor for CH_4 (Chapter 7). Given the larger Gibbs free energy change of these conversions, compared to AOM coupled to SR (Chapter 5 and 7), higher growth rates can be expected on those substrates. Therefore, if the same microorganisms are responsible for AOM coupled to SR, they could be enriched faster on those alternative substrates. However, to do so; sulfate-reducing bacteria, methanogens, homoacetogens and fermentative bacteria (which are not involved in AOM coupled to SR but are able to utilize the alternative substrates), should not be present in the inoculum, otherwise they might outcompete the microorganisms that are involved in AOM coupled to SR. These microorganisms might be lost during long-term enrichments with CH_4 and sulfate as sole substrates, after which experiments should be done aseptically.

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Summary

Methane-dependent sulfate reduction (SR) and anaerobic oxidation of methane (AOM) are not necessarily indications for AOM coupled to SR. Both processes occurred in anaerobic granular sludge, while AOM and SR were not coupled (Chapter 2).

Unlike granular sludge, Eckernförde Bay sediment (Baltic Sea) does contain microorganisms mediating AOM coupled to SR. These organisms could be enriched in a well-mixed ambient-pressure submersed-membrane bioreactor (MBR) operated at 15°C and fed with methane and sulfate as sole substrates, the AOM rate doubled every 3.8 months (Chapter 3). During the enrichment, the anaerobic methanotroph (ANME) of type 2a became the dominant archaea. Carbon derived from methane was incorporated both in archaeal and bacterial lipids, indication that bacteria were also involved in AOM coupled to SR (Chapter 4).

During incubation of Eckernförde Bay sediment in a high-pressure vessel at 10.1 MPa CH_4, the AOM rate increases over time (Chapter 6). However, this increase is not or hardly faster than in a MBR operated at ambient-pressure (Chapter 3). Since a MBR is a more practical technique, it is more suited to grow CH_4-oxidizing sulfate-reducing biomass.

The AOM and SR by the enrichment were optimal at a temperature, pH and salinity of 20°C, 7.5 and 30‰, respectively. Sulfate was removed almost completely (to 0.04 mM) and sulfide accumulated to a maximum concentration of 2.4 mM. Thiosulfate and sulfite were utilized as alternative electron acceptors (Chapter 5).

The presence of acetate (1.0 mM), formate (1.0 mM), methanol (1.0 mM), hydrogen (7.2 kPa) and carbon monoxide (7.2 kPa), did not or hardly inhibit AOM by the Eckernförde Bay enrichment, and can therefore be excluded as interspecies electron carriers between ANME and sulfate reducers (Chapter 7). Sulfate reduction with methane as electron donor can be applied in bioreactors for the removal of sulfate and the production of sulfide, which can subsequently be used to precipitate metals. However the extreme low growth rate and low growth yield makes it difficult to combine sulfide production and metal precipitation in one system. Further research should focus on obtaining faster growth rates, e.g. by growing anaerobic methanotrophs on alternative substrates.

Samenvatting

Methaan-afhankelijke sulfaat reductie (SR) en anaërobe oxidatie van methaan (AOM) zijn niet altijd geschikte indicatoren voor AOM gekoppeld aan SR. Beide processen treden op in anaëroob granulair slib terwijl AOM en SR niet waren gekoppeld (Hoofdstuk 2).

In tegenstelling tot korrel slib, bevat Eckernförde Bay sediment (Oostzee) micro-organismen die AOM gekoppeld aan SR katalyseren. Deze micro-organismes kunnen worden opgehoopt in een goed gemengde membraan bioreactor (MBR) bedreven bij atmosferische druk en 15°C en gevoed met methaan en sulfaat als enige substraten, de AOM snelheid verdubbelde iedere 3.8 maanden (Hoofdstuk 3).

Gedurende de verrijking werd de anaërobe methanotroof (ANME) van het type 2a de dominante archaea. Koolstof uit methaan werd ingebouwd in lipiden van zowel archaea als bacteriën, wat erop wijst dat ook bacteriën zijn betrokken by AOM gekoppeld aan SR (Hoofdstuk 4).

Gedurende de incubatie van Eckernförde Bay sediment in een hoge druk vat bij 10.1 MPa methaan steeg de AOM snelheid (Hoofdstuk 6). Die stijging ging echter niet of nauwelijks sneller dan in de MBR bij normale druk (Hoofdstuk 3). Een MBR is een praktischere techniek, daarom is een MBR het meest geschikt om methaan-oxiderende sulfaat-reducerende biomassa te kweken.

AOM en SR door de ophoping waren optimaal bij een temperatuur, pH en zoutgehalte van 20°C, 7.5 and 30‰, respectievelijk. Sulfaat werd nagenoeg complete verwijderd (tot 0.04 mM) en sulfide accumuleerde tot een maximale concentratie van 2.4 mM. Thiosulfaat en sulfiet werden gebruik als alternatieve elektron acceptor (Hoofdstuk 5)

AOM door de Eckernförde Bay ophoping werd niet of nauwelijks geremd door de aanwezigheid van acetaat (1.0 mM), formiaat (1.0 mM), methanol (1.0 mM), waterstof (7.2 kPa) en koolstof monoxide (7.2 kPa), daarom kunnen deze verbindingen worden uitgesloten als elektronen drager tussen ANME en sulfaat reduceerders (Hoofdstuk 7).

Sulfaat reductie met methaan als elektron donor kan worden toegepast in bioreactoren voor de verwijdering van sulfaat en de productie van sulfide, wat vervolgens kan worden gebruikt om metalen neer te slaan. De extreem lage groeisnelheid en lage "growth yield" vormen een obstakel voor een toepassing van sulfide productie en het neerslaan van metalen in een systeem. Verder onderzoek zou zich moeten richten op het verkrijgen van hogere groeisnelheden, bijvoorbeeld door het kweken van anaërobe methanotrofen op alternatieve substraten.

About the author

Roel Johannes Wilhelmus Meulepas was born on April 21, 1979 in Schaijk (The Netherlands). In 1997, he obtained the “vwo” diploma at the Mondriaan College in Oss, after which he started his study Environmental Hygiene at Wageningen University. During this study he specialized in Environmental technology. The thesis research was done at the laboratory of Microbiology, on the characterization of a sulfate-reducing microorganism isolated from a full-scale bioreactor, and at the Sub-department of Environmental Technology, on the biological conversion of carbon monoxide and water to hydrogen and carbon dioxide. His internship was done at “Universidad Autonoma Metropolitana” in Mexico City, on a sulfur-producing bioreactor. In January 2004, he obtained his MSc degree, after which he started his PhD research at the Sub-department of Environmental Technology at Wageningen University.

From January 2009, he works at the Environmental Resources department of UNESCO-IHE in Delft as researcher on metal removal by microorganisms.

List of Publications

Meulepas, R.J.W., Jagersma, C.G., Gieteling, J., Buisman, C.J.N., Stams, A.J.M. and Lens, P.N.L. Enrichment of Anaerobic Methanotrophs in a Sulfate-reducing Membrane Bioreactor. Biotechnol. Bioengin. Accepted for publication

Meulepas, R.J.W., Jagersma, C.G., Zhang, Y., Petrillo, M., Cai, H., Buisman, C.J.N., Stams, A.J.M. and Lens, P.N.L. Anaerobic Oxidation of Methane and Methane-Dependent Sulfate Reduction by Anaerobic Granular Sludge. Submitted.

Meulepas, R.J.W., Jagersma, C.G., Khadem, A.F., Buisman, C.J.N., Stams, A.J.M. and Lens, P.N.L. Effect of Environmental Conditions on Sulfate reduction with Methane as Electron Donor by an Eckernförde Bay Enrichment. Submitted.

Meulepas*, R.J.W., Jagersma*, C.G., Khadem, A.F., Buisman, C.J.N., Stams, A.J.M. and 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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Roel

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CERTIFICATE

The Netherlands Research School for the
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Research and Management Skills:

- Learning an analytical technique called MAR-FISH, and using it to analyse own samples, Department of Biotechnology at Aalborg University, Denmark
- Elaboration of project proposal: Anaërobe Methaan Oxidatie voor Biologische Sulfaat Reductie (AMethOx for SuRe)
- Organization Seminar: Trends in Environmental Biotechnology, Innovation in waste gas treatment
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- NVA/TCA Symposium: anaerobic Waste Water Treatment and N, P and S removal, 16 November 2005, Wageningen, The Netherlands
- Symposium: Sensible Water Technology (SENSE meeting), 12-13 April 2007, Leeuwarden, The Netherlands
- Seminar: Anaerobic Oxidation of Methane, 21-22 February 2008, Herzlake, Germany
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Cover picture: Methane oxidation, sulfate reduction and sulfate removal rates in time, derived from Figure 3.2C (between day 330 and 822).