Anaerobic oxidation of methane

Evaluation of alternative conditions

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CONTENTS

CHAPTER 1

General introduction

1.1 The microbial sulphur cycle

Most of the Earth's sulphur is present as sulphate and sulphide minerals. However, the oceans represent the most important source of sulphate (Madigan *et al.,* 2012). The main three forms of sulphur in nature are sulphide, elemental sulphur and sulphate (oxidation states -2, 0 and +6 respectively) and many biological and chemical conversions can transform one form into another. Figure 1.1 presents a simplified representation of the microbial sulphur cycle. (Tang *et al.,* 2009; Madigan *et al.,* 2012).

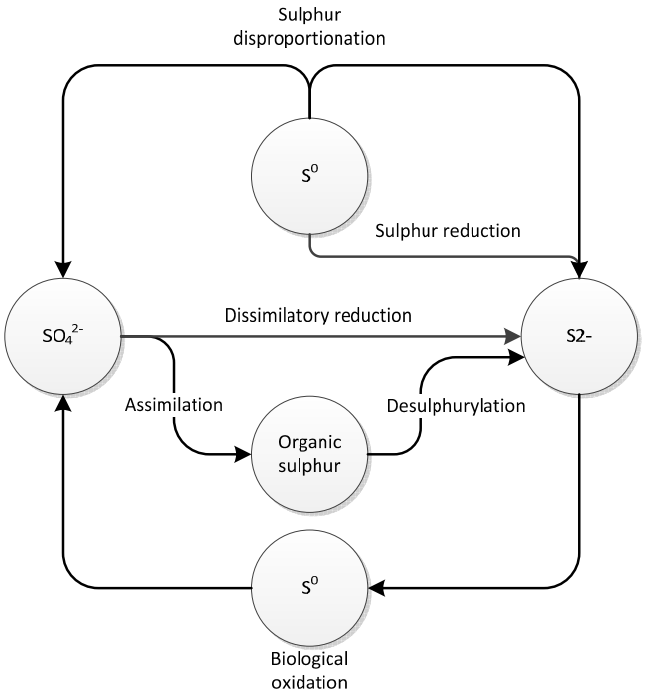

Figure 1.1 Simplified representation of the microbial sulphur cycle
(Adapted from Tang *et al*., 2009; and Madigan *et al*., 2012).

Sulphate is consumed either via assimilative or dissimilative metabolism. Mostly sulphur containing aminoacids are built via assimilative metabolism. Alternatively, mainly sulphide is produced when dissimilative sulphate reduction (SR) takes place. Hydrogen sulphide exists in different forms in solution (H_2S, HS^- and/or S^{2-} depending on the pH).

Sulphide can also be produced from reduction of elemental sulphur. However, many of the microorganisms that can perform this reaction are incapable of performing SR. Furthermore, as these sulphur and sulphate reducers do not compete with each other, it is

common to find them in the same ecosystem (Madigan *et al.,* 2012). Moreover, sulphur reducers can form synergistic interactions with sulphide oxidizers such as green/purple sulphur bacteria. In this way, the sulphide produced from elemental sulphur by the former can be oxidized back to its original form by the latter. Additionally, besides the green/purple sulphur bacteria, sulphur can also be oxidized by chemolitrophs. This occurs especially when sulphide, which would give more energy from the conversion to sulphate, is already consumed.

Disproportionation is another important sulphur conversion. It occurs when different atoms from the same molecules are simultaneously oxidized and reduced. For example, sulphur can be disproportionated into sulphate (oxidized form) and sulphide (reduced form) generating 25.4 $KJ \cdot mol^{-1}$ under biological conditions (Figure 1.1)

1.2 Sulphate reduction

Sulphate is one of the most common ions in the environment (Liamleam and Annachhatre 2007). It is generated and discharged in the effluent from many industrial processes (edible oil, food production, coal burning power plants, processing tannery, pulp and paper, textiles, fermentation and sea food processing industry) (Austin, 1984; Shin *et al.,* 1997, Lens *et al*., 1998). Sulphuric acid, which is commonly used for pH control, is reported to be the main source of sulphate contamination (Lens *et al.,* 2008) and it is estimated that every year over $150 \cdot 10^6$ Ton of sulphate is released by industry (Kirk-Othmer, 2000).

As easily biodegradable organic compounds are commonly available in water bodies receiving the sulphate, and sulphate reducing bacteria (SRB) are ubiquitous, sulphate reduction is widespread in anaerobic zones such as lake and sea sediments. Because of this, industrial effluents containing sulphate can create not only odour but also health and ecotoxicity problems in the receiving water bodies. Treatment of such streams can be performed with biological sulphate removal technologies. These technologies are based on the reduction of sulphate to sulphide in anaerobic reactors followed by partial oxidation of the sulphide to elemental sulphur in a separate reactor controlled at microaerophilic conditions. This not only avoids contamination of the receiving water body; but also allows to recover sulphur which can be reused in a chemical process or as fungicide and fertilizer (Weijma *et al*., 2006).

In order to treat sulphate-containing effluents with biological sulphate removal technologies, an electron donor must be added if the waste stream does not contain sufficient. The selection of the type of electron donor is determined by technical and economic factors. Donors such as ethanol or hydrogen offer the possibility of having

efficient systems with high SR rates (van Houten, 1996.). However, they are relatively expensive (Meulepas *et al.* 2010). On the other hand, cheaper but more complex substrates such as vegetal compost, cow manure or molasses are inefficient; and, as some are not completely oxidized, produce high amounts of residual waste material (Liamleam and Annachhatre 2007 and references therein).

Different to conventional chemical methods for metal removal, the biological sulphate reduction process technology can remove the sulphate simultaneously with the metals. This is because they can be easily separated from the liquid phase by precipitation caused by their reaction with the produced sulphide. Furthermore, this technology can also be used to convert the sulphate from wastewater into elemental sulphur (Janssen *et al.,* 2001), which is a product with increasing economic value. This elemental sulphur can be obtained either by sulphide oxidation on microaerobic conditions or by sulphide conversion by green/purple sulphur bacteria. The former being more widely applied.

1.3 Anaerobic oxidation of methane coupled to sulphate reduction

Methane is the simplest and most stable hydrocarbon. It is the most reduced formed of carbon (oxidation state -4) and it is a colourless and odourless gas at standard conditions. Furthermore, it is the main compound in natural gas and it can be biologically produced from the degradation of organic matter. Its production can occur in a diversity of ecosystems that vary from marine and fresh water sediments, to wetlands, digestive tracts of animals and insects, engineered methanogenic systems and agricultural waste (Meulepas *et al*., 2010)

The anaerobic oxidation of methane (AOM) is a process that occurs in marine sediments where methane meets the sulphate from the water (Figure 1.2) (Reaction 1) (Hinrichs and Boetius, 2002; Meulepas *et al*., 2010). AOM is the dominant anaerobic terminal metabolic process in marine sediments. And, as it removes large amounts of methane, it has an important effect in the global carbon cycle (Crutzen, 1994; Reeburgh, 1996).

Reaction 1 $CH_4 + SO_4^{-2} + 2H^+ \rightarrow CO_2 + H_2S + 2H_2O$ −16.5 kJ·mol^{-1} CH_4

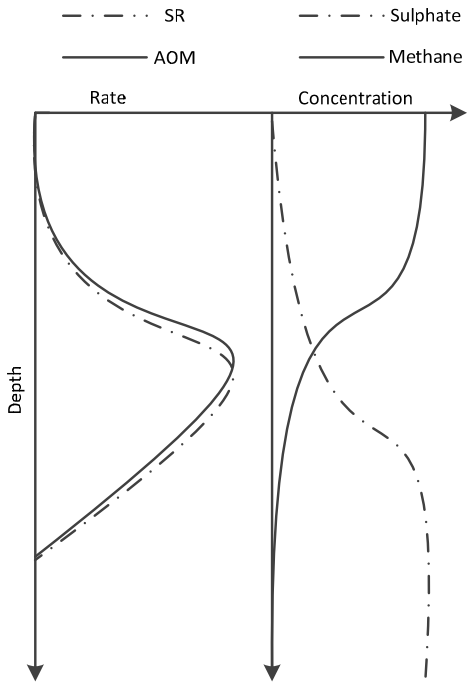

Figure 1.2. Profiles of rates of sulphate reduction and anaerobic oxidation of methane (A) and of methane and sulphate concentrations (B) in marine sediments Adapted from Knittel and Boetius 2009 and Meulepas *et al*.,2010.

1.3.1 Mechanisms

The existence of microbial populations that use methane as electron donor for the reduction of sulphate has been known for years (Anthony, 1982; Amaral and Knowles, 1995) and the necessary conditions for this reaction are abundantly present in nature (availability of sulphate together with methane and a low redox potential) (Reeburgh, 1976). However, the mechanisms involved in this conversion are not fully understood yet.

This phenomenon has been traditionally understood as a syntrophic relationship between sulphate reducers and archaea that are phylogenetically related to methanogens. This syntrophic relationship has been reported to occur in both, communities of single growing cells and aggregates. In the AOM coupled to SR, it is hypothesized that the archaea microorganisms (known as ANME for anaerobic methanotrophs) consume methane while producing an intermediate compound. This compound is in its turn used by sulphate reducing bacteria as electron donor to reduced sulphate to hydrogen sulphide (Zehnder

and Brock, 1980; Alperin and Reeburgh, 1985; Hoehler *et al.,* 1994, Boetius *et al*., 2000; DeLong, 2000, Valentine and Reeburgh, 2000) (Figure 1.3).

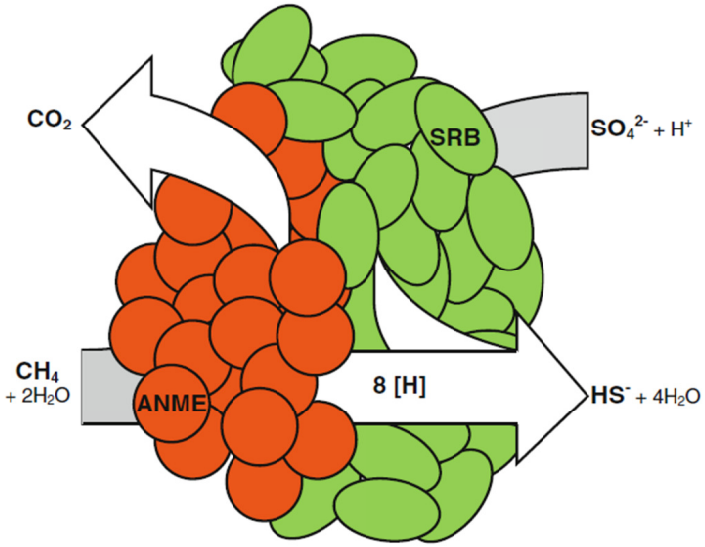

Figure 1.3. Schematic representation of the proposed interspecies electron transfer in consortia composed of methane oxidizing archaea (left) and sulphate reducing bacteria (right) (from Meulepas *et al*, 2010)

There is strong evidence indicating that the metabolic pathway of the AOM is somehow similar to reverse methanogenesis (Hallam *et al.,* 2004; Shima and Thauer, 2005; Knittel and Boetius, 2009). Therefore, methanogenic substrates have been tested as possible intermediates. However, it is still unknown which is the interspecies electron carrier (Stams and Plugge, 2009 and reference therein). Because of this, three possibilities have been suggested; the first one is that electrons are not transferred as a compound but they are directly transferred to the sulphate reducer, possibly via nanowires (Stams and Plugge, 2009). The second possibility is that the ANME are also capable of performing the SR. In this case, the sulphate reducing bacteria commonly found around them would metabolize complex organic compounds released by the ANME microorganisms (Thauer and Shima, 2008). And finally, as Milucka *et al*., (2012) proposed, AOM could be carried out by ANME releasing zero valent sulphur which is in turn disproportionated by Deltaproteobacteria (Figure 1.4).

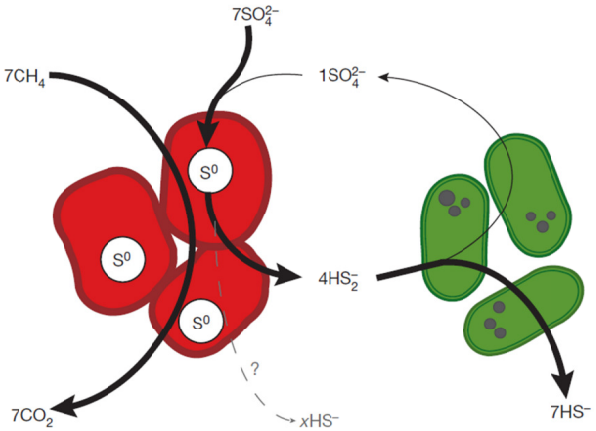

Figure 1.4. Proposed model of anaerobic oxidation of methane coupled to sulphate reduction. ANME-2 oxidize methane with a concomitant reduction of sulphate to zero-valent sulphur (S^0, Elemental reacts with sulphide to form polysulphides (disulphide, among others). Disulphide is taken up by the associated Deltaproteobacteria and is disproportionated to sulphate and sulphide. Sulphate produced during disproportionation might be re-used by the ANME and the ANME may also reduce some of the sulphate all the way to sulphide (grey dotted line). Dark circles in the bacteria represent intracellular precipitates rich in iron and phosphorus. Taken from Milucka *et al* 2014.

1.3.2 Biotechnological research

The use of methane as electron donor in SR processes saves energy when compared to hydrogen. As one molecule of methane can donate eight electrons while one of hydrogen can only donate two, four times less gas must be transferred from the gas to the liquid phase. Additionally, methane (as natural gas) is 4 times cheaper than hydrogen or ethanol (Meulepas *et al.,* 2010). Furthermore, as this biological conversion not only produces a cleaner effluent than conventional chemical methods for sulphate removal but it also allows to recover sulphur; it has obtained a great deal of attention over the last years. However, increasing the volumetric conversion capacity of a bioreactor based in this conversion has proven to be very difficult. The main reasons are that only 1% of the consumed methane is destined to the production of biomass (Thauer and Shima, 2008; Knittel and Boetius, 2009) and that doubling times for anaerobic oxidation of methane lie between 1.2 and 7.5 months (Deusner *et al.,* 2010; Girguis *et al.,* 2005; Krüger *et al.*, 2008; Meulepas *et al.*, 2009a; Nauhaus *et al.*, 2007; Zhang *et al.*, 2010).

In order to obtain industrially feasible conversion rates of AOM coupled to SR several factors must be considered. These vary from pH and temperature to salinity and type of bioreactor to be used. Therefore, as this is an extremely slow process; actions must be taken in order to try to rapidly enrich for the appropriate microorganisms.

Previous research performed at Wageningen University (Meulepas *et al.,* 2009b) found that the optimal pH, salinity and temperature for biomass originated in Eckernförde bay were 7.5, 30‰ and 20°C, respectively. Furthermore, it was also described how the biomass was completely inhibited at 2.4 (±0.1) mM of sulphide. However, the biomass managed to recover activity after the sulphide concentration was lowered. Because of this, the capability to continuously remove sulphide from the system is one of the criteria to properly select a bioreactor in which AOM takes place. That study also showed how not only sulphate but also thiosulphate and sulphite could be used by microorganisms capable of performing AOM. This is important for two reasons. The first is that processes in which sulphite or thiosulphate must be removed from industrial effluents are not uncommon (*e.g.* paper production, fracking processes). The second reason is that as The Gibbs free energy change that can be obtained from methane oxidation coupled to thiosulphate or sulphite reduction is larger than that with sulphate, it could be hypothesized that these electron acceptors might allow higher conversion rates and therefore higher grow rates.

Conditions to grow of the microorganisms in bioreactors have been reported to be similar to those present in marine ecosystems (salinity 30 $\%_o$ and neutral pH) (Meulepas 2009). It has also been reported that there is a direct relation between the methane partial pressure and the AOM rates (Kruger *et al.* 2005; Nauhaus *et al.* 2005; Meulepas *et al.,* 2009a; Kallmeyer and Boetius 2004, Zhang *et al.*, 2010, Deusner *et al.,* 2010); implying that methane acts as limiting substrate under standard conditions. However, the use of high pressure reactors is not economically feasible and other options must be looked into. One alternative to enrich the microorganisms and obtain higher conversion rates in bioreactors is to use membranes. In such system complete biomass retention is obtained, which is particularly important when dealing with slowly growing microorganisms as those involved in AOM. This is the case of the membrane bioreactor used by Meulepas *et al.,* (2009a). It not only allowed continuous removal of hydrogen sulphide and carbon dioxide in the gas phase but it also kept all the biomass in the reactor; permitting to increase the AOM and SR rates to 0.60 $mmol \cdot L^{-1} \cdot day^{-1}$, 150 times higher than at the beginning of the reactor operation.

1.4 Interspecies electron transfer

As mention above, electron transfer not only in AOM consortia but also in granular biomass has been thought to rely on the transfer of compounds which carry the electrons between the different kinds of microorganisms in the consortia.

Several studies for AOM consortia have been carried out in order to determine which compound it might be. However, no evidence that points toward one specific electron

carrier has been found (Stams and Plugge, 2009 and reference therein). Furthermore, studies performed with granular biomass suggest, that in some cases, the theoretical interspecies electron carrier compounds can not be the only electron carriers. This is due to the fact that those compounds are dependent on diffusion and are theoretically not able to reach the electrons transfer rates measured in practice (Reguera *et al.,* 2005; Morita *et al*., 2011, Cruz *et al.,* 2014).

It might be hypothesized that this electron transfer is carried out in conductive structures through which the electron transfer occurs. Such mechanisms have been previously described in co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* (Reguera *et al*., 2005; Summers *et al*., 2010; Morita *et al*., 2011). Furthermore, *G. metallireducens* has also been shown to build electrically conductive structures with methanogenic archaea (Summers *et al.,* 2010; Morita *et al.,* 2011; Rotaru *et al.,* 2014). Moreover, the study of Morita *et al*., (2011) revealed evidence that these aggregates were not using hydrogen, further supporting the idea that direct interspecies electron transfer took place. It might be possible that this kind of electron transfer also occurs in AOM systems. As it has been reported that not to be limited to granular biomass nor nanodistances, since Pfeffer *et al*., (2012) presented evidence of it happening across centimetres distances in marine sediments by filaments of bacteria.

1.5 Scope and organization of this thesis

An introduction of the sulphur cycle and the AOM coupled to SR is presented in this chapter. Furthermore, some of the issues that currently surround the still poorly understood AOM process are raised. In order to achieve industrially feasible conversion rates of SR using methane as electron donor the microorganisms involved in this conversion must be identified and their role understood. However, due to their slow metabolism, any study that addresses these topics is complicated because many factors that are difficult to control might play a role (trace elements and micronutrients, light, other microorganisms present, etc.). For this reason, different strategies that seem capable of raising conversion rates and therefore grow rates are worth evaluating.

Chapter 2 attempts to do this by testing different alternative electron donors (ethane and propane) and electron acceptors (elemental sulphur and thiosulphate) for sediments originated in Eckernförde and Aarhus bay. Results show that even though thiosulphate disproportionation plays and important factor; the methane oxidation rates obtained with this electron acceptor are higher than sulphate or sulphur. Because of this, it is decided to add thiosulphate to a 5 L methane fed membrane bioreactor (Chapter 3). Before starting this system, it is expected that not only the addition of thiosulphate, but also of acetate (which has been previously reported to selectively enrich for methanotrophic microorganisms), results in a rapid enrichment of an AOM consortia. However, the conditions achieved in the reactor (partial stripping of sulphide along with small amounts of light), created a completely different ecosystem. Physicochemical results and microbiological analysis showed that neither methanogenic nor methanonotrophic microorganisms were enriched. Instead, a community of thiosulphate disproportionating microorganisms working in a mutualistic relationship with bacteria capable of oxidizing sulphate into elemental sulphur was enriched. Such system however, could be used to efficiently recover elemental sulphur from water containing thiosulphate.

Chapter 4 describes the finding of methane oxidation associated with sulphate reduction in a freshwater environment containing ANME microorganisms and sulphate reducers. This could have important repercussions in a potential industrial application; as previous studies have always indicated that the highest AOM conversion rates occur at sea water conditions. This is expected as most of those studies have used biomass originated in sea sediments. Using fresh water would potentially allow to reduce conversion and environmental costs as lower quantities of raw material would be required in reactors dedicated to this conversion.

Chapter 5, on other hand, deals in an indirect way with the still unknown topic of the interspecies electron transfer between the methanotrophic archaea and the sulphate reducing microorganisms. In this chapter, a simple methodology for the measurement of conductance in granular biomass is evaluated. This methodology, or a modified version of it, could be used in future research to determine if the electron transfer in AOM systems occurs via direct electron transfer. Finally, the results obtain in this research, their impact and suggestions for further research are discussed in Chapter 6.

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CHAPTER 2

High rates of anaerobic oxidation of methane, ethane and propane coupled to thiosulphate reduction

Anaerobic methane oxidation coupled to sulphate reduction and the use of ethane and propane as electron donors by sulphate-reducing bacteria represent new opportunities for the treatment of streams contaminated with sulphur oxyanions. However, growth of microbial sulphate-reducing populations with methane, propane or butane is extremely slow, which hampers research and development of bioprocesses based on these conversions. Thermodynamic calculations indicate that the growth rate with possible alternative terminal electron acceptors such as thiosulphate and elemental sulphur may be higher, which would facilitate future research. Here, we investigate the use of these electron acceptors for oxidation of methane, ethane and propane, with marine sediment as inoculum. Mixed marine sediments originating from Aarhus Bay (Denmark) and Eckernförde Bay (Germany) were cultivated anaerobically at a pH between 7.2 and 7.8 and a temperature of 15 °C in the presence of methane, ethane and propane and various sulphur electron acceptors. The sulphide production rates in the conditions with methane, ethane and propane with sulphate were respectively 2.3, 2.2 and 1.8 $\mu mol \cdot S \cdot L^{-1} \cdot day^{-1}$. For sulphur, no reduction was demonstrated. For thiosulphate, the sulphide production rates were up to 50 times higher compared to those of sulphate, with 86.2, 90.7 and 108.1 $\mu mol \cdot S \cdot L^{-1}$ day^{-1} for methane, ethane and propane respectively. This sulphide production was partly due to disproportionation, 50 % for ethane but only 7 and 14 % for methane and propane respectively. The oxidation of the alkanes in the presence of thiosulphate was confirmed by carbon dioxide production. This is, to our knowledge, the first report of thiosulphate use as electron acceptor with ethane and propane as electron donors. Additionally, these results indicate that thiosulphate is a promising electron acceptor to increase start-up rates for sulphate-reducing bioprocesses coupled to short-chain alkane oxidation.

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2.1 Introduction

Removal of sulphate and recovery of dissolved metals from mining and metallurgical waste and process streams can be accomplished by microbiological sulphate reduction processes. In such processes, already applied on full-scale, sulphate is reduced to hydrogen sulphide, which is then either partially bio-oxidized to elemental sulphur or it is used to precipitate base metals like Copper, Nickel or Zinc as insoluble metal sulphides (Weijma *et al*., 2006).

The wastewaters and process streams of concern often contain insufficient electron donor for the targeted sulphate reduction capacity. Ethanol and hydrogen are the most common electron donors that are added to support sulphate reduction, but these add substantial operational costs. These costs would be drastically reduced by using methane, ethane or propane as electron donors. Additionally, these alkane gases are more easily available than ethanol and hydrogen, allowing the application on a much wider range.

There is microbiological evidence of sulphate reduction with these alkane gases. Anaerobic oxidation of methane (AOM) coupled to sulphate reduction in marine waters and sediments has been reported in geochemical studies (Table 2.1, conversion 1) (Alperin *et al*., 1988; Barnes *et al*., 1976; Reeburgh, 1980; Reeburgh, 1976). It is thought to be performed by an obligate syntrophic interaction between a methanogenic archaeon carrying out reversed methanogenesis and a sulphate-reducing bacterium (Boetius *et al*., 2000; Hinrich *et al*., 2000; Valentine and Reeburgh, 2000, and reference therein). Reported AOM conversion rates in different marine sediments are between 0.001 (North Sea) and 20.9 $\mu mol \cdot gdry\ weight^{-1} \cdot day^{-1}$ (Black Sea) (Krüger *et al*., 2005; Treude *et al*., 2007). Reported doubling times of AOM consortia are between 1.2 and 7.5 months (Deusner *et al*., 2010; Girguis *et al*., 2005; Krüger *et al*., 2008; Meulepas *et al*., 2009a; Nauhaus *et al*., 2007; Zhang *et al*., 2010). This slow growth hampers the research and development of sulphate reduction processes. For example, in previous work carried out in membrane bioreactors in our laboratory, it took 884 days to increase the sulphate reduction rate from 0.0015 to 0.6 $mmol \cdot L^{1} \cdot day^{-1}$ (Meulepas *et al*., 2009a), which is still 100 times too low for practical applications.

Table 2.1 Reduction and disproportionation conversions of oxidized sulphur compounds, and their standard (ΔG°) and biological (ΔG°') Gibbs free energy changes. pH 7.0, and the following concentrations: CH_4 0.9 mM,C_2H_6 1.3 mM, C_3H_8 1.0 mM, HCO_3^- 7.0 mM and H_2S 0.5mM. All Gibbs free energy changes are given in Kilojoule per mole of the first compound of the reaction equation

	Stoichiometry	ΔG^o	$\Delta G^{o'}$
Methane			
1	$CH_4 + SO_4^{2-} + H^+ \rightarrow HCO_3^- + H_2S + H_2O$	-16.5	-21.3
2	$CH_4 + S_2O_3^{2-} + H^+ \rightarrow HCO_3^- + 2H_2S$	-39.0	-60.3
3	$CH_4 + 4S^0 + 3H_2O \rightarrow HCO_3^- + 4H_2S + H^+$	$+23.4$	-8.1
Ethane			
4	$4C_2H_6 + 7SO_4^{2-} + 6H^+ \rightarrow 8HCO_3^- + 7H_2S + 4H_2O$	-63.8	-166.1
5	$4C_2H_6 + 7S_2O_3^{2-} + 3H_2O + 6H^+ \rightarrow 8HCO_3^- + 14H_2S$	-103.1	-325.3
6	$C_2H_6 + 7S^0 + 6H_2O \rightarrow 2HCO_3^- + 7H_2S + 2H^+$	$+6.2$	-133.9
Propane			
7	$2C_3H_8 + 5SO_4^{2-} + 4H^+ \rightarrow 6HCO_3^- + 5H_2S + 2H_2O$	-103.2	-192.7
8	$2C_3H_8 + 5S_2O_3^{2-} + 3H_2O + 4H^+ \rightarrow 6HCO_3^- + 10H_2S$	-159.4	-334.5
9	$C_3H_8 + 10S^0 + 9H_2O \rightarrow 3HCO_3^- + 10H_2S + 3H^+$	-3.3	-211.5
Disproportionation			
10	$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S$	-22.5	-39.6
11	$4S^0 + 4H_2O \rightarrow SO_4^{2-} + 3H_2S + 2H^+$	$+40.0$	-25.4

In addition, the use of ethane, propane and butane (short chain alkanes, SCA) as electron donors by marine microorganisms has been reported recently (Jaekel *et al*., 2013; Kniemeyer *et al*., 2007; Savage *et al*., 2010) and a strain capable of using propane and butane as electron donors coupled to sulphate reduction has been isolated (Jaekel *et al*., 2013; Kniemeyer *et al*., 2007). This strain (BuS5) also grows slowly, with a doubling time of 4-5 days, which is 6 times lower than the fastest doubling time found for microorganisms capable of performing anaerobic methane oxidation (Girguis *et al*., 2005).

The main bottleneck when studying the use of alkane gases for sulphate reduction processes is the slow growth of the involved microbiological consortia. To overcome this,

we hypothesize that the use of alternative electron acceptors that lead to thermodynamically more favourable reactions result in higher growth rates and biomass yields. This approach is realistic, as it is known that many sulphate reducers can use alternative terminal electron acceptors (Meulepas *et al*., 2009b; Widdel *et al*., 2007). Table 2.1 shows the standard Gibbs free energy change for the conversions of methane, ethane and propane with sulphate, thiosulphate and sulphur. It reveals that thiosulphate reduction is thermodynamically more favourable than sulphate reduction. Therefore, it could be expected that reactors started with these "alternative" substrates (thiosulphate, ethane and propane) might be able to achieve industrially feasible conversion rates faster than reactors that run with methane/sulphate as electron donor/acceptor couple. On the other hand, and unlike thiosulphate, the sulphur conversion shows an energetically unfavourable behaviour under standard conditions. However, under biological conditions and with the mentioned electron donors, it presents favourable energy gains (Table 2.1); making sulphur and interesting electron acceptor to be studied.

2.2 Materials and methods

2.2.1 Source of microorganisms

The biomass used for inoculation originated from sediment of Aarhus bay (Baltic Sea, Denmark) and Eckernförde Bay (Baltic Sea, Germany). The sampling site and sampling method for the Eckernförde Bay sediment has been described previously by Meulepas *et al*., (2009a). The Aarhus Bay sediment was obtained at the central part of the bay, at sampling station M1 and M5, which have been described previously (Dale *et al*.,2008). Cores were taken at Station M1 (56°07.0762' N 10°20.8078' E) at a water depth of 15 m, and M5 (56°06.1977' N 10°27.4822' E) at a water depth of 27.5 m, during a research cruise in May 2011 on the RV Tyra. Samples from station M5 were taken using a Rumohr core sampler (80 cm long, 7.5 cm diameter) and reached 60 cm into the sediment bed. Samples from station M1 were taken using a gravity core sampler (3 m long, 12 cm diameter). Directly after collection, the cores were cut into 10 cm slices and kept at 4 °C in sealed gas tight anaerobic bags with oxygen consuming AnaeroGen sachets (Thermo Scientific, Hampshire, UK). Back in the laboratory, the samples were placed in an anaerobic chamber. From every 10 cm section of core M5 and only from sections of core M1 that were determined to belong to the sulphate methane transition zone (SMTZ) by methane and sulphide pore water determination, 5 ml subsamples were taken using a cut-off syringe. These samples were pooled and mixed 1:1 (v/v) with synthetic sea water medium. This mixed slurry was kept at 4 °C and the headspace was filled with 100% 5.5 grade methane (99.999% purity) to a pressure of 1.5 bar. The samples from Aarhus Bay were

collected 4 months before starting the experiment; and the samples from Eckernförde Bay 87 months before. Previous to experimental start-up, both Aarhus and Eckernförde Bay sediment slurries were mixed in a ratio of 1:1 (v/v). This mixture was inoculated in synthetic sea water medium with a ratio of 1:3 (v/v).

2.2.2 Standard incubations procedure

The batch experiments were done under anaerobic conditions in serum bottles. They were closed with butyl rubber stoppers and aluminium caps and the weight and volume of the bottles were determined. Before the medium and biomass were injected into the bottles with a syringe and needle, the oxygen in the bottles was removed by flushing ten times with helium gas. The total liquid volume for each bottle was half of the bottle volume. After adding medium and biomass, the bottles were flushed and filled with 1.7± 0.2 bar of methane, ethane or propane. The experiments were conducted in triplicate for each electron donor. In addition, two control bottles were filled with helium instead of alkanes for each sulphur compound. Cultures were grown anaerobically at a pH between 7.2 and 7.8. The bottles were incubated in the dark at 15^{o}C and shaken at 80 rpm. In the conditions in which inhibition of the microorganisms due to high sulphide concentrations was observed, sulphide was removed by flushing with helium gas and the corresponding alkane was added again to each bottle.

2.2.3 Medium

Synthetic seawater medium was used for the batch incubations (Meulepas *et al*., 2009a). The initial concentration of sulphate was 28 mM, of thiosulphate 14 mM (this, in order to add the same amount of sulphur atoms as for sulphate), and of sulphur 0.26 mM which is high above the possible amount that can be dissolved. All the conditions contained 1 ml$\cdot$L^{-1} of a resazurin solution and 1 ml$\cdot$L^{-1} of a 0.9 M sodium sulphide solution. As the original sediment was kept in medium with sulphate, the medium in the thiosulphate and sulphur bottles contained approximately 3-4 mM of sulphate on the beginning of the experiment.

2.2.4 Analyses

Methane and carbon dioxide were analysed using a gas chromatograph (GC-2010A, Shimadzu, Japan). The GC used two columns connected in parallel (Porabond Q (50m x

0.53mm x 10µm) and Molsieve 5A (25m x 0.53mm x 50µm)). Helium, at a flow rate of 14 $ml \cdot min^{-1}$ was used as carrier gas. A thermal conductivity detector was operated at 175°C and the injector at 120°C. The temperature of the oven was kept on 75°C for 2.75 minutes, time during which methane and carbon dioxide were measured. In the samples that contained either ethane or propane, a longer cycle was required in order to elute these compounds from the column. In this case, right after the initial 2.75 minutes, starts a 15 minutes ramp ($1^{o}C \cdot min^{-1}$) followed by 13.25 minutes of constant temperature. Next, there was a 1.5 minutes ramp ($40^{o}C \cdot min^{-1}$). Finally, the obtained 150°C were maintained for 0.1 minutes. 50 µl of headspace gas from the batch bottles were injected to the GC with a helium pre-washed syringe. Results were quantified with the software Chromeleon 6.8 SR7. The rates and concentrations that are presented on this study were calculated considering all the relevant equilibria for methane, ethane, propane and carbon dioxide.

Hydrogen sulphide was quantified colorimetrically in a reaction yielding methylene blue using standard kits (LCK 653) and a photo spectrometer (XION 500), both from Hach Lange (Dusseldorf, Germany). This method measures all the dissolved sulphide compounds (H_2S, HS^- and S^{2-}). Liquid samples were taken with syringe and needle that were previously flushed with helium. The pressure in the headspace of the bottles was measured using a GHM 3150 – Greisinger electrode (Regenstauf, Germany). The pH was determined with pH paper test strips from Dosatest, Prolabo (Fontenay, France). As for the alkanes and carbon dioxide, the rates and concentrations for hydrogen sulphide are calculated taking into account its concentration and relevant equilibria in liquid and gas phase.

Fatty acids were analysed with a gas chromatograph (Hewlet Packard 5890 series II, Palo Alto, USA). The method description has been previously described previously by Lindeboom *et al*. (2013). The measurement of alcohols was also done in the same chromatograph. The description of the method can be found in Steinbusch *et al*., (2008).

2.3 Results

2.3.1 General

In all the tested conditions between 0.1-0.5 mM of sulphide was produced during the first two weeks of the experiments. This sulphide presumably originated from the use of residual organic matter present in the sediment for sulphate reduction. Additionally, carbon dioxide in the head space was analysed in all the incubations. However, in the conditions in which sulphate and sulphur were used as electron acceptors, the expected increase in the carbon dioxide concentration was in the same order of magnitude as the

error of the measurement. Therefore, for those conditions, the analysis of the results is based on the hydrogen sulphide production.

2.3.2 Sulphate

Batch incubations were done in triplicate or duplicate (controls) to determine the ability of the biomass to reduce sulphate coupled to anaerobic oxidation of methane, ethane and propane. Figure 2.1 displays the change in the sulphide concentration in the batch bottles.

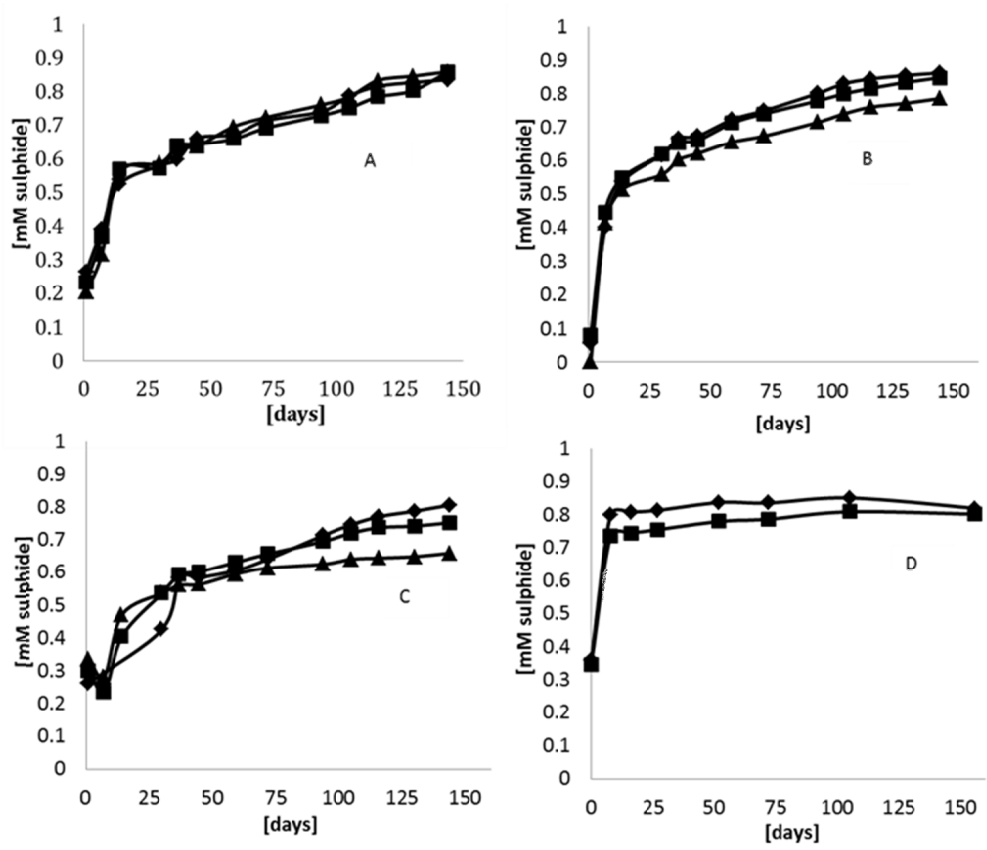

Figure 2.1 Sulphide production in bottles with sulphate A. Methane B. Ethane C. Propane and D. Control.

Figure 2.2 shows the maximum sulphide production rate (SPR), which is calculated after the initial endogenous sulphide production period (days 14 to 144). Clearly, the presence of alkanes results in increased rates of sulphide formation.

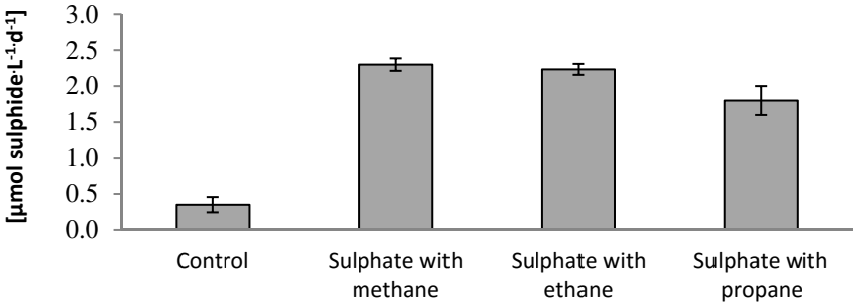

Figure 2.2 Maximum sulphide production rate in bottles with sulphate as electron acceptor.

2.3.3 Sulphur

The mixed sediments from Aarhus and Eckernförde Bay were incubated with sulphur during 120 days. Due to an error in the preparation in one of the propane bottles only two are presented here. As can be seen in Figure 2.3, the initial endogenous sulphide production period was followed by a minor increase in the sulphide concentration.

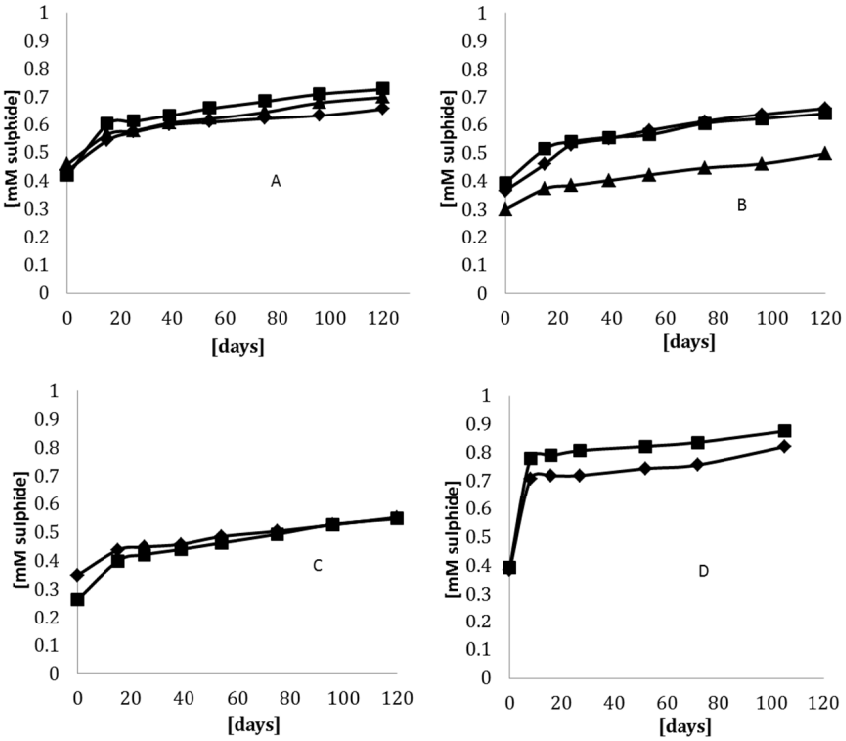

Figure 2.3 Sulphide production in bottles with sulphur A. Methane B. Ethane C. Propane and D. Control.

Figure 2.4 shows the SPR in the bottles with sulphur. It was calculated from day 15 to 120. It can be observed that the presence of alkanes barely enhances the maximum SPR. However, as these bottles contained between 3 and 4 mM of sulphate in the beginning of the experiment, it is possible that the sulphide originated from sulphate instead of sulphur.

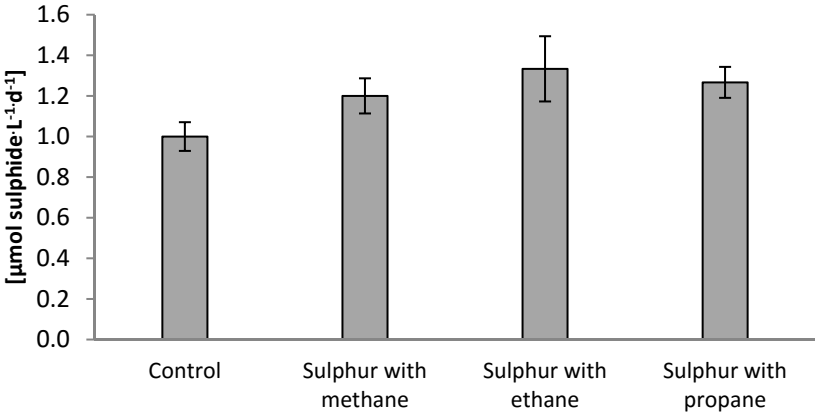

Figure 2.4 Maximum sulphide production rate in bottles with sulphur as electron acceptor.

2.3.4 Thiosulphate

The capacity of the microorganisms present in the sediments to reduce thiosulphate was tested with methane, ethane and propane as electron donors. As can be seen in Figure 2.5, in the conditions in which the alkanes were present as electron donors, higher maximum SPRs were obtained compared to the other tested sulphur compounds. Furthermore, the SPR slowed down when the sulphide concentration reached 5.4 (±0.1) mM, indicating that the microorganisms became inhibited. For this reason, after day 100, part of the sulphide was removed. And, as result of this, sulphide production resumed. The controls showed a continuous sulphide production (up to 4.1 mM). This can be explained by thiosulphate disproportionation (equation 10); which has been reported previously under marine conditions (Habicht *et al*., 1998; Holmkvist *et al*., 2011; Jørgensen, 1990; Jørgensen and Bak, 1991).

The maximum SPRs in the thiosulphate bottles are compared in Figure 2.6. Rates were calculated between days 39 and 81. It can be observed that the maximum SPR is enhanced by the presence of alkanes and even though 4.1 mM of sulphide was produced in the controls, its maximum production rate is significantly lower than in the presence of the alkane gases.

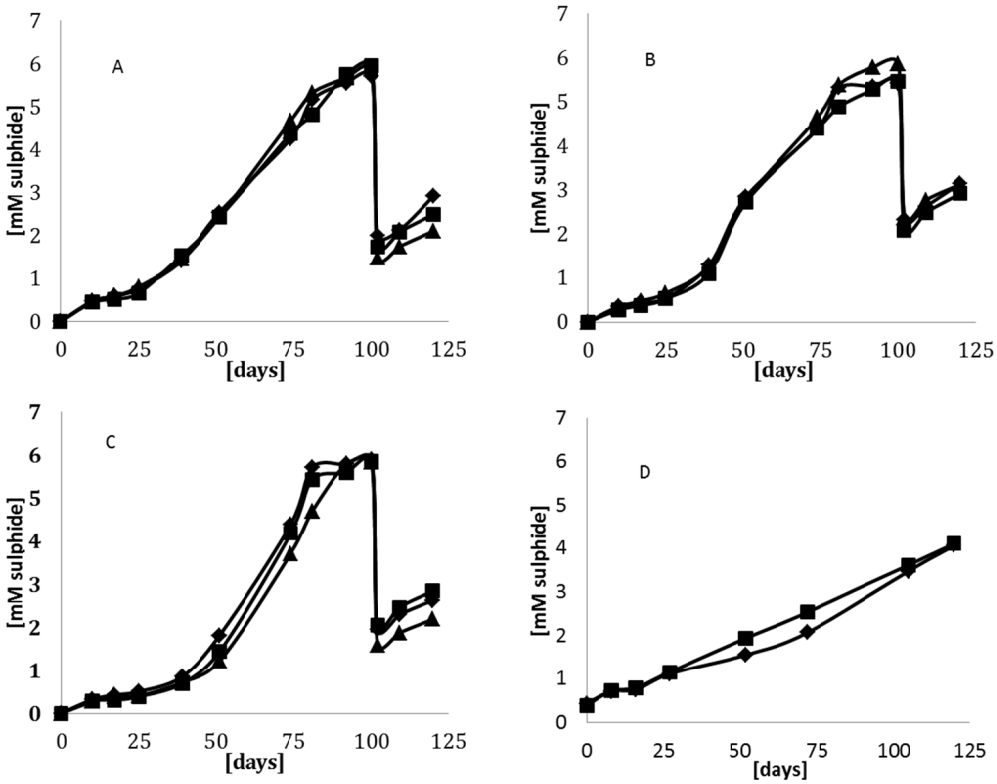

Figure 2.5 Sulphide production in bottles with thiosulphate A. Methane B. Ethane C. Propane and D. Control.

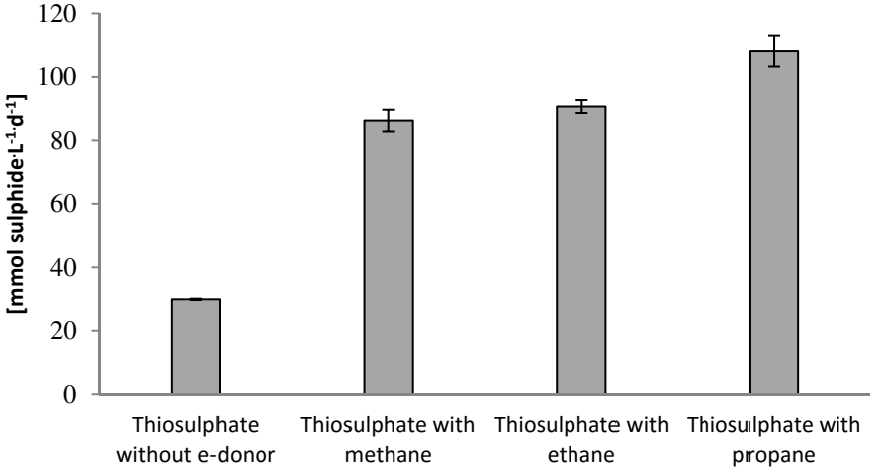

Figure 2.6 Maximum sulphide production rate in bottles with thiosulphate as electron acceptor.

As Figure 2.7 presents, there was no carbon dioxide production in the controls. Confirming the oxidation of the alkanes, carbon dioxide was produced in the bottles with methane, ethane and propane during the highest SPR period (days 39 to 92). Assuming that the electron equivalents derived from alkane oxidation to carbon dioxide were used for thiosulphate reduction according to equations 2, 5 and 8 (Table 2.1), the resulting amount of sulphide produced from that conversion was calculated. This assumption is realistic as the biomass yield is low under anaerobic conditions and because neither volatile fatty acids nor alcohols were produced in any of the experiments. The sulphide formed via disproportionation can then be calculated by subtracting, the calculated amount due to thiosulphate reduction, from the measured total sulphide production. The results of these calculations, as shown in Figure 2.7, reveal that most of the sulphide generated in the bottles with methane and propane originates from thiosulphate reduction. For methane, this corresponded to 94% of the total sulphide produced (3.4 mM of 3.6 mM), while for propane it corresponded to 86 % (4.2 mM of 4.9 mM). In contrast, disproportionation plays an important role particularly when ethane is used, as it accounts for 50% of the produced sulphide (2.0 mM of 4.0 mM).

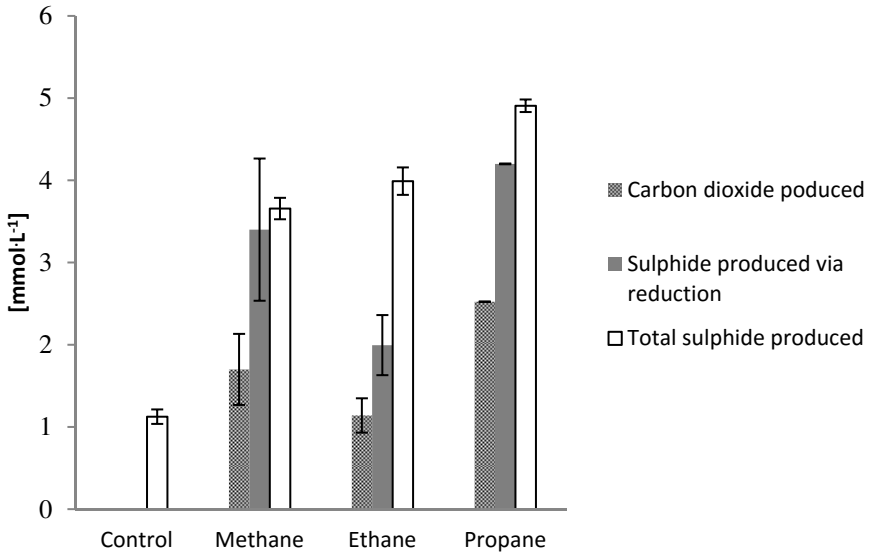

Figure 2.7 Carbon dioxide production related to sulphide production using different electron donors in conditions with thiosulphate during highest SPR period (day 39-81).

2.4 Discussion

The maximum SPRs in $\mu mol \cdot L^{-1} \cdot d^{-1}$ in all experiments are summarized in Table 2.2. It shows that the highest rates that are obtained with the used mixture of sediments correspond to

the conditions in which thiosulphate is used as electron acceptor. Additionally, these results show that the presence of the alkanes enhances the maximum SPR in the conditions with sulphate, and especially in the ones with thiosulphate. On the other hand, no clear increase in the activity of the microorganisms was observed in the conditions in which sulphur was present as electron acceptor. To our knowledge, this is the first study that reports the use of ethane and propane as electron donors with thiosulphate as electron acceptor. However, methane oxidation in the presence of different sulphur compounds has been previously reported by Meulepas *et al*. (2009b).

Table 2.2 Maximum sulphide production rates [$\mu mol \cdot L^{-1} \cdot d^{-1}$]

	Control	Methane	Ethane	Propane
Sulphate	0.35±0.21	2.30±0.17	2.23±0.15	1.80±0.40
Sulphur	1.00±0.14	1.20±0.17	1.33±0.32	1.27±0.15
Thiosulphate	29.90±0.42	86.23±6.86	90.67±4.07	108.13±9.73

Table 2.3 presents the maximum electron transfer rates obtained. It displays how barely any electron transfer is obtained when sulphur is used as electron acceptor. On the other hand, the rates obtained with thiosulphate are between 28 and 45 times higher than the rates obtained with sulphate.

Table 2.3 Maximum electron transfer rate [$\mu mol\ electron \cdot L^{-1} \cdot d^{-1}$]

	Control	Methane	Ethane	Propane
Sulphate	2.80±1.68	18.40±1.36	17.84±1.20	14.40±3.20
Sulphur	2.00±0.18	2.40±0.34	2.66±0.64	2.54±0.30
Thiosulphate	179.40±2.52	517.38±41.16	544.02±24.42	648.78±58.38

As can be seen in Table 2.2 and Table 2.3, the reduction of thiosulphate in the presence of the alkanes proceeded faster than sulphate reduction. This supports our hypothesis that indeed, the use of thermodynamically more favourable alternative electron acceptors result in increased microbial rates (Table 2.1).

When comparing the different electron donors for the sulphate and thiosulphate conditions, there is no large difference on the SPRs; this might indicate that similar processes are involved in the degradation of the different alkanes. A possible explanation is that the organisms able to oxidize SCA's, use thiosulphate as electron acceptor, and that this process can proceed faster than SCA oxidation coupled to sulphate reduction.

For AOM, it has been hypothesized that the process is carried out by two microorganisms in consortia, a methane oxidizer and a sulphate reducer. The presence of thiosulphate

would then render growth of the methane oxidizer independent of the possible rate limiting electron transfer to the sulphate reducer. This would mean that the methane oxidizers are enriched in the presence of thiosulphate. Sulphate reducers still may be enriched by energy conservation through thiosulphate disproportionation.

For ethane and propane, the situation might be different, as sulphate reducers have been isolated from marine sediments that couple their oxidation to sulphate reduction. This shows that these conversions do not have to rely on a syntrophic relation. Our results indicate that these sulphate reducers might be more rapidly enriched by growth on thiosulphate.

Finster *et al*., (1998) reported elemental sulphur disproportionation by *Desulfocapsa sulfoexigens sp* with a SPR of 0.1 $mmol \cdot L^{-1} \cdot day^{-1}$. Meulepas *et al*., (2009b) reported that sulphur was reduced in the presence of methane as sole added electron donor with a maximum SPR of 0.04 $mmol \cdot L^{-1} \cdot day^{-1}$. In our study, sulphur reduction coupled to methane oxidation could not be proven.

The SPRs obtained in the thiosulphate conditions from this study are comparable with the results found by Meulepas *et al*. (2009b)., That study enriched sediments were used and a SPR of 0.11 $mmol \cdot L^{-1} \cdot day^{-1}$ after over 800 days using methane as electron donor was obtained. Our results suggest that for this mixture of microorganisms, and under the evaluated conditions, thiosulphate is the most promising electron donor for methane, ethane and propane degradation. It is still to be determined which were the responsible microorganisms for this conversion and if they can be enriched.

2.5 Conclusions

The mixture of Aarhus Bay and Eckernförde Bay sediments used in this study showed to be capable of performing SCA oxidation couple to sulphate and thiosulphate reduction. SCA oxidation coupled to reduction of sulphur could not be demonstrated. Our results suggest that methane, ethane and propane oxidizers might be more rapidly enriched in the presence of thiosulphate. Therefore, thiosulphate might help as start-up aid for bioreactors dedicated to alkanes degradation coupled to sulphate reduction; a process that has been characterized by long doubling times. However, sulphate reducers might be deprived of substrate in mixed cultures in the presence of thiosulphate. This requires attention when further developing the start-up strategy with thiosulphate. For wastewater containing thiosulphate, SCA's may represent feasible electron donors for full scale applications.

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CHAPTER 3

Thiosulphate conversion in a methane and acetate fed membrane bioreactor

The use of methane and acetate as electron donors for biological reduction of thiosulphate in a 5 L laboratory membrane bioreactor was studied and compared to disproportionation of thiosulphate as competing biological reaction. The reactor was operated for 454 days in semi-batch mode; 30% of its liquid phase was removed and periodically replenished (days 77, 119, 166, 258, 312 and 385). Although the reactor was operated under conditions favourable to promote thiosulphate reduction coupled to methane oxidation, thiosulphate disproportionation was the dominant microbial process. Pyrosequencing analysis showed that the most abundant microorganisms in the bioreactor were phototrophic green sulphur bacteria (GSB) belonging to the family *Chlorobiaceae* and thiosulphate disproportionating bacteria belonging to the genus *Desulfocapsa*. Even though the reactor system was surrounded with opaque plastic capable of filtering most of the light; the GSB used it to oxidize the hydrogen sulphide produced from thiosulphate disproportionation to elemental sulphur. Interrupting methane and acetate supply did not have any effect on the microbial processes taking place. The ultimate goal of our research was developing a process that could be applied for thiosulphate and sulphate removal and biogenic sulphide formation for metal precipitation. Even though the system achieved in this study did not accomplish the targeted conversion using methane as electron donor, it does perform microbial conversions which allow to directly obtain elemental sulphur from thiosulphate.

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3.1 Introduction

Sulphur compounds like sulphate, sulphite and thiosulphate are common constituents of aqueous effluents from chemical, mining and metallurgical industries. Biological treatment of these streams not only allows to remove the sulphur oxy-anions from the aqueous effluents, but also to recover sulphur which can be (re)used in chemical industry, or as fungicide and fertilizer. Various industrial processes in which sulphur oxyanions are biologically reduced to sulphide have been developed and optimized (Stams *et al.,* 2008). After reduction, the produced sulphide is either oxidized to elemental sulphur or used to precipitate metals. The latter not only allows to recover sulphur but also the used metals (Weijma *et al*., 2006).

Because industrial wastewaters often lack sufficient electron donor for biological reduction of the sulphur-oxyanions, these need to be added from external sources. The most commonly added electron donors are ethanol and hydrogen (Meulepas *et al.* 2010). However, their use adds considerable operational and investment costs to the process. Industrial grade ethanol is fairly expensive for waste water treatment purposes. Furthermore, although the feedstock for hydrogen production (methane) is relatively cheap, the required methane reformer is expensive. Using methane as direct electron donor for sulphate reduction through the microbiological process of anaerobic oxidation of methane (AOM) would allow to reduce operational costs (Meulepas *et al*., 2010). AOM is thought to rely on an obligate syntrophic relationship between anaerobic methanotrophic (ANME) archaea and sulphate reducers (Boetius *et al*., 2000; Hinrich *et al.,* 2000; Valentine and Reeburgh, 2000). Because AOM consortia have doubling times between 1.2 and 7.5 months on methane and sulphate (Deusner *et al.,* 2010; Girguis *et al.,* 2005; Krüger *et al*., 2008; Meulepas *et al.,* 2009a; Nauhaus *et al.,* 2007; Zhang *et al.,* 2010), economically feasible conversion rates in bioreactors have not been obtained yet (Suarez *et al*., 2014). The highest specific AOM rate (370 $\mu mol \cdot g_{dry\_weight}^{-1} \cdot day^{-1}$) was obtained in a high pressure-continuous incubation (Deusner *et al*.,2010). Furthermore, the highest volumetric sulphate reduction rate (0.6 $mmol \cdot L^{-1} \cdot day^{-1}$ or 286 $\mu mol \cdot g_{dry\_weight}^{-1} \cdot day^{-1}$) was reported by Meulepas *et al.* (2009a) in an 884 days experiment using a 2-L membrane bioreactor. However, these rates are still low considering that up to 100 times higher sulphate reduction rates can be achieved with hydrogen or ethanol.

To increase AOM rates for practical applications, we aim to stimulate the growth of AOM consortia. Jagersma *et al.,* (2012) found that incubating Eckernförde Bay sediment in the presence of thiosulphate and acetate increased the number of ANME microorganisms as compared to all Archaea. Furthermore, a higher standard Gibbs free energy can be obtained when using thiosulphate (Reaction 1, Table 3.1) instead of sulphate (Reaction 2,

Table 3.1) for methane oxidation (-39.0 $KJ \cdot mol^{-1}CH_4$ vs. -16 $KJ \cdot mol^{-1}CH_4$). It has been previously shown that this higher energy gain leads to higher AOM rates (Suarez *et al*., 2014, Chapter 2). However, in that study, part of the thiosulphate was disproportionated to sulphate and hydrogen sulphide (Reaction 3, Table 3.1).

Table 3.1. Sulphate and thiosulphate reduction and thiosulphate disproportionation and their standard (ΔG°) and biological (ΔG°′) Gibbs free energy changes. pH 7.0, and the following concentrations: CH_4 0.9 mM, HCO_3^- 0.5 mM, SO_4 28 mM, S_2O_3 14 mM and HS^- 0.5 mM. All Gibbs free energy changes are given in Kilojoule per mole of the first compound of the reaction equation

	Stoichiometry	ΔG°	ΔG°′
Reaction 1	$CH_4 + S_2O_3^{2-} \rightarrow HCO_3^- + 2HS^- + H^+$	−39.0	−66.6
Reaction 2	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	−16.5	−27.6
Reaction 3	$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$	−22.5	−39.6

In this work we aimed to rapidly enrich for microorganisms capable of performing AOM coupled to thiosulphate reduction. For this, we supplied acetate and thiosulphate as additional substrates besides methane and sulphate. This was done in a 5-L semi-batch membrane bioreactor inoculated with mixed sediments from Eckernförde Bay and Aarhus Bay. This bioreactor type not only allows complete biomass retention, but also continuous removal of hydrogen sulphide. This is important as low concentrations of hydrogen sulphide can inhibit AOM in Eckernförde bay sediments or enrichments (Meulepas *et al.,* 2009b). For the biomass used in this study; Suarez et al., (2014) reported this concentration to be 5.4 mM.

3.2 Materials and methods

3.2.1 Source of microorganisms

The biomass used for inoculation originated from sediments of Aarhus (Baltic Sea, Denmark) and Eckernförde Bay (Baltic Sea, Germany). The sampling site and method for the Eckernförde Bay sediment has been previously described by Meulepas *et al*., (2009a) and for the Aarhus Bay sediment it was described in Suarez *et al*., (2014). Both sediments were mixed in a ratio of 1:1 and 180 $g_{dry\_weight} \cdot L^{-1}$ was added to the reactor.

3.2.2 Medium

Synthetic sea water medium was prepared as described previously by Meulepas *et al*., (2009a). The initial sulphate concentration was 24 mM. Sodium thiosulphate (17 ± 2 mM) was added on days 0, 77, 120, 166, 258, 316 and 385 while acetate (0.6 ± 0.1 mM) was added on days 0, 57, 77, 82, 105, 120, 126 and 134. 1 $ml \cdot L^{-1}$ of a 2 mM resazurin solution and 1 $ml \cdot L^{-1}$ of a 0.9 M sodium sulphide solution were added to the medium during preparation.

3.2.3 Reactor set up

The reactor set-up is shown in Figure. The bioreactor (R-201) was a cylindrical glass vessel (height: 50 cm, internal diameter: 12 cm, total liquid volume: 5 L) and it was operated as a fed-batch system, which means the reactor was operated in batch mode and 30% of the medium was replaced on days 77, 119, 166, 258, 312 and 385. Removal of the medium was done through 4 polysulphone membranes (M-201) (Triqua BV, Wageningen, The Netherlands), with a total effective surface area of 0.028 m^2 and a filter pore size of 0.2 µm to achieve complete cell retention. The extraction of the media was performed using a peristaltic pump (P-301) (Watson Marlow 520U, Cornwall, UK). The transmembrane pressure was monitored with a pressure transmitter (PTX 1400, Druck, Leicester, UK). Redox potential was determined via a QR402X sensor (Prosense-Qis.Oosterhout, The Netherlands). The pH was monitored with a sulphide resistant pH electrode (QP108X, Prosense-Qis. Oosterhout, The Netherlands) and it was controlled by manual addition of sodium hydroxide (0.2 M). A PT100 sensor was used to monitor the temperature. The reactor was equipped with a water jacket and temperature was controlled with a Julabo F25-ME (Seelbach, Germany) water cooling system. The reactor was partially protected from day light by adding a blue screen to the reactor room. Light protection varied between 93% and 99.9% depending on the light intensity available in the reactor surroundings. This variation was measured with a portable light meter (Li-Cor LI-250A) equipped with Quantum sensor. Additionally, on day 385, the reactor was covered with isolation foil to ensure a completely dark reactor environment.

Methane (99.9995%) The Linde Group (Munich, Germany) from a gas cylinder (V-101) was continuously added to the reactor via a gas sparger at a flow rate of 2.2 $L \cdot Lreactor^{-1} \cdot day^{-1}$. This was done in order to supply methane to the microorganisms, to (partially) remove carbon dioxide and hydrogen sulphide via stripping and to prevent membrane fouling. Additionally, as a water lock (V-301) was connected to the bioreactor gas vent, a slight overpressure was maintained during the whole operation time. The methane flow was

measured and controlled by a thermal mass flow controller (MFC-101) (5850E, Brooks, Veenendaal, The Netherlands). Nitrogen (V-102) (99.5%, The Linde Group) was also added via a mass flow controller (MFC-102). Nitrogen fed was done either to maintain anaerobic conditions in situations in which the reactor had to be opened (*e.g.*, electrodes calibration or sampling) or after day 312 when the methane fed was stopped.

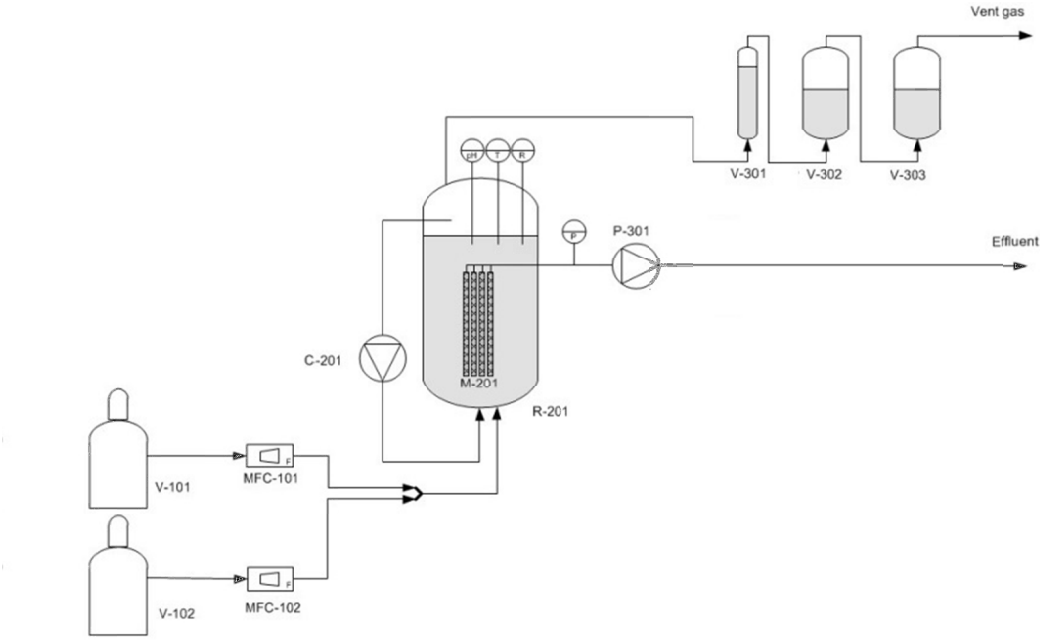

Figure 3.1. Reactor set-up. V stands for the vessels , MFC for the mass flow controllers, P for pumps, C for the compressor and R for the reactor. pH, T and R represent the pH, temperature and redox sensors respectively..

The reactor was mixed twice a week via a laboratory compressor (P-201) (N 86 KT.18, KNF Laboport, Rowville, Australia).

A 1 M zinc chloride bottle (V-302) was located situated after the water lock from the beginning of the reactor operation. Another zinc chloride bottle (V-303) was added on day 77. As hydrogen sulphide was continuously stripped out of the reactor, V-302 and V-302 allowed to capture it as zinc sulphide

Temperature, pH and redox where monitored in real time and data were logged into the computer by LabView 7.1 software. Mass flow controllers were regulated through that software as well.

3.2.4 Physicochemical analyses

To ensure reproducible and representative aqueous reactor samples, the samples were taken after at least 30 minutes of mixing with the pump P-201.

Dry weight was determined according to Standard Methods (APHA 2005).

Acetate was measured by gas chromatography (Hewlett Packard 5890 series II, Palo Alto, USA) according to Lindeboom *et al*. (2013). Methane and carbon dioxide were also measured using a gas chromatograph (GC-2010A, Shimadzu, Japan) according to Suarez *et al*., 2014.

Sulphate and thiosulphate were quantified using a Dionex ICS2100 ionic ion chromatography system (Dionex, Salt Lake City, USA). Liquid samples of 5 µl were injected using a gradient separated over a guard column (Dionex IonPac AS19, 4x50mm) and an analytical column (Dionex IonPac AS19, and 4x250 mm). The columns were operated at a temperature of 30°C. A potassium hydroxide solution at a flow rate of 1.0 $ml \cdot min^{-1}$ was used as eluent. It was prepared on-line using the EG40 Eluent Generator (Dionex) equipped with a KOH cartridge (Dionex P/N 58900) and milliQ water. The KOH concentration gradient was programmed as follows: 10 mM from 0 to 10 minutes. Followed by a 15 minutes gradient in which the concentration was linearly raised to 50 mM and kept there for 5 minutes. Finally, the KOH concentration was decreased to 10 mM during the last minute. An electrochemical detector (ECD) was used. Sample preparation prior to measurement consisted on centrifuging the sample at 10,000 rpm during 10 minutes. This was followed by mixing 0.2 ml of liquid sample with 0.2 ml of a 1M zinc acetate solution to capture hydrogen sulphide. After a second centrifugation to remove the captured zinc sulphide, 0.9 ml of a 1M mannitol solution was mixed with 0.1 ml of the supernatant. Mannitol was added to stabilize sulphur compounds. The standards for sulphate had a concentration between of 0.25 and 2 mM while for thiosulphate standards with a concentration range between 0.4 and 1.8 mM were used. Results were quantified using the software Chromeleon 6.8 SR7.

Hydrogen sulphide was quantified colorimetrically in a reaction yielding methylene blue using standard kits (LCK 653) and a photo spectrometer (XION 500), both from Hach Lange (Düsseldorf, Germany). This method measures all the dissolved sulphide compounds (H_2S, HS^- and S^{2-}).

Total sulphur in the samples was analysed by a combined microwave and Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Vista-MPX CCD simultaneous,

Varian Inc. Palo Alto, USA). The method has been previously described by Hageman *et al*. (2013). The standard deviation in all measurements was equal or lower than 1.8%.

Elemental sulphur was determined by high performance liquid chromatography (HPLC). For this measurement, the sample was first centrifuged and extraction with acetone was performed to the pellet. The exact conditions for sample preparation and measurement were described in Jansen *et al*., (1995).

Samples for scanning electron microscopy - energy-dispersive X-ray spectroscopy (SEM-EDX) measurements were washed three times with sulphur free medium before analysis. Washing was done by centrifuging at 10.000 rpm for 10 minutes after which the supernatant was removed and the pellet was resuspended in sulphur-free medium. Repeating this procedure 3 times warranted that the only sulphur present in the sample corresponded to sulphur in solid form. The samples were placed on SEM sample holders by carbon adhesive tabs (EMS, Washington, USA) and subsequently coated with about 15 nm Iridium. Samples were analysed for morphology at 2 KV, 6 pA, WD 5 mm at room temperature, in a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, The Netherlands). EDX analyses were accomplished using the same electron microscope by a X-Max/AZtec X-ray analyser (Oxford Instruments Analytical, High Wycombe, England) at an acceleration voltage of 15 kV, 200pA, WD 5mm.

Several methods were used to detect polysulphide. The first one consisted on measuring the absorbance of the centrifuged sample at 285 nm as described by Teder (1967). Second, as described by Teder (1971) and Jørgensen *et al*. (1979), the zero valent sulphur atoms in the polysulphide chains were separated by acidifying the sample from which other solids have been previously separated; in this case by sedimentation. After this, elemental sulphur was measured as described above.

Batch tests with biomass taken from the reactor were done in order to determine if methanotrophic or methanogenic activity was observed. Biomass for these tests was taken on day 105 of the reactor operation. The tests were done in serum bottles which were made anaerobic before adding the medium and the biomass. Cultures were incubated in the dark at 15 °C. The complete description of how this was done has been previously reported in Suarez *et al*., 2014.

3.2.5 Microbial community analysis

Genomic DNA was extracted using the Fast DNA Kit for Soil (MP Biomedicals, Ohio, USA) according to the manufacturer's protocol with two 45-second beat beating steps using a

Fastprep Instrument (MP Biomedicals, Ohio, USA). Quantity and quality was checked using NanoDrop® ND-2000 (Thermo Sientific, Wilmington, DE, USA).

Extracted DNA from the sampling point at 105 days was used for archaeal clone library construction. To amplify almost full-length archaeal 16S rRNA genes for cloning, primers A109f (Grosskopf *et al*., 1998) and 1492R (Lane, 1991) were used (primers are given in Table 3.S1). PCR amplification was done with the GoTaq Polymerase kit (Promega, Madison, Wisconsin, USA) using a G-Storm cycler (G-storm, Essex, UK) with a pre-denaturing step of 120 s at 95^{o}C followed by 35 cycles of 95^{o}C for 30 s, 40 s at 52^{o}C and 90 s at 72^{o}C. Lastly, a post-elongation step of 5 min at 72^{o}C was done. PCR products were pooled and purified using the PCR Clean & Concentrator kit (Zymo Research Corporation, Irvine, CA, USA) and product size was checked on a 1.5% agarose gel. DNA quantification was done using NanoDrop® ND-2000 (Thermo Sientific, Wilmington, DE, USA). Pure products were ligated into a pGEM-T Easy plasmid vector (pGEM-T Easy Vector System I, Promega, Madison, Wisconsin, USA) and transformed into *E. coli* XL1-Blue Competent Cells (Stratagene/Agilent Technologies Santa Clara, CA, USA). Both ligation and transformation were performed according to the manufacturer's instructions. Nucleotide sequence data reported are available in the European Nucleotide databases under the accession nos. LN626804-LN626890.

Reactor samples for quantitative real-time PCR (qPCR) were taken after 15 days and after 90 days of reactor operation. Extracted DNA was purified using the OneStep PCR inhibitor removal kit (Zymo Research Corporation, Irvine, CA, USA). The DNA concentration was determined with Nanodrop (Thermo Fisher Scientific, MA, USA). Amplifications were done in triplicate in a BioRad CFX96™ system (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 25 µl using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 5 ng of template DNA and primers with optimal concentrations and annealing temperatures for highest efficiency and specificity (Table 3.S2), all according to the manufacturer's recommendations. Triplicate standard curves were obtained with 10-fold serial dilutions ranged from 2.10^{5} to 2.10^{-2} copies per µl of plasmids containing 16S rRNA archaeal inserts of ANME-1a, ANME-2a/b. The efficiency of the reactions were up to 91% and the R^2 of the by real time Q-PCR obtained standard curves were up to 0.997. All used primers were extensively tested for specificity with cloned archaeal inserts of ANME-1a, ANME-2a/b and with genomic DNA of *Methanosarcina mazei* TMA (DSM-9195) and *Desulfovibrio sp*. G11 (DSM-7057). PCR conditions consisted of a pre-denaturing step for 5 min at 95^{o}C, followed by 5 touch-down cycles of 95^{o}C for 30 s, annealing at 65^{o}C for 30 s with a decrement per cycle to reach the optimized annealing temperature, and extension at 72^{o}C (times are shown in Table 3.S2). This was followed by 40 cycles of denaturing at 95^{o}C for 20 s, 30 s of annealing (temperatures are shown in Table 3.S2) and extension at 72^{o}C (times are shown in Table 3.S2). PCR products were checked for specificity by a

melting curve analysis (72-95^{o}C) after each amplification step and gel electrophoresis. Quantification of specific archaeal and bacterial groups was expressed as number of copies/gram dry weight and relative to the amount of general archaeal products.

Extracted DNA from the sampling point at 105 days was used for bacterial 16S rRNA gene pyrosequencing. Barcoded amplification of the V1-V2 region of the 16S rRNA gene was done using forward primer 27F-DegS (van den Bogert *et al*., 2011) that was extended with the titanium adapter A and an eight-base sample specific barcode (Hamady *et al*. 2008) at the 5'-end, and an equimolar mix of reverse primers 338R-I and 338R-II (Daims *et al.* 1999) that were appended with the titanium adapter B at the 5'-end. All primers used are given in Table 3.S1. PCR amplification was performed in a SensoQuest labcycler (SensoQuest GmbH, Goettingen,Germany) in a total volume of 100 µl containing 2 µl DNA (20 ng/ul), 500 nM of barcoded forward primer and reverse primermix (Biolegio BV, Nijmegen, The Netherlands), 2 U of Phusion Hot start II High-Fidelity DNA polymerase (Finnzymes, Vantaa, Finland), 20 µl of 5x HF buffer, 2 µl PCR grade nucleotide mix (Roche, Diagnostics GmbH, Mannheim, Germany), and 65 µl nuclease free sterile water. PCR amplification conditions were a pre-denaturing step of 3 min at 98^{o}C followed by 30 cycles of 98^{o}C for 10 s, 56^{o}C for 20 s and 72^{o}C for 20 s. Lastly, a post-elongation step of 10 min at 72^{o}C was done. PCR products were loaded on a 1% (v/v) agarose gel containing 1x SYBR Safe (Invitrogen) to check the PCR product size and purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, MA, USA). The concentration was determined using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA). All samples for pyrosequencing were pooled in equimolar amounts afterwards. Pooled samples were loaded on a 1% (v/v) agarose gel containing 1x SYBR Safe (Invitrogen) and bands of the correct size were excised and purified with the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, MA, USA) using 25 ul elution buffer for collecting the DNA. Pooled samples were quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA) and submitted for pyrosequencing on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC Biotech, Germany).

The pyrosequencing data were analysed with a workflow based on Quantitative Insights Into Microbial Ecology (QIIME) v1.2 (Caporaso *et al*. 2010), and reads were filtered for chimeric sequences using the usearch algorithm (Edgar, R.C. 2010). OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th, 2010 using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. The SILVA reference database was used for taxonomic classification (Quast *et al*.,(2013). After picking representative OTUs, we quantified the absolute and relative amount of reads of every OTU and we only considered OTUs that are highly abundant (>100 reads/OTU).

3.3 Results and discussion

3.3.1 Reactor operation until day 152

The concentrations of sulphate (Figure 3.2a), thiosulphate (Figure 3.2b), acetate (Figure 3.2c) and sulphide (Figure 3.2d) in the reactor up to day 152 are presented. Additionally, Figure 3.2e shows the net sulphide production, which accounts not only for the sulphide present in the reactor but also for the sulphide that is stripped out and captured as zinc sulphide. The pH of the reactor was kept around 7.3 (±0.4) during the whole experiment (data not shown). The redox potential was -375 mV (±75 mV) (data not shown).

During phase I (Figure 3.2), the initial 0.5 mM of added acetate was consumed while the thiosulphate concentration slightly decreased (1.1 mM at a rate of 0.028 $mmol \cdot L^{-1} \cdot day^{-1}$). This could have been either due to reduction with acetate (Reaction 4) and organic compounds that might be present in the sediments, or disproportionation (Reaction 3). However, as the sulphate concentration did not change, there was no clear indication for the latter. Furthermore, acetate might have been used as carbon source for anabolic use.

Reaction 4 $$CH_3CO_2^- + S_2O_3^{2-} + H_2O \rightarrow 2HCO_3^- + 2HS^- + H^+$$

After the depletion of acetate on day 40 (phase II, Figure 3.2), the thiosulphate consumption rate increased at least 29-fold (13.4 mM consumed in 17 days, 0.79 $mmol.L^{-1}.day^{-1}$). Concomitantly, 13.2 mM of sulphate was formed, resulting in a ratio thiosulphate decrease: sulphate increase close to 1:1. This indicated that thiosulphate was disproportionated according to reaction 3. Apparently, as shown before by Pikaar *et al*., 2013, thiosulphate reducing bacteria shifted to disproportionation after acetate was depleted. However, there was only 0.45 mM of sulphide formed and it was unlikely that it escaped through the gas vent. This suggested than an unknown sulphur compound was being formed from thiosulphate.

Acetate (0.50 mM) was again added on day 57 and it was consumed by day 77 (Phase III, Figure 3.2). During this 20 day period, in which thiosulphate was not present in the reactor, 4.3 mM of sulphate was consumed. According to reaction 5, and assuming that the acetate was only used for metabolic purposes, the consumed acetate could only account for about 12% of the sulphate removal.

Reaction 5 $$CH_3CO_2^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$$

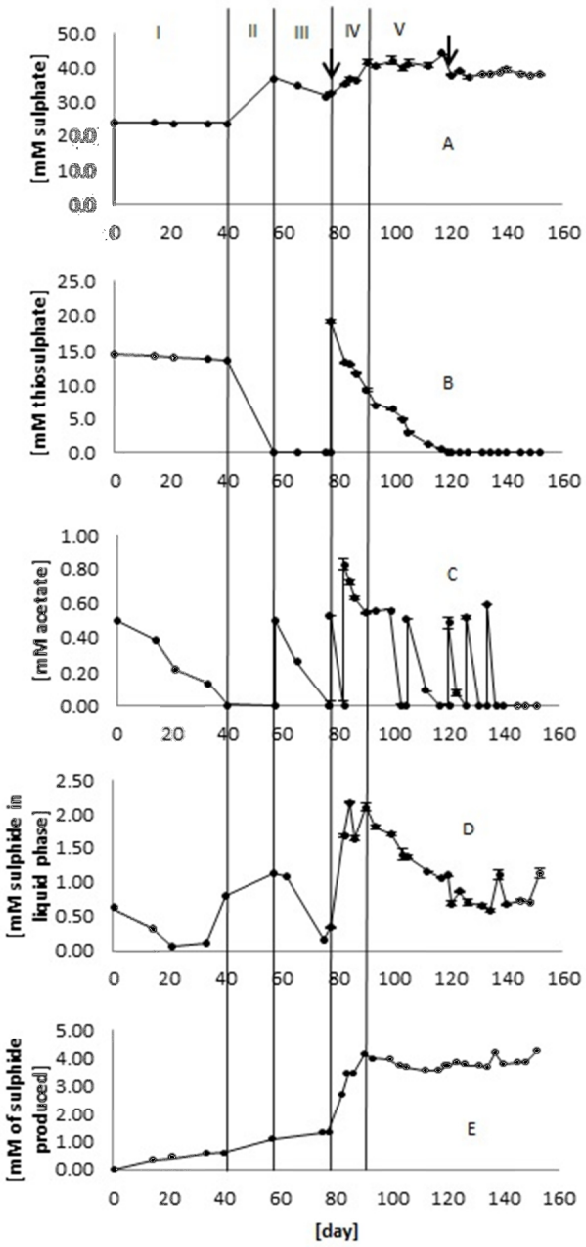

Figure 3.2. Measured compounds concentrations and net sulphide production. A, sulphate. B thiosulphate. C, acetate. D, sulphide E. net sulphide production. The arrows indicate the days in which medium was partially replaced. Error bars represent the standard deviation of duplicate measurements. The vertical lines indicate the limit of the time phases that are used to explain the Figure. I (day 0-40), II (day 40-57), III (day 57-77), IV (day 77-90), V (day 90-152)

As it can be expected that the inoculum was depleted of easily biodegradable electron donors by this time, this result pointed to AOM (reaction 2). However, only 0.27 mM of sulphide production was measured (Figure 3.2e), which accounts for only about 6% of the sulphur species removed as sulphate, indicating again that an unidentified sulphur compound had formed. The nature of this compound will be looked into in the following sections.

On day 77, 30% of the medium was replaced. On this day also thiosulphate (19.1 mM) and acetate (0.53 mM) were added. From that moment and up to day 90 (Phase IV, Figure 3.2), 9.8 mM thiosulphate was steadily consumed, while 2.8 mM sulphide and 9 mM sulphate were produced. The ratio of -1:+0.9 for depleted thiosulphate to formed sulphate indicates that at least 90% of the depleted thiosulphate was disproportionated. However, the ratio of thiosulphate depleted : sulphide formed of –1:+0.3 indicates a maximum of only 30% of sulphide produced. In addition, of the 19.6 mM of sulphur removed as thiosulphate only 11.8 mM of sulphur species were recovered as sulphate and sulphide, indicating again the formation of an unidentified sulphur compound(s).

Finally, phase V (day 90 to 152) was characterized by thiosulphate consumption (9.3 mM) with barely any net sulphate or sulphide production (2.4 mM and 0.1 mM, respectively). This indicates that both sulphate and sulphide were being removed from the system. Sulphate removal was possibly done through sulphate reduction, but to understand the mechanism of sulphide removal several additional analyses were carried out.

Solid sulphur species were measured by total sulphur analyses (ICP, performed on day 99) of an untreated reactor sample (60.2 mM_S ±1.8%) and a centrifuged reactor sample (54.7 mM_S ±1.8%). Furthermore, polysulphide in the supernatant was detected by spectrophotometry and HPLC analysis. As the used sulphide measurement only accounts for one sulphur atom per polysulphide chain; not only solid sulphur but also polysulphide might account for the sulphur gap in the mass balance. However, results show that the total sulphur in the supernatant corresponded fairly well with the sum of sulphide, sulphate and thiosulphate concentrations (Table 3.2, day 99). Therefore, polysulphide and other unidentified soluble sulphur compounds could only contribute slightly, if at all, to the sulphur balance.

Table 3.2. Soluble sulphur compounds concentrations (day 99)

	[mM Sulphur]
H_2S	1.7±0.04
SO_4	42.3±2.2
S_2O_3	12.6±0.4
TOTAL	56.6±2.6

Green aggregates were observed under the microscope from around day 99 onwards. Furthermore, the presence of elemental sulphur in the pellet of the reactor sample and the green aggregates was not only confirmed by HPLC measurements (data no shown) but also by SEM-EDX analysis (Figure 3.3).

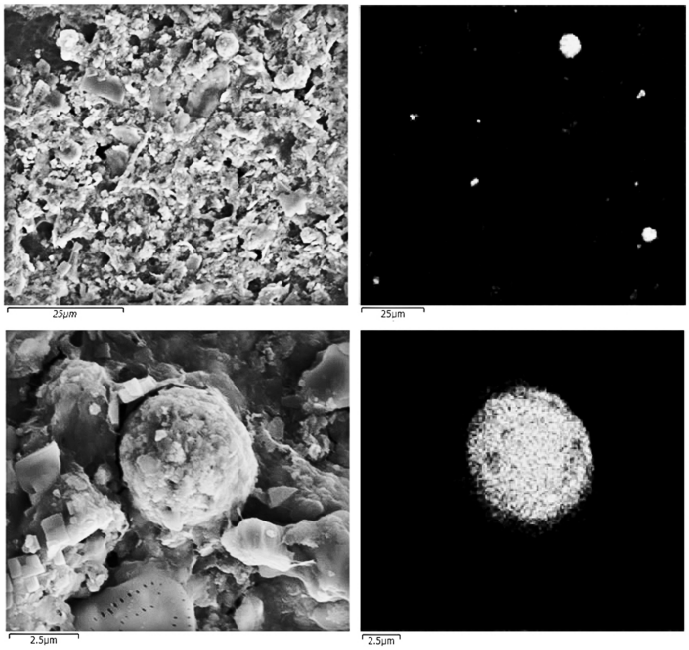

Figure 3.3 SEM-EDX pictures of the reactor contents. The left side pictures show to the original SEM image while the clear areas in the right side images represent the sulphur that was identified in the samples.

The results obtained did not show evidence of AOM taking place. Instead, the dominant microbial processes that seem to be taking place in the reactor were thiosulphate disproportionation into sulphate and sulphide, followed by sulphide oxidation into elemental sulphur. Microbial community analyses were performed in order to obtain a better understanding of the conversions occurring in the reactor.

3.3.2 Microbial community analysis

Biomass samples taken from the reactor at day 105 were subjected to 16S rRNA pyrosequencing for bacterial community analysis and 16S rRNA gene cloning for archaeal community analysis.

Pyrosequencing results show that the most abundant group of microorganisms belonged to the *Chlorobiaceae* family (Figure 3.4) of which all sequences show at least 98% similarity to *Prosthecochloris aestuarii* (Gorlenko 1970). These green sulphur bacteria (GSB) comprised 86% of all reads retrieved from the reactor. GSB are strictly anaerobic microorganisms that assimilate carbon dioxide only in the presence of light while oxidising sulphide to elemental sulphur (Reaction 6) (Gorlenko 1970, Frigard and Dahl 2009). GSB use bacteriochlorophyll c for this phototrophic sulphide oxidation (Gorlenko 1970), which explains the green aggregates in the reactor. Furthermore, GSB are highly efficient photoautotrophic microorganisms; they have been found at 100 m depth in the Black Sea (Manske *et al*., 2005) and at a deep-sea hydrothermal vent (Beatty *et al*., 2005), explaining why GSB were able to grow in the reactor even though it was kept in a nearly-dark environment.

Reaction 6 $$2HS^- + CO_2 \xrightarrow{\text{light}} 2S^o + CH_2O\,(\text{carbohydrate}) + H_2O$$ (Tang, 2009)

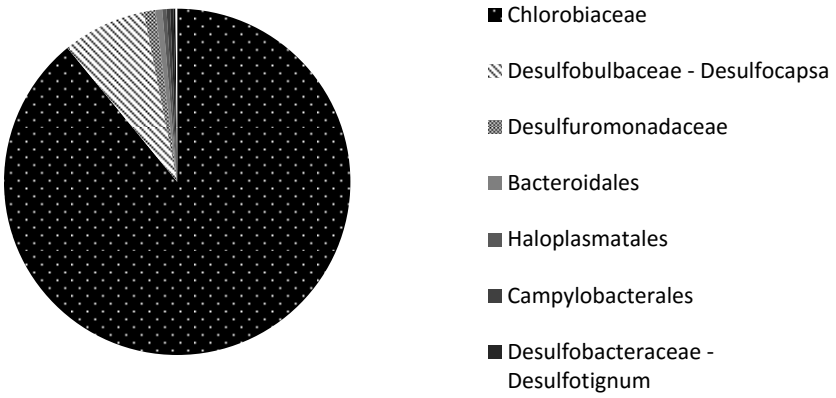

Figure 3.4 Bacterial 16S rRNA gene pyrosequencing results of the reactor inoculated with Eckernförde bay sediment and Arhus bay sediment. Sample was taken at 105 days of incubation. Relative abundance of only major groups with >100 reads/OTU at the order level are shown**.**

Bioreactor studies in which AOM was performed, such the one of Meulepas *et al*., (2009a), did not report presence of GSB. However, that reactor was covered with opaque plastic in order to protect it from light and avoid the interference of phototrophic microorganisms. Our results indicate that partially filtering light is not enough and it is of extreme importance to keep bioreactors targeted to cultivate slow growing microorganisms such as those involved in AOM in complete darkness.

The second most abundant group detected by the pyrosequencing analysis shows a 100% similarity with *Desulfocapsa sulfexigens* strain DSM 10523. *Desulfocapsa* spp. are known to be capable of performing thiosulphate disproportionation (Finster *et al*., 2008).

Moreover, the sulphide removal by GSB creates a thermodynamically favourable condition for *Desulfocapsa* (Finster *et al*. 1998) which can produce more sulphide for the GSB to consume, creating a mutualistic symbiotic relationship. Furthermore, acetate has been reported to stimulate growth of not only *Desulfocapsa* (Janssen *et al*., 1996), but if sulphide and carbon dioxide are present, also of GSB (Gorlenko 1970). Therefore, as *Desulfocapsa sulfexigens* has not been previously associated with AOM, the presence of thiosulphate and acetate and the continuous sulphide removal were the most probable reason for its enrichment.

Finally, also sequences of sulphate reducing bacteria were found. They belong to the family of *Desulfuromonadaceae*, with 97-100% similarity to *Desulfuromusa bakii* strain Gyprop and sequences related to *Desulfotignum toluenicum* strain H3 (99% similarity) (Ommedal and Torsvik, 2007). The number of reads of these reducing bacteria was low (<1.5%) with sulphate reduction probably fuelled by acetate and organic compounds released by cell debris.

Archaeal clone library analysis revealed low diversity of archaeal sequences (Figure 3.5) and the most abundant sequences found belonged to anaerobic methanotrophic archaea from the ANME-2a/b subtype (37% of sequences). Other anaerobic methanotrophic archaea found belonged to ANME-1b comprised only a minor fraction of the archaeal community (3.4%). These results suggest that the conditions provided to the reactor did promote enrichment of ANME-2a/b microorganisms. PCR was therefore performed to determine if these methanotrophic ANME 2a/b archaea were actively growing.

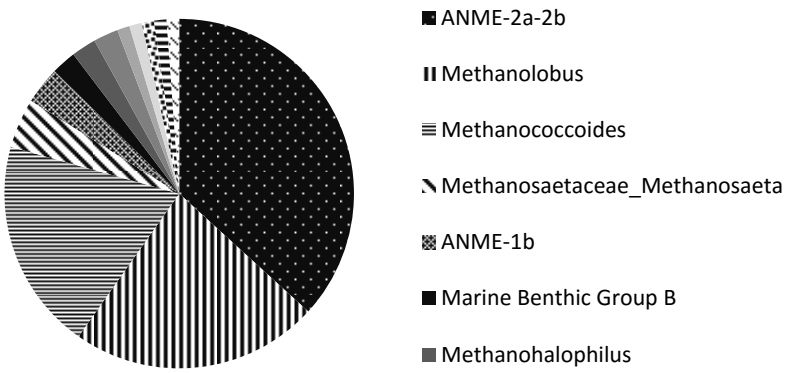

Figure 3.5 Archaeal 16S rRNA gene clone library results of the reactor inoculated with Eckernförde bay sediment and Arhus bay sediment. Sample was taken at 105 days of incubation. Relative abundance of only major groups with >100 reads/OTU at the order level are shown.

Reactor samples for qPCR were taken after 15 days and after 90 days of reactor operation. Results show that ANME-2a did not increase in absolute copies/g dry weight nor in relative amount to total Archaea during reactor operation between these days (Figure 3.6). ANME-2a/b and total archaeal numbers decreased during reactor run, which is consistent with the sulphide oxidation and thiosulphate disproportionation as dominant processes while sulphate reduction was barely observed. The relative abundance of ANME-2a/b stayed fairly stable when compared to the total archaea. This could be due to the constant feeding of methane and sulphate, which could sustain some viability of anaerobic methanotrophs. These results provide evidence that even though the clone library results show that the ANME-2a/b were the most abundant sequences found; net growth of ANME archaea was not achieved. Other archaea that showed a relatively high presence in the clone library belonged to *Methanolobus* (23%) and *Methanococcoides* (20%), which are both methylotrophic methanogens commonly found in marine sediments. However, methanogenic activity was not observed in batch tests made with biomass taken from the reactor, meaning that these Archaea were already present in the inoculum.

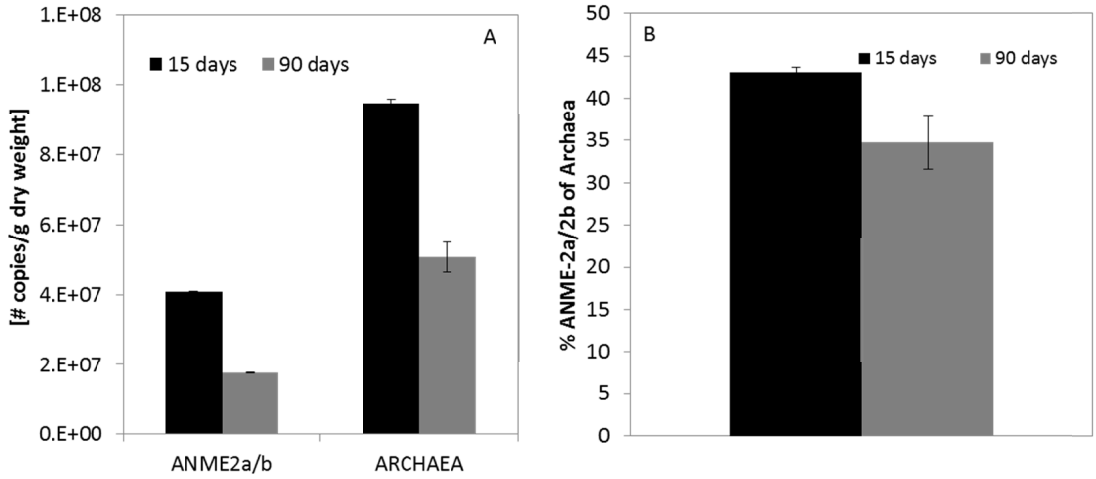

Figure 3.6 Left: qPCR results of ANME-2a specific primers and general archaeal primers copies/g. Right: ratio of ANME-2a over archaea. Samples taken at 15 and 90 days. Standard deviation for triplicate measurements.

3.3.3 Overall reactor operation

On day 152, an ICP measurement was performed. Not only was the sulphur concentration in the total sample (44.4 mM_S ±1.8%), and its supernatant measured (40.0 mM_S ±1.8%),

but also its pellet (5.4 mM_S±1.8%) and the pellet from the green reactor content (GSB) (2.0 mM_S ±1.8%). A sulphur mass balance for the reactor operation until day 152 (Table 3.3) was obtained using the results from this ICP analysis plus the total change in concentration of the measured soluble sulphur compounds. The sulphur in the polysulphide chains (0.7 mM) was estimated from the difference between the sulphur measured in the supernatant of the total sample by ICP analysis and the total sulphur obtained by adding the measured sulphate (38.2 mM), sulphide (1.1 mM) and thiosulphate (0 mM). The sulphur balance closes on about 90% (33.2 mM_S consumed vs 29.8 mM_S produced). The missing 10% (3.4 mM) can be accounted to solid sulphur settled in the bottom of the reactor.

Table 3.3. Sulphur balance for the reactor operation until day 152.

	[mM Sulphur]
Sulphate	19.4
Thiosulphate	-33.2
Sulphide	4.3
Zero- valent sulphur in polysulphide	0.7
Sulphur in GSB	2.0
Solid sulphur forms	3.4
Non measured sulphur	-3.4

Methane continued to be fed to the bioreactor and 30% of the medium was replenished on day 166. However, up to day 257 sulphate was not reduced and AOM activity was not demonstrated. Furthermore, neither evidence of methanotrophic nor methanogenic activity was found in batch experiments performed with biomass taken from the reactor. Additionally, as mention in section 3.2, qPCR analysis did not show increase in either ANME-1 or ANME-2 methanotrophic archaea. These results indicate that AOM was not enhanced in our study. Our reactor was designed and operated under conditions thought to be favourable to increase methanotrophic activity (pH 7.3, redox -375 mV and temperature 15^{o}C). However, neither this nor sulphate consumption was observed. Instead growth of GSB and *Desulfocapsa* in a presumably symbiotic relationship took place. Perhaps, if complete darkness had been provided to the reactor and sulphide concentration had been kept closer to zero, GSB would not have been able to grow and this might have avoided that *Desulfocapsa* and GSB outgrow the microorganisms capable of performing AOM. Another reason might be that unlike the reactor from Meulepas *et al*., (2009a) and other reactors in which AOM has been increased; our system was thoroughly mixed and this might have created shear stress which the ANME microorganisms could not stand. Finally, although the sulphide concentration was kept

below inhibition levels, the lack of continuous mixing might have created zones in the reactor with high sulphide concentrations hindering the growth of ANME archaea.

To further analyse the contribution of the microorganisms found in the pyrosequencing analysis, the reactor was subjected to two additional operational phases; 30% of the medium was replaced at the beginning of each. Results are presented in Figure 3.7 and Table 3.4 presents the mass balance.

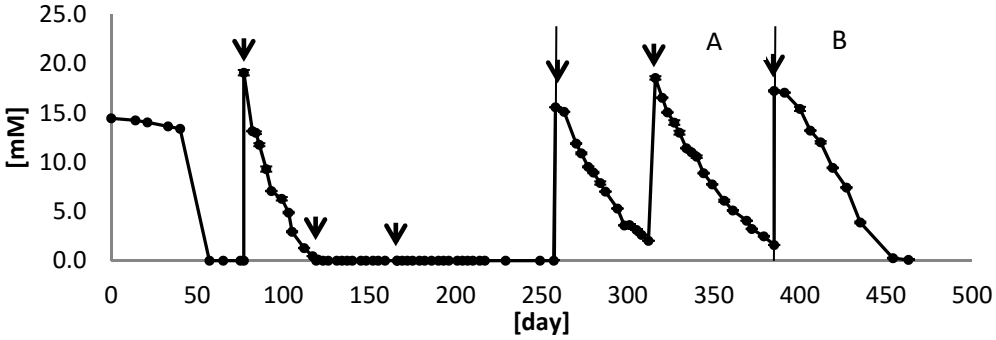

Figure 3.7 Thiosulphate concentration in liquid phase. Acetate was not added in either phase A nor B. Methane fed was interrupted on day 316. The reactor was completely covered from light on day 385. The arrows indicate the days in which medium was replaced. Error bars represent the deviation of duplicate measurements. The vertical lines indicate the time phases that are used to explain the Figure. A (day 258-385), B (day 385-454).

Table 3.4. Sulphur mass balance in the liquid phase between days 258 and 385. Acetate was not added in either phase A or B of Figure 3.7. Methane fed was interrupted on day 316. The reactor was completely covered from light on day 385. The numbers between brackets correspond to the fraction of sulphate and sulphide produced from the consumed thiosulphate which stoichiometrically should be 1.0 (Reaction 3).

	Days 258-385 (nearly-dark) Delta compound [mM]	**Days 385-454 (dark) Delta compound [mM]**
Thiosulphate	-30.5	-17.0
Sulphate	29.5 (0.97)	17.5 (1.03)
Sulphide	8.1 (0.26)	7.5 (0.44)

In the first period (day 258-385) (Phase A, Figure 3.7), thiosulphate was added and methane was initially fed, but no acetate was supplemented. Thiosulphate was added again day 316. But on this day, the methane fed was stopped until the end of the reactor run. As expected, ending the methane and acetate fed did not have an influence of the observed metabolism and results were similar to those previously discussed (section 3.1).

Thiosulphate disproportionation did occur. But, as the GSB continued producing sulphur from sulphide, the latter did not match the stoichiometry (Table 3.4).

On the last phase (days 385-454) (Phase B, Figure 3.7), the reactor was completely covered with isolation foil to ensure complete darkness. As could be predicted, the complete lack of light affected the GSB metabolism (reaction 6). The percentage of sulphide produced related to the disproportionated thiosulphate was raised from 26% to 44% (Table 3.4). However, the sulphide production did not match the thiosulphate disproportionation. It could be hypothesized that acetate was able to support growth of GSB in the dark (Tang *et al*., 2011). However, it was complete depleted in the reactor when it was covered. Nevertheless, even though the net amount of produced sulphide did not increase as much as expected, the concentration of sulphide in liquid phase did double (data no shown) providing another confirmation that the green sulphur bacteria were affected by complete lack of light. A summary of the reactions that occurred in the reactor is presented in figure 3.8.

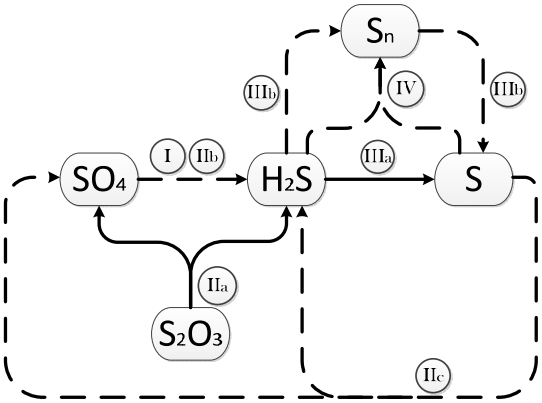

Figure 3.8 Sulphur compounds reactions (possibly) occurring in the reactor. The continuous lines are conversions that occurred in the reactor. The dotted lines are some of the conversions that, from the pyrosequencing results, theoretically could have occurred but no evidence was found. (I) corresponds to conversions made by sulphate reducers. (II) to *Desulfocapsa* conversions; (IIa) was demonstrated; (IIb) might occur when short chain alcohols are available (IIc) might occur when the sulphide concentration is zero (Jansen *et al* 1996, Finster *et al*., 1998). (iii) are the GSB conversions; sulphide might have been oxidized to sulphur directly (IIIa) or via polysulphide formation (IIIb)

It is relevant to realize that it is likely that in periods without mixing, different sections of the reactor might have been exposed to different light intensities. Thus, resulting in varying microbial activity which could concurrently have led to concentration, redox and microbial community gradients. However, as the reactor content was thoroughly mixed before sampling, the samples used for the different analyses are considered homogeneous and representative of the average composition/concentration present in the reactor.

To our knowledge, only one more reactor study dealing with thiosulphate disproportionation has been reported previously (Pikaar *et al*, 2013). Like in our study, they used a submerged membrane bioreactor system. However, they obtained higher thiosulphate disproportionation rates (8.4 $mmol \cdot L^{-1}.day^{-1}$ vs. 0.79 $mmol \cdot L^{-1} \cdot day^{-1}$ in our reactor) using granular and thermophilic biomass operating at 65^{o}C. Our system operates at a lower temperature and it allows direct conversion to elemental sulphur. Both of these characteristics make the sulphur recovery easier and the process costs lower. Nevertheless, more research would be required if industrially relevant thiosulphate conversion rates need to be obtained.

3.4 Conclusions

A bioreactor in which rates of anaerobic methane oxidation were increased was not obtained in this study. However, we obtained a system in which thiosulphate disproportionation and sulphide oxidation yielded sulphate, sulphide and sulphur out of thiosulphate. This process could be used to recover solid sulphur from thiosulphate. This thiosulphate could be generated from photographic fixing, pulp bleaching, gold leaching or fracking processes. However, additional studies should be performed to optimize this conversion by GSB. Such process would potentially allow cost reduction as other technologies used for this conversion rely in the use of additional reactors and raw materials such as air and/or metallic compounds.

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ADDITIONAL INFORMATION

Table 3.S1 Primers used in this study for pyrosequencing and cloning of bacterial and archaeal 16S rRNA gene fragments.

Primer	Primer sequence (5'-3')
Adapter A[1]	CCATCTCATCCCTGCGTGTCTCCGACTCAG
Adapter B[1]	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG
27F-DegS[2]	GTTYGATYMTGGCTCAG
338R-I[3]	GCWGCCTCCCGTAGGAGT
338R-II[3]	GCWGCCACCCGTAGGTGT
A109f[4]	ACKGCTCAGTAACACGT
1492R[5]	GYTACCTTGTTACGACTT

[1] Provided by GATC Biotech, [2] Van den Bogert *et al*., 2011, [3] Daims *et al*., 1999, [4] Grosskopf *et al.,* 1998, [5] Lane 1991.

Table 3.S2 Primers used for qPCR in this study with the corresponding concentrations. Extension time of 40 s. Annaeling temperature of 55.4 °C for the archaeal primers and 57.5 °C for the bacterial ones.

Target	Primer name	Sequence (5'-3')	Amplicon size [bp]	Primer concentration [µM]
Archaea[1]	Arch-787f	*ATTAGATACCCSBGTAGTCC*	272	10
	Arch-1059r	*GCCATGCACCWCCTC*		10
ANME-2a/b[2]	ANME2a-26F*	*TGT TGG CTG TCC GGA TGA*	833	10
		TGT TGG CTG TCC AGA TGA		10
		TGT TGG CTG TCC AGA TGG		10
	ANME2a-1242R	*AGG TGC CCA TTG TCC CAA*		10
ANME-1[2]	ANME1-395F*	*AAC TCT GAG TGC CTC CAA*	1039	10
		AAC TCT GAG TGC CTC CTA		10
		AAC TCT GAG TGC CCC CTA		10
	ANME1-1417R*	*CCT CAC CTA AAC CCC ACT*		10
		CCT CAC CTA AAT CCC ACT		10

*These primers are a mixture of each of the stated primers inequimolar amounts, as previously described[2]

[1] Yu et al., 2005, [2] Miyashita et al., 2009

CHAPTER 4

Anaerobic oxidation of methane associated with sulphate reduction in a natural freshwater gas source

The occurrence of anaerobic oxidation of methane (AOM) and trace methane oxidation (TMO) was investigated in a freshwater natural gas source. Sediment samples were taken and analysed for presence of potential electron acceptors coupled to AOM. Long term incubations of these samples with ^{13}C-labeled CH_4 ($^{13}CH_4$) and different electron acceptors showed that both AOM and TMO occurred. In conditions with ferrihydrite, ferrihydrite + humic acids and the control without electron acceptor, ^{13}C-labeled CO_2 ($^{13}CO_2$) simultaneously increased with methane formation, which is typical for TMO. In the presence of nitrate, neither methane formation nor methane oxidation occurred. Net AOM was measured only with sulphate as electron acceptor. Here, sulphide production occurred simultaneously with $^{13}CO_2$ production and no increase in ^{12}C-methane (produced during methanogenesis) accompanied methane oxidation. In control incubations with sulphate but without $^{13}CH_4$, little methane production was observed, excluding TMO as possible source for $^{13}CO_2$ production from $^{13}CH_4$. Pyrosequencing analysis showed presence of ANME-2a/b (anaerobic methane oxidizing archaea) and AAA (AOM associated archaea) in the incubations with methane and sulphate. Higher abundance of ANME-2a/b in incubations with methane and sulphate was confirmed with qPCR analysis. Bacterial pyrosequencing showed presence of sulphate reducing bacteria belonging to SEEP-SRB1 in the incubations with methane and sulphate. This report explicitly shows that methane oxidation is associated with sulphate reduction in a low-saline freshwater environment, containing ANME-2a/b and AAA methanotrophs and SEEP-SRB1 sulphate reducers.

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4.1 Introduction

Anaerobic oxidation of methane (AOM) coupled to the reduction of nitrate and nitrite is a process found in freshwater environments (Deutzmann and Schink 2011, Ettwig *et al*., 2008, Ettwig *et al*., 2009, Haroon *et al*., 2013, Hu *et al*., 2009, Raghoebarsing *et al*., 2006). Microbial methane oxidation coupled to iron and/or manganese reduction was described to occur in marine sediments (Beal *et al* 2009, Riedinger *et al*., 2014), brackish sediments (Egger *et al*., 2015), a terrestrial mud volcano (Chang *et al*., 2012), and also in freshwater environments (Amos *et al*., 2012, Crowe *et al*., 2011, Sivan *et al*., 2011). Recently, humic acids (HAs) were also hypothesized to act as electron acceptor for AOM (Gupta *et al*., 2013). AOM coupled to sulphate reduction (SR) was discovered to occur in marine sediments (Martens and Berner 1974, Reeburgh 1976), and the responsible microorganisms were first identified to be communities of anaerobic methanotrophic archaea (ANME) and sulphate reducing bacteria (SRB) of the *Deltaproteobacteria* (Boetius *et al*., 2000). AOM coupled to SR in freshwater is likely limited by the low sulphate concentrations, which are around 10 to >500 μM (Holmer and Storkholm 2001). Presence of AOM and SR in freshwater has been observed but both processes could not be coupled and the responsible microorganisms were not identified (Grossman *et al*., 2002, Schubert *et al*., 2011, van Breukelen and Griffioen 2004). Sulphate dependent AOM and ANME archaea were detected in a freshwater lake (Eller *et al*., 2005) and in a high-salinity terrestrial mud volcano (Alain *et al*., 2006). Methane oxidation was also shown in a wide range of soils, but methanogenesis was occurring simultaneously (Gauthier *et al*., 2015). ANME-1 related Archaea have been found in a terrestrial subsurface (Takeuchi *et al*., 2011), but oxidation of labelled methane was also apparent in the control incubation where no external electron acceptor was added. This was also the case in other studies (Beal *et al*., 2009, Egger *et al*., 2015, Sivan *et al*., 2011).

In stable isotope labeling experiments, ^{13}C-labeled methane ($^{13}CH_4$) can be converted to ^{13}C-labeled carbon dioxide ($^{13}CO_2$) during methanogenesis, in a process called trace methane oxidation (TMO) (Zehnder and Brock 1979). TMO was demonstrated to occur in pure cultures of different methanogens (Harder 1997, Moran *et al*., 2005, Moran *et al*., 2007, Zehnder and Brock 1979), in granular sludge (Harder 1997, Meulepas *et al*., 2010, Zehnder and Brock 1980) and in freshwater and terrestrial environments (Blazewicz *et al.,* 2012, Zehnder and Brock 1980). Differentiation between AOM and TMO is therefore difficult because both processes can produce $^{13}CO_2$ at comparable rates. Also, at elevated methane partial pressure, TMO rates increase (Smemo and Yavitt 2007, Zehnder and Brock 1980) and methanogenesis is repressed, which subsequently positively affects SR when sulphate is present (Meulepas *et al*., 2010). Moreover, ferrous sulphate addition may result in enhanced trace methane oxidation rates (Zehnder and Brock 1980). This

means that with elevated $^{13}CH_4$ pressure and high concentrations of sulphate, an increase in $^{13}CO_2$ production and elevated SR cannot be taken as evidence for AOM coupled to SR unless net methane consumption is measured. Although there is convincing evidence that ANME archaea are capable of net AOM, detecting ANME sequences or cells in mixed communities containing also methanogens does not prove that AOM takes place. Especially in view of the fact that ANME could potentially perform methanogenesis (Bertram *et al*., 2013, Lloyd *et al*., 2011) and as a consequence could perform TMO.

In this study we investigated the occurrence of both TMO during net methanogenesis and AOM in samples taken from a freshwater natural gas source. AOM was distinguished from TMO by simultaneous detection of $^{13}CH_4$, $^{12}CH_4$ (produced during methanogenesis) and $^{13}CO_2$. We investigated methane oxidation and production activity in long term slurry incubations (>168 days) with different electron acceptors that are possibly involved in AOM and $^{13}CH_4$ as the sole electron donor. Control incubations without addition of methane were done to accurately distinguish between net methane oxidation and net methanogenesis. Incubations with sulphate and with and without methane were monitored for an extended period of 728 days.

4.2 Materials and methods

4.2.1 Sampling

Samples were taken in spring of 2011 from two natural gas sources in Berkhout, Noord Holland, The Netherlands (52° 38’31’’ N, 4° 59’49’’ E). These gas sources were used for domestic purposes by capturing natural gas from groundwater pockets, using a 30 m long pipe (Figure 4.1). Different locations were sampled: the effluent of an active gas source (‘tank’) and the sediment of the ditch where the effluent is collected (‘ditch 1’), the sediment of the ditch where the effluent of the storage tank is collected (‘ditch 2’), and from the sediment inside a gas source that was no longer in use (‘tank 2’, not in Figure 4.1). Sediment samples were collected in nitrogen flushed bottles with an inversed pump. In the laboratory, the gas phase of the bottles was flushed with 100% 5.5 grade methane (99.999%) and stored at 4°C for ±21 months. All samples were pooled 1:1:1 (v/v/v) in an anaerobic chamber prior to inoculation.

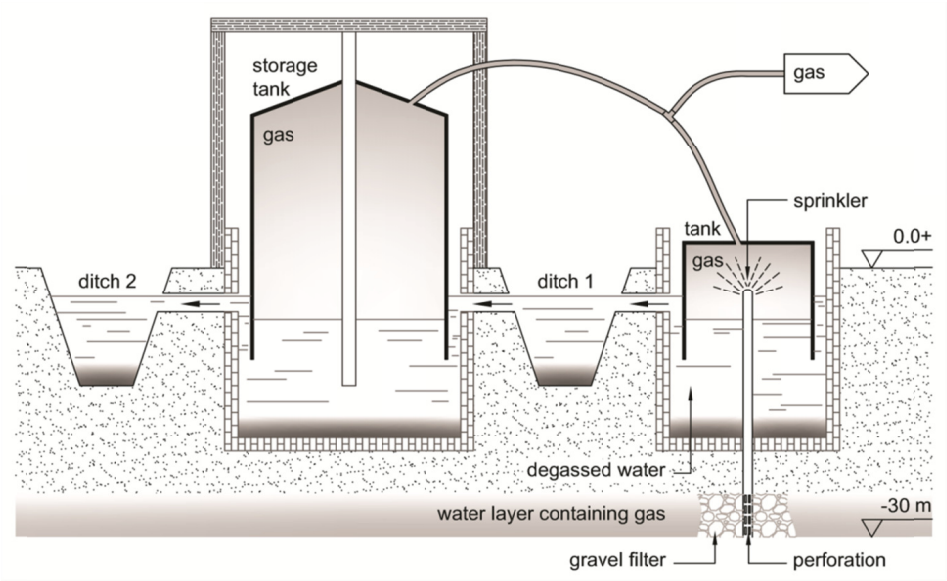

Figure 4.1 Schematic presentation of the system that is used for capturing natural gas at Berkhout, Noord-Holland, The Netherlands (adapted from Bartstra, 2003). A 30 m deep pipe reaches the pressurized groundwater pockets containing natural gas. Degasification occurs at lower pressure inside the gas source tank where the sprinkler facilitates the process. Gas can be transported to the house or to a storage tank floating on the water ditch. Sampling locations were inside an inactive tank (tank 2, not on picture), from the effluent of the active gas source (tank) and the sediment of the ditch where the effluent is collected (ditch 1) and from the sediment of a ditch where the effluent of the storage tank was collected (ditch 2).

4.2.2 Media composition

Media were prepared as described previously (Stams *et al.*, 1993) using 1 ml·L^{-1} of the vitamin stock solution.

4.2.3 Experimental set-up

15 ml aliquots of the pooled sediments (0.07 g_{vss}) were incubated in triplicate in bicarbonate buffered medium (1:1 v/v) with sulphate (20 mM), iron (as ferrihydrite, 10 mM), humic acids (20 g·l^{-1}), iron combined with humic acids (iron as ferrihydrite, 10 mM and humic acids 2 g·l^{-1}), and nitrate (20 mM). Iron was combined with humic acids to facilitate electron transfer from insoluble iron to soluble humic acids that can act as an

electron shuttle (Kappler *et al*., 2004). A control without electron acceptor was included. All triplicate conditions were tested with and without $^{13}CH_4$ in the headspace. All experiments were done in 60 ml serum bottles closed with butyl rubber stoppers and aluminium caps. After 10 cycles of exchanging the headspace gas with N_2, it was changed to N_2/CO_2 (1:1) to a pressure of 1.5 bar. When $^{13}CH_4$ was added, N_2/CO_2 was added to a pressure of 1.3 bar and 99.99% $^{13}CH_4$ gas (Campro Scientific, Veenendaal, The Netherlands) was added to a final pressure of 1.8 bar. The serum bottles were incubated at 15°C in the dark.

4.2.4 Preparation of ferrihydrite

Ferrihydrite (simplified as $Fe(OH)_3$) was produced in nanoparticle size (<10 nm) as described (Schwertmann and Cornell 1991). After preparing, the mineral was repeatedly washed and centrifuged for 3 times and subsequently dialyzed to remove electrolytes. The precipitate was then freeze-dried to remove access water and immediately added to the incubations.

4.2.5 Analytical measurements

Nitrate and sulphate were analysed by an Ion Chromatography system equipped with an Ionpac Ionpac AS9-SC column and an ED 40 electrochemical detector (Dionex, Sunnyvale, CA, USA). The system was operated at a column temperature of 35°C, and a flow rate of 1.2 $ml \cdot min^{-1}$. Eluent consisted of a carbonate/bicarbonate solution (1.8 and 1.7 mM respectively) in deionized water.

Headspace gas composition was measured on a gas chromatograph-mass spectrometer (GC-MS) composed of a Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Rt-QPLOT column (Restek, Bellefonte, PA, USA), and a DSQ MS (Thermo Fisher Scientific). Helium was used as a carrier gas with a flow rate of 120 $ml \cdot min^{-1}$ and a split ratio of 60. The inlet temperature was 80°C, the column temperature was set at 40°C and the ion source temperature at 200°C. CH_4 and CO_2 in the headspace were quantified from the peak areas in the gas chromatographs. The fractions of $^{13}CO_2$, $^{13}CH_4$ and $^{12}CH_4$ were derived from the mass spectrum as previously done (Shigematsu *et al*., 2004). Validation of the method was done using standards with known mixture of $^{13}CO_2$, $^{12}CO_2$, $^{13}CH_4$ and $^{12}CH_4$. The concentrations of total CO_2, total CH_4, $^{13}CO_2$, $^{12}CH_4$ (produced during methanogenesis in incubations with $^{13}CH_4$) and $^{13}CH_4$ were calculated as described previously (Timmers *et al*., 2015). Headspace CO_2 and CH_4 after 168 days of incubation was

quantified from the peak areas recorded with a CompactGC gas chromatograph (Global Analyser Solutions, Breda, The Netherlands).

The concentrations of iron(II) and iron(III) were measured with the ferrozine colorimetric method (Stookey 1970). Prior to analysis, samples were acidified with 2 M HCl (1:1 v/v) and centrifuged for 5 min at 15,700 rcf to precipitate humic acids. Absorbance at 562 nm was measured in a U-1500 spectrophotometer (Hitachi, Chiyoda, Tokyo, Japan).

Sulphide concentration was measured with the methylene-blue colorimetric method. Samples were directly diluted in 1:1 (v/v) in a 5% (w/v) zinc acetate solution to bind all sulphide. Deionised water wa added to a volume of 4.45 ml and 500 µl of reagent A (2 $g \cdot l^{-1}$ dimethylparafenyldiamine and 200 $ml \cdot l^{-1}$ H_2SO_4) and 50 µl of reagent B (1 $g \cdot l^{-1}$ $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ and 0.2 $ml \cdot l^{-1}$ H_2SO_4) were added concurrently and mixed immediately. After 10 minutes, samples were measured with a Spectroquant Multy colorimeter (Merck Millipore, Darmstadt, Germany) at 660 nm.

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) using a Vista-MPX CCD simultaneous (Varian Inc., Palo Alto, CA, USA) was used to quantify the elemental composition of all samples, as previously done (Hageman *et al.*, 2013). The standard deviation in all measurements was ≤1.8%.

The pressure of the serum vials was determined using a portable membrane pressure unit GMH 3150 (Greisinger electronic GmbH, Regenstauf, Germany). The pH was checked by pH paper. Conductivity was measured using a standard electrode. The VSS contents were analyzed according to standard methods (American Public Health Association, 1995).

4.2.6 DNA extraction

Genomic DNA was extracted at 323 days of incubation from the triplicate incubations with methane and sulphate (CH_4+SO_4^{2-}), sulphate only (SO_4^{2-}-only), and methane only (CH_4-only) and from the original sediment (BHori). DNA was extracted in triplicate for every separate incubation using the Fast DNA Kit for Soil (MP Biomedicals, Santa Ana, CA ,USA) according to the manufacturer's protocol with two 45-second beat beating steps using a Fastprep Instrument (MP Biomedicals). Triplicate extracted DNA for every separate incubation was pooled and DNA concentrations were determined with the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

4.2.7 Bacterial community profiling

Genomic DNA extracted from samples at 323 days of incubation and from the original sediment (BHori) was used for bacterial profiling. A PCR amplification replicate of BHori (BHoriA and BHoriB) was done to correct for technical biases. Barcoded amplification of the V1-V2 region of the 16S rRNA gene was done using forward primer 27F-DegS (van den Bogert *et al.,* 2011) that was extended with the titanium adapter A and an eight-base sample specific barcode (Hamady *et al.,* 2008) at the 5'-end, and an equimolar mix of reverse primers 338R-I and 338R-II (Daims *et al.,* 1999) that were appended with the titanium adapter B at the 5'-end. PCR amplification was performed in a thermocycler GS0001 (Gene Technologies, Braintree , UK) in a total volume of 100 µl containing 2 µl DNA (20 ng/ul), 500 nM of barcoded forward primer and reverse primermix (Biolegio BV, Nijmegen, The Netherlands), 2 U of Phusion Hot start II High-Fidelity DNA polymerase (Finnzymes, Vantaa, Finland), 20 µl of 5x HF buffer, 2 µl PCR grade nucleotide mix (Roche Diagnostics GmbH, Mannheim, Germany), and 65 µl nuclease free sterile water (Promega Corporation, Madison, WI, USA). PCR amplification conditions were a pre-denaturing step of 3 min at 98°C followed by 30 cycles of 98°C for 10 s, 56°C for 20 s and 72°C for 20 s. Lastly, a post-elongation step of 10 min at 72°C was done. PCR products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific) and the concentration was determined using the Qubit 2.0 fluorometer (Thermo Fisher Scientific). All samples for pyrosequencing were mixed in equimolar amounts. Pooled samples were loaded on a 1% (v/v) agarose gel containing 1x SYBR Safe (Invitrogen, Thermo Fisher Scientific) and bands of approximately 340 bp were excised and purified with the GeneJet Gel Extraction Kit (Thermo Fisher Scientific) using 25 ul elution buffer for collecting the amplified DNA. Mixed samples were quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific) and submitted for pyrosequencing on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC Biotech AG, Konstanz, Germany).

4.2.8 Archaea community profiling

Genomic DNA extracted from samples at 323 days of incubation and from the original sediment (BHori) was used for archaeal profiling. A PCR amplification replicate of BHori (BHoriA and BHoriB) was done to correct for technical biases. A method adapted from Jaeggi *et al*., (2014) was used. Barcoded amplification of 16S rRNA genes was done by using forward primer 340F (Gantner *et al*., 2011) that was extended with the titanium adapter A and a ten-base sample specific barcode at the 5'-end, and reverse primer 1000R (Gantner *et al.,* 2011) that was appended with the titanium adapter B at the 5'-end. PCR

amplification was performed in a total volume of 50 µl containing 1 µl DNA, 200 nM of each forward and reverse primer (Biolegio BV), 1 U of KOD Hot Start DNA Polymerase (Merck Millipore), 5 µl of 10x KOD-buffer, 3 µl MgSO4 (25 mM), 5 µl dNTP mix (2 mM each), and 33 µl nuclease free sterile water. PCR amplification conditions were a pre-denaturing step at 95°C for 2 minutes followed by 35 cycles of 95°C for 20 s, 5°C for 10 s, and 70°C for 15 s. The approximately 660 bp PCR amplicon was subsequently purified using the MSB Spin PCR apace kit (STRATEC Biomedical AG, Birkenfeld, Germany) and the concentration was checked with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Purified PCR products were mixed in equimolar amounts. The mixed sample was further purified using the Purelink PCR Purification kit (Thermo Fisher Scientific), with high-cutoff binding buffer B3, and submitted for pyrosequencing on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC Biotech AG).

4.2.9 Pyrosequencing analysis

The pyrosequencing data was analysed with a workflow based on Quantitative Insights Into Microbial Ecology (QIIME) v1.2 (Caporaso *et al*., 2010), and reads were filtered for chimeric sequences using the usearch algorithm. OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. The SILVA reference database was used for taxonomic classification (Quast *et al*., 2013). After picking representative OTUs, the relative amount of reads of every OTU to the total amount of reads per sample was quantified. Afterwards, the average relative amount of reads per condition from the biological triplicate samples were calculated. For analysis of the original sample BHori, the average of the PCR duplicates (BHoriA and BHoriB) was calculated. Then, the significant differences of every representative OTU between the conditions CH_4+SO_4^{2-} and CH_4 -only and between CH_4+$SO_4$2- and SO_4^{2-}-only were calculated separately, using the Kruskal-Wallis test ($p<0.05$). We then selected only representative OTUs that were significantly higher in conditions with CH_4+SO_4^{2-} as compared to CH_4 -only and SO_4^{2-}-only.

4.2.10 Quantitative real-time PCR

Genomic DNA extracted from samples at 323 days of incubation and from the original sediment (BHori) was used for qPCR analysis. The DNA was purified with the DNA clean and concentrator-5 kit (Zymo Research, Irvine, CA, USA) and the concentration was

determined with the Qubit 2.0 fluorometer (Thermo Fisher Scientific). Amplifications with specific primers for ANME2a/b were done as described previously (Timmers *et al*., 2015). Quantification was expressed as the total number of 16S rRNA gene copies per gvss extracted from the incubations.

4.2.11Nucleotide sequences

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers LN795911-LN796465 for archaeal sequences and LN796466-LN808676 for bacterial sequences.

4.3 Results and discussion

4.3.1 AOM and TMO

Methane production was observed in most conditions, but was negligible in the presence of sulphate and did not occur in the presence of nitrate (Figure 4.2). Methane production in conditions with and without added methane showed a similar pattern, but the amount of methane produced was lower in incubations where methane was added (data no shown). This was probably caused by the increase in TMO due to a higher methane concentration (Smemo and Yavitt 2007, Zehnder and Brock 1980). Production of $^{13}CO_2$ was apparent in all incubations with $^{13}CH_4$ in the headspace, except in the conditions with nitrate and HAs (Figure 4.2).

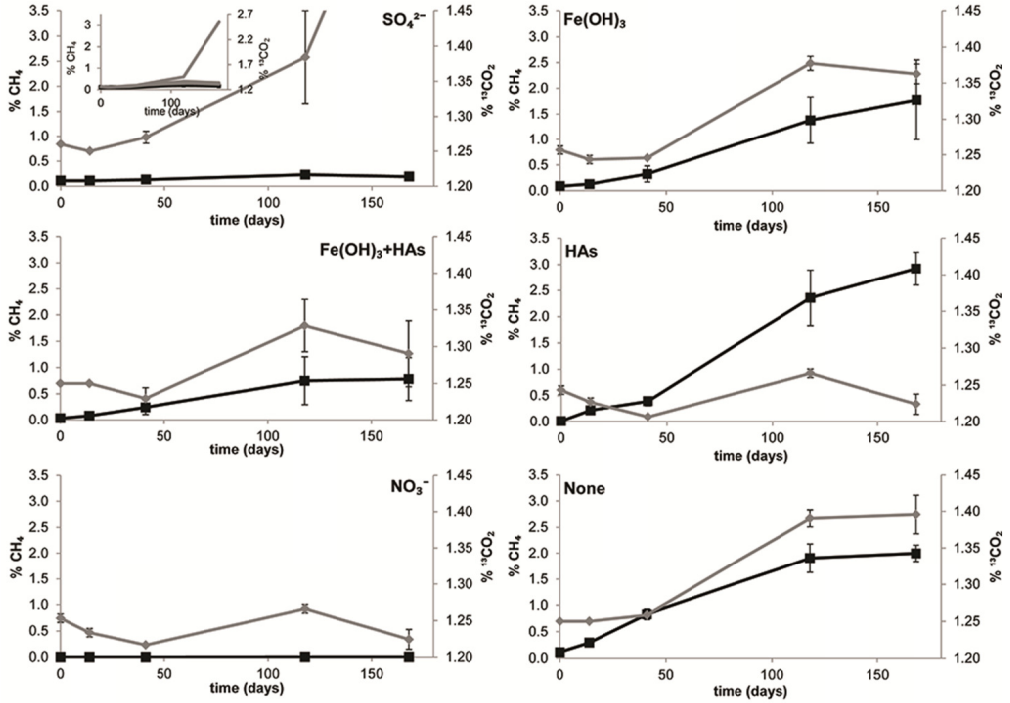

Figure 4.2 Percentage of methane (black lines) and percentage of $^{13}CO_2$ of total CO_2 (grey lines) during 168 days of incubation in bottles with 100% $^{13}CH_4$ in the headspace and with different electron acceptors. Standard deviations represent triplicate incubations. Note the different scale in the condition with sulphate where one of the triplicates '1A-2' highly increased in $^{13}CO_2$.

Typical for TMO, $^{13}CO_2$ simultaneously increased with methane formation in the conditions with ferrihydrite, ferrihydrite + HAs and the control without electron acceptor (Figure 4.2). In incubations with nitrate, no methane or $^{13}CO_2$ was produced, but in the incubations with sulphate, an increase in $^{13}CO_2$ with a minor increase in $^{12}CH_4$ was observed. The ratio of methane oxidized per methane produced was only >1 for conditions with sulphate, which is indicative for AOM (Table 4.1). The $^{13}CO_2$ production in all other conditions was not substantially different between ferrihydrite, ferrihydrite + HAs and the control conditions, indicating that TMO was not influenced by the electron acceptor added. Ferrous sulphate addition has been shown to increase the percentage of methane oxidized to methane produced and reached up to 15% in lake sediments and to a maximum of even 98% in digested sludge (Zehnder and Brock 1980). Addition of sodium sulphate inhibits not only methane formation, but also TMO in sludge (Meulepas *et al.*, 2010, Zehnder and Brock 1980) and in freshwater slurries (Segarra *et al.*, 2013), but stimulates methane oxidation in brackish slurries (Segarra *et al.*, 2013). In this study, the incubations with sulphate showed substantial methane oxidation at low salinity.

Table 4.1. CH_4 oxidized per CH_4 formed after 168 days of incubation. Calculated from the amount of CH_4 formed in incubations without CH_4 in the headspace and the amount of $^{13}CO2$ formed in incubations with a headspace of 100% $^{13}CH_4$ with different electron acceptors.

Condition	Bottle #	$^{13}CO_2$ formed (µmol)	$^{12}CH_4$ formed (µmol)	CH_4 oxidized/CH_4 formed
SO_4^{2-}	1A-1	6.2	1.8	>1
	1A-2	20.3	1.5	>1
	1A-3	10.4	0.9	>1
$Fe(OH)_3$[a]	2A-1	2.2	14.8	0.15
	2A-2	4.8	34.5	0.14
	2A-3	8.1	41.7	0.19
$Fe(OH)_3$+HAs[a]	3A-1	1.5	13.8	0.11
	3A-2	2.5	19.6	0.13
	3A-3	0	5.8	n/a
HAs	4A-1	0	51.4	n/a
	4A-2	0	56.0	n/a
	4A-3	0	47.4	n/a
NO_3^-	5A-1	0	0.0	n/a
	5A-2	0.2	0.0	n/a
	5A-3	0	0.0	n/a
None[a]	6A-1	6.8	33.0	0.21
	6A-2	6.1	34.9	0.18
	6A-3	4.3	37.5	0.11

Means with same letters in superscript are not significant (Wilcoxon Rank Sum test, $p<0.05$). n/a means not applicable.

The ICP measurements of all samples prior to mixing showed that from all possible electron acceptors for methane oxidation, sulphur and iron are present whereas selenium and manganese were nearly absent. The pooled inoculum contained no nitrate and an average of around 2 mM sulphate. Sulphate was not detectable after 41 days of incubation in most conditions (except where sulphate or nitrate was added) while $^{13}CO_2$ production continued. When ferrihydrite was added, the $^{13}CO_2$ production seemingly also continued when all iron was reduced to Fe(II) after 300 days, even when it was less bioavailable in the absence of HAs. Iron reduction did occur faster in incubations with ferrihydrite + HAs than in the incubations with only ferrihydrite. The incubations with 20 $g \cdot l^{-1}$ HAs contained an average of 28.8 (±1.0) mM acid soluble Fe(II) and did not show any detectable $^{13}CO_2$ increase (Figure 4.2 and Table 4.1). Conditions with ferrihydrite and 2 $g \cdot l^{-1}$ of HAs thus contained around 2.9 mM of Fe(II) derived from HAs and also showed much

less $^{13}CO_2$ production as compared to the control. The HAs batch we used contained calcium which could scavenge produced CO_2 to form calcium carbonate. After acidification of the samples, we did see an increase in total CO_2 but the detected fraction of $^{13}CO_2$ did not increase. Previously, reduced methane emission after addition of HAs to peat ecosystems has been observed and was presumably caused by increased methane oxidation (Blodau and Deppe 2012). We however did not find methane oxidation activity after addition of HAs.

4.3.2 AOM associated with SR

Reduction of the electron acceptors sulphate, ferrihydrite and nitrate occurred in all conditions with and without addition of methane (Table 4.2). The reduction rates of nitrate and sulphate with and without added methane in the first 168 days were similar (2-tailed t-test with unequal variance, $p<0.05$) which was probably due to endogenous SR masking sulphate-dependent AOM. However, due to endogenous substrate depletion, the SR rate in incubations with only sulphate had substantially decreased after 343 days of incubation. In conditions with methane and sulphate, there was no difference in SR rates probably due to coupling to AOM. In this time period, we could not completely link AOM with SR and sulphide production as the abundance of the green sulphur bacteria (GSB) *Chlorobiaceae* could be responsible for the fluctuations in sulphide and sulphate levels, even in the dark with limited exposure to light during sampling. This also explained the green coloration only in incubations amended with sulphate, derived from the bacteriochlorophyll of GSB (Gorlenko 1970). GSB are strictly anaerobic autotrophic sulphide oxidizers and have been found to be active when exposed to very little light (Beatty *et al*., 2005, Manske *et al*., 2005). Their activity during sampling probably kept the sulphide concentration low and sulphate concentrations high. After maintaining complete darkness in the slurries, the $^{13}CO_2$ production continued throughout incubation time and free sulphide was eventually measured. In bottle 1A-2 that showed the highest $^{13}CO_2$ production after 168 days of incubation (Table 4.1), sulphide production increased simultaneously with $^{13}CO_2$ production during the last period between 343 and 728 days (Figure 4.3), which indicated AOM associated with SR.

Table 4.2. Reduction rates of the electron acceptors. Sulphate, iron in the form of ferrihydrite with and without humic acids (HAs), and nitrate in each incubation with and without methane in the headspace during the first 168 days of incubation and sulphate reduction rates in incubations with sulphate after between 343 and 728 days of incubation (in µmol $gVSS^{-1}\cdot d^{-1}$). Standard deviations for biological triplicates.

Condition	Reduction rates (µmol $g_{VSS}^{-1}\ d^{-1}$)
0-168 days	**Sulphate**
CH_4+SO_4^{2-ac}	5.14 (±3.04)
SO_4^{2-a}	7.58 (±0.50)
CH_4^{b}	0
343-728 days	
CH_4+SO_4^{2-ac}	5.94 (±0.83)
SO_4^{2-c}	5.02 (±0.16)
0-168 days	**Iron**
CH_4+Ferrihydrite[a]	0.12 (±0.01)
Ferrihydrite[a]	0.15 (±0.02)
CH_4+Ferrihydrite+HAs[b]	10.58 (±1.95)
Ferrihydrite+HAs[b]	4.29 (±7.10)
	Nitrate
CH_4+NO_3^{-a}	28.02 (±1.38)
NO_3^{-a}	25.32 (±1.02)

Means with different letters in superscript are significant (independent 2-tailed t-test with unequal variance, $p<0.05$).

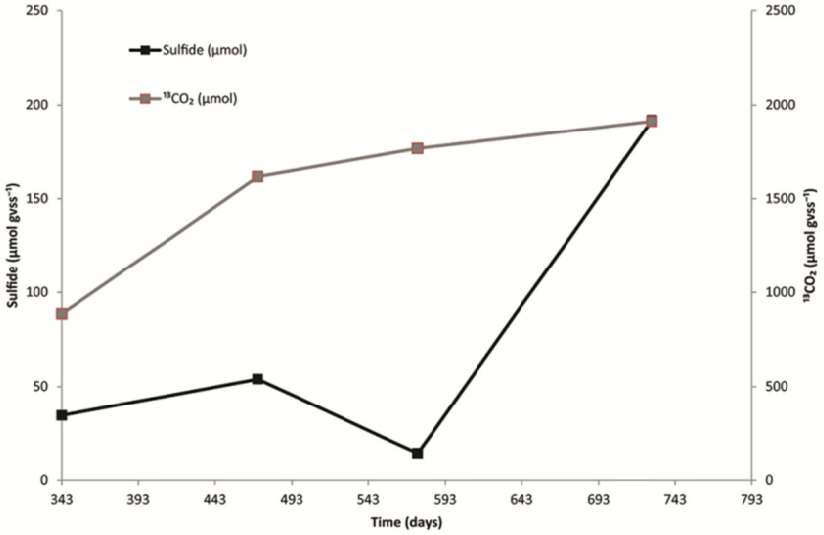

Figure 4.3 Amount of sulphide (µmol·gvss^{-1}) and $^{13}CO_2$ (µmol·gvss^{-1}) in one of the triplicates '1A-2' with sulphate and 100% $^{13}CH_4$ in the headspace after 343 days of incubation. Technical standard deviations of measurements between time points were on average 7.7% for $^{13}CO_2$ and 4.3% for sulphide as determined from replicate measurements of standards with known composition.

4.3.3 Microbial community profiling

For all samples, the highest average percentage of 16S rRNA reads for Archaea clustered with the *Methanosarcinaceae*, *Methanoregulaceae*, *Methanosaetaceae*, *Methanobacteriaceae*, and the Miscellaneous *Crenarchaeota* Group (MCG). Archaeal OTUs that showed a significantly higher percentage of reads (Kruskal Wallis, $p<0.05$) in condition CH_4+SO_4^{2-}, as compared to CH_4-only (Figure 4.4A) and SO_4^{2-}-only (Figure 4.4B) make up a less than 8% of all reads. In condition CH_4+SO_4^{2-}, ANME2a/b sequences represented 0.16% of all reads and were much more abundant than in the condition CH_4-only. Higher abundance of ANME2a/b in conditions CH_4+SO_4^{2-} compared to SO_4^{2-}-only was shown by qPCR analysis. This indicates the involvement of ANME2a/b in AOM coupled to SR, as shown before in marine environments (Orphan *et al.*, 2001a). The ANME2a/b OTU showed 98% identity with ANME2a/b from both marine and freshwater environments and do not form a monophyletic cluster with ANME2a/b found in other low-salinity and low-sulphate environments. A marine enrichment of ANME-2a/b species that share 98% identity was previously shown to be completely inhibited in AOM activity at a salinity of 5‰ (Meulepas *et al* 2009), indicating that the ANME2a/b detected in this study could be adapted to low salinity.

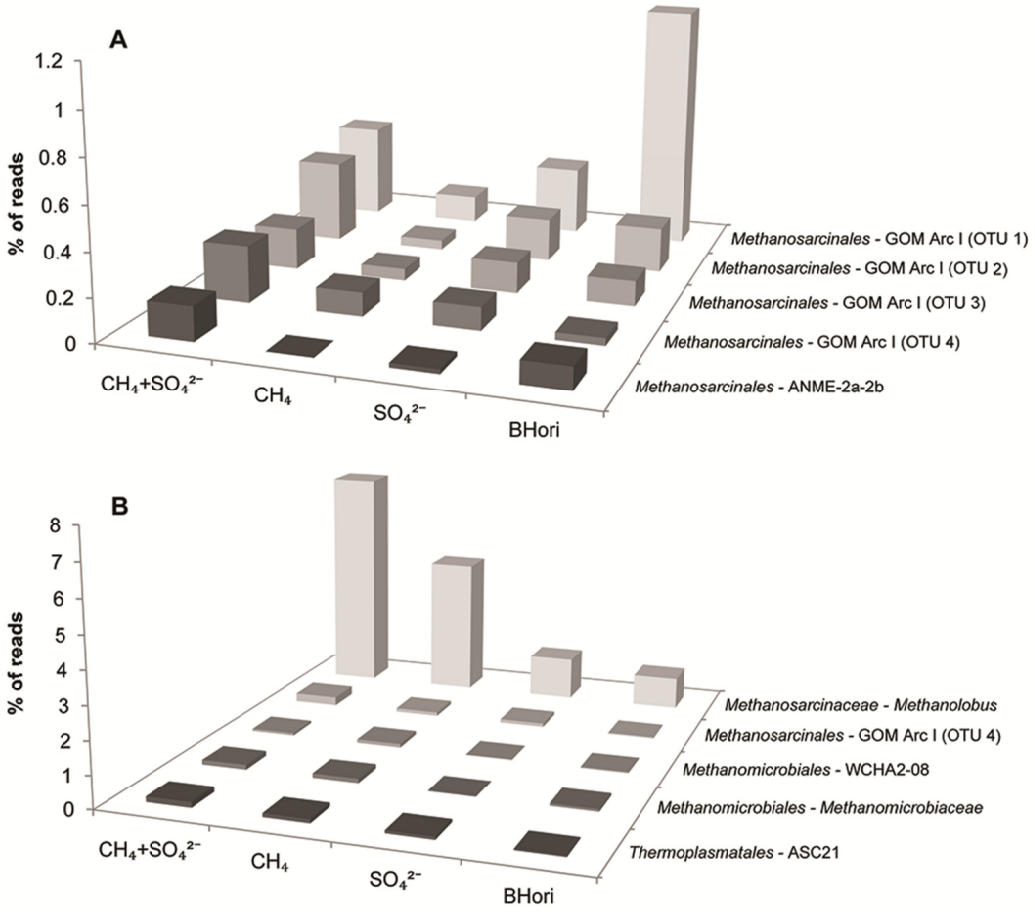

Figure 4.4. Archaeal 16S rDNA pyrosequencing results showing the representative OTUs that were significantly higher in the conditions CH_4+SO_4^{2-} as compared to CH_4-only (A) and SO_4^{2-}-only (B) (Kruskall-Wallis, $p<0.05$). Also displayed is the original pooled sample (BHori). Displayed is the average percentage of reads per representative OTU of the three biological triplicates per condition.

A higher percentage of reads was also found for 1 OTU of *Methanosarcinales* GOM Arc I (OTU 4) in CH_4+SO_4^{2} compared to both CH_4-only and SO_4^{2-}-only (Figure 4.4). This GOM Arc I group has been related to ANME microorganisms. It was found to be 97% identical to "*Methanoperedens nitroreducens*", which performed AOM coupled to nitrate reduction (Haroon *et al.*, 2013). Furthermore, it was 99% identical to other named "AOM associated archaea AAA" (Knittel and Boetius 2009) members that were thought to be responsible for freshwater AOM coupled to SR in Lago di Cadagno sediments (Schubert *et al.,* 2011).

It was already shown that "*M. nitroreducens*" uses the complete reverse methanogenesis pathway (Haroon *et al*., 2013). We did not find nitrate-dependent AOM activity, which leaves open the possibility that the AAA in this study could perform AOM coupled to SR in the presence of sulphate reducing bacteria. Sulphate addition in presence of methane also had a positive effect on more GOM Arc I related OTUs (Figure 4.4A), which could make the possible contribution to AOM activity substantial. The higher percentage of reads of *Methanolobus* in conditions CH_4+SO_4^{2-} compared to SO_4^{2-}-only (Figure 4.4B) implied that methane addition had an effect on *Methanolobus* abundance. The reason for this effect is unclear, since this genus is known to be able to utilize methylated compounds (Zhang *et al*., 2008), but not methane. However, *Methanolobus* was also found in a marine methane-oxidizing bioreactor (Girguis *et al*., 2003).

Bacterial diversity was high in all samples, with the highest relative number of reads for all samples clustering with the *Deltaproteobacteria* (*Syntrophobacteriacaea* and *Desulfobacteraceae*) and *Gammaproteobacteria* (*Methylococcaceae*), *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Chlorobi* (family *Chlorobiaceae*). Bacterial OTUs that showed a substantially higher percentage of reads (Kruskal Wallis, $p<0.05$) in condition CH_4+SO_4^{2-} as compared to both CH_4-only and SO_4^{2-}-only make up less than 0.5% of all reads and are given in Figure 4.5. These OTUs were clustering with the *Desulfobacteraceae*, *Clostridiales* and *Planctomycetaceae*. The latter OTU of SEEP-SRB1 was only found in condition CH_4+SO_4^{2-}. However, other SEEP-SRB1 OTUs that were detected did not show a difference in read abundance between the conditions CH_4+SO_4^{2-},CH_4-only and SO_4^{2-}-only. The SEEP-SRB1 clade has been detected in several marine AOM mediating environments (Harrison *et al*., 2009, Knittel *et al*., 2003, Losekann *et al*., 2007, Orphan *et al*., 2001b, Pernthaler *et al*., 2008, Vigneron *et al*., 2013, Yanagawa *et al*., 2011) and enrichments (Jagersma *et al*., 2009, Zhang *et al*., 2011). The SEEP-SRB1a has been identified as the dominant bacterial partner of ANME-2a/b in different marine AOM mediating environments (Schreiber *et al*., 2010). From the other OTUs that showed a higher percentage of reads in CH_4+SO_4^{2-}, little is known about their role in AOM coupled to SR. It has been shown before that different SRB besides SEEP-SRB1 belonging to *Desulfobacteraceae* form consortia with different ANMEs (Orphan *et al*., 2002, Vigneron *et al*., 2013) and even non-SRB were found to aggregate with ANMEs (Pernthaler *et al*., 2008). We did not find any information related to the nitrate-dependent methanotrophic bacterium *Methylomirabilis oxyfera* (Ettwig *et al* 2010.,), confirming the lack of AOM coupled to denitrification.

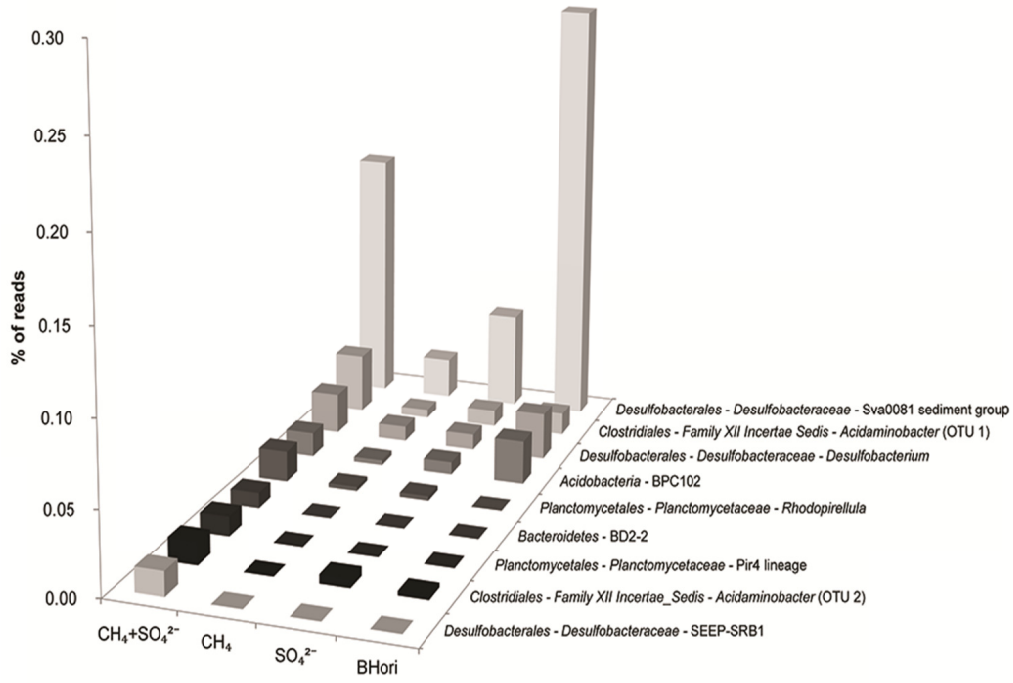

Figure 4.5. Bacterial 16S rDNA pyrosequencing results showing the representative OTUs that were significantly higher in the conditions CH_4+SO_4^{2-} as compared to both CH_4-only and SO_4^{2-}-only (Kruskall-Wallis, $p<0.05$). Also displayed is the original pooled sample (BHori). Displayed is the average percentage of reads per representative OTU of the three biological triplicates per condition.

4.3.4 Low-salinity sulphate-dependent AOM

The sulphate concentration was between 0.07 and 0.28 mM in the gas source effluent and about 2 mM in the pooled inoculum. The measured conductivity (2.0-2.6 $mS \cdot cm^{-1}$) and chloride concentration (128-254 $mg \cdot mL^{-1}$) of the gas source effluent and pooled inoculum samples indicate a somewhat higher salinity than typical freshwater but a much lower salinity than typical brackish environments. This could correspond to the historical marine influence of the sampling site before lake formation due to dike construction, as described for proximal sites (van Diggelen *et al.*, 2014). In marine environments, the sulphate:chloride ratio is around 1:19. The sulphate:chloride ratio of the lake surface water was around 1:2.6, with 1.7 mM sulphate and 4.4 mM chloride. Therefore, marine influences cannot explain the high sulphate concentrations. The sulphate concentration in deeper layers of the gas source could be even higher than measured in the gas source effluent before AOM took place. In marine systems, sulphate-dependent AOM occurred

even below 0.5 mM of sulphate, but at lower rates than at higher sulphate concentrations (Yoshinaga *et al*., 2014; Meulepas *et al*., 2009; Beal *et al*., 2011). In typical freshwater environments, the sulphate concentration is generally lower, making AOM-SR less feasible. This could explain why AOM-SR, and presence of ANME, was previously only detected in freshwater and terrestrial environments with relatively high sulphate concentrations (Eller *et al*., 2005; Alain *et al*., 2006), as could be the case this study. The finding of AOM and ANME/SEEP-SRB1 sequences in the freshwater gas source suggests that these syntrophic clades are active and ubiquitously distributed in marine and in low-salinity environments.

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CHAPTER 5

Electrical conductivity of granular biomass: methodology and characterization

Biological oxidation-reduction reactions in granular biomass require electrons transfer between the involved bacterial and/or archaeal cells. This electron transfer between cells can occur through different mechanisms, and in some cases it may rely on the creation of electrically conductive paths in the granular aggregates. A better understanding of the interspecies electron transfer mechanisms in granular biomass would help in the design, development and optimization of processes for anaerobic wastewater treatment. In this study, a methodology for the measurement of the electrical conductance of granular biomass was assessed. The electrical conductance of granular biomass was measured using a probe consisting of two electrodes separated by a non-conductive gap. We show how the probe design is a critical factor for performing this measurement. In addition, we also present data which indicates that from the measured biomass types, only ethanol oxidizing methanogenic granules are conductive.

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Diego A. Suarez-Zuluaga, Leire Caizan, Sebastian Canizales-Gomez, Doekle Yntema, Jan Weijma, Annemiek ter Heijne, Cees J.N. Buisman. Electrical conductivity of granular biomass: methodology and characterization

5.1 INTRODUCTION

The up-flow anaerobic sludge blanket (UASB) reactor is commonly used for wastewater treatment. One of the keys to the UASB performance is the spontaneous formation of granular biomass. Such biomass is characterized for having high biological activity and sedimentation velocity. This kind of behaviour is the result of different kinds of microorganisms coexisting in a balanced granular ecosystem (Lettinga *et al*., 1980; Angenent *et al.,* 2004).

The microbial composition of the granules is highly dependent on the composition of the wastewater in which they are cultivated. These granular aggregates are generally comprised by a diverse community of hydrolytic-fermentative bacteria, hydrogen-producing acetogenic bacteria and methanogens, which cooperate to degrade complex organic compounds to methane and carbon dioxide. The redox reactions carried out in these granules require electron transfer between the different microorganisms present (Schmidt and Ahring, 1995). However, this process is not completely understood and it has been traditionally treated as a "black box" (Alphenaar, 1994).

Electron transfer in granular biomass has been thought to rely on the transfer of compounds which carry the electrons between the different kinds of microorganisms present (*e.g.* hydrogen). However, studies suggest that in some cases, these compounds can not be the only electron carriers as they are dependent on diffusion and are theoretically not able to reach the electrons transfer rates measured in practice (Reguera e*t al.,* 2005; Morita *et al*., 2011, Cruz *et al*., 2014).

Co-cultures of *Geobacter metallireducens* and *Geobacter Sulfurreducens* have been shown to form electrically conductive aggregates through which the electron transfer occurs. This transfer might be occurring via conductive appendages or redox proteins (Reguera *et al*., 2005; Summers *et al*., 2010; Morita *et al*., 2011). Furthermore, *G. metallireducens* has also been shown to build electrically conductive structures with methanogenic archaea (Summers *et al.,* 2010; Morita *et al.,* 2011; Rotaru *et al.,* 2014). In the study of Morita *et al*., (2011) it is shown how aggregates from a UASB reactor treating brewery waste presented metallic-like conductivity 3-fold higher than that showed by *Geobacter* species (Summers *et al*., 2010). They also revealed that these aggregates were not using hydrogen, further supporting the idea that direct interspecies electron transfer took place.

Direct electron transfer has been reported to not to be limited to nanodistances. Pfeffer *et al*., (2012) presented evidence of it happening across centimetres distances in marine sediments by filaments of bacteria of the *Desulfobulbaceae* family. That study shows that

the oxygen available at the surface of the sediments was reduced with electrons originated from sulphide oxidation at the subsurface's anoxic layers while charge balance was maintained by ions present in the environment surroundings. In this case, and possibly also in granular biomass, the competitive advantage of building these conductive structures would be the capability to connect electron donors and acceptors separated in space.

Present knowledge about the composition and mechanisms developed by granular biomass indicates that large organic molecules are degraded in the outside layers and the resulting compounds are degraded in the inner layers until carbon dioxide and methane are the products in the centre of the granule. Direct electron transfer would present competitive advantage because it would effectively transfer electrons without requiring hydrogen diffusion. Or, as with the filamentous microorganisms described by Pfeffer *et al.,* (2012), because it would allow to connect electron donors and acceptors which are separated in space.

Understanding how and in which microbial systems direct interspecies electron transfer occurs would lead to a change to the current approach to modelling, designing and optimizing wastewater treatment processes. As an example, selected systems have already being optimized in order to stimulate the electron transfer between cells by adding conductive materials *e.g*. activated carbon (Liu *et a*l., 2012) or magnetite (Kato *et al*., 2010, 2012a, 2012b; Aulenta *et al.,* 2013, Cruz *et al*., 2014).

The possibility that electrons are directly transferred in methanogenic granular biomass might create a change in the way the mass transfer mechanisms inside these granules are currently understood. Using conductive paths to transfer electrons in granular biomass might be more efficient than interspecies hydrogen transfer. Up until now, direct interspecies electron transfer has only been studied in methanogenic sludge and it is not known to which extent other types of granular biomass are conductive. Therefore, the main objective of the current work is the evaluation of the methodology developed by Summers *et al*., (2010) for the measurement and analysis of the electrical conductance of different types of granular biomass. For this, we compare the results obtained from two probes with different material and dimension characteristics; and use them to measure several types of granular biomass.

5.2 MATERIAL AND METHODS

5.2.1 Probes

Electrical conductance measurements were performed in two different probes. The first probe (Small/Pt probe, Figure 5.1-I) consisted of three platinum (Pt) electrodes separated by 50 µm non-conductive gaps. The materials used were silicon, with platinum on top (thickness 100nm) and an adhesion layer of 10 nm chromium. The second probe was larger (Large/Au Probe, Figure 5.1-II); and it was built as reported in Malvankar *et al.,* (2011). It was constructed on a glass slide and it consisted of two gold electrodes separated by a 50 µm non-conductive gap. The gold layer had a thickness of 100 nm and was located on top of a 10 nm chromium adhesion layer. Figure 5.1-III helps to visualize the difference in the dimensions of the probes. It shows the two gold electrodes from the Large/Au probe and the Small/Pt Probe inside the drawn black circle.

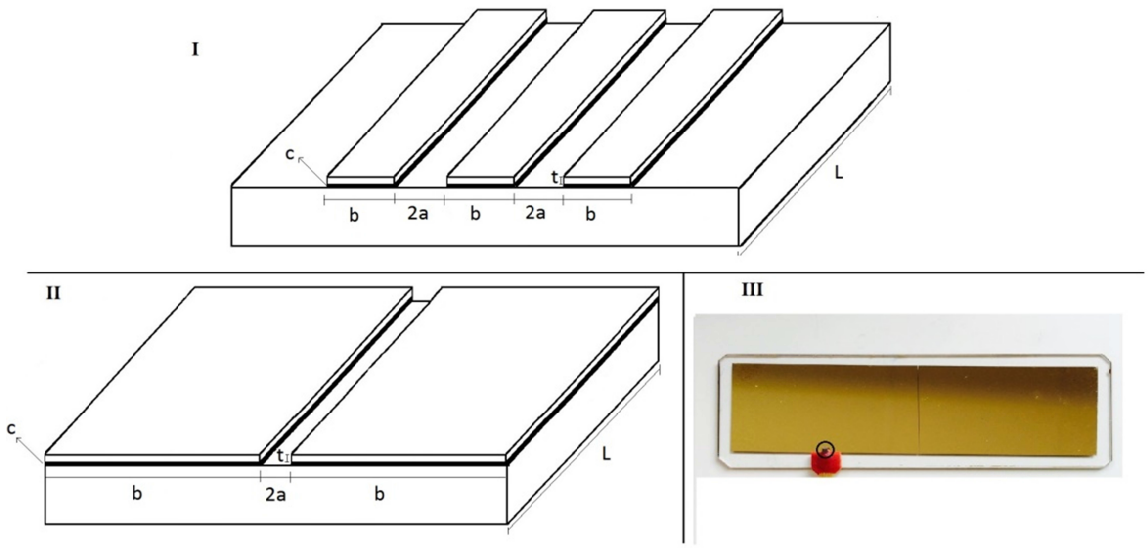

Figure 5.1. I, Dimensions of Small/Pt probe. L=0.4mm, b=0.3mm 2a=50µm, c=10nm, t=100 nm. **II, Dimensions of Large/Au Probe.** L≈2.5cm, b≈1.3cm, 2a=50µm, c=10nm, t=40 nm**. III, Picture of the electrodes.** The Small/Pt probe is inside the black circle.

5.2.2 Measurement

Chronoamperometry was performed applying voltages ranging from +0.3V to -0.3V in intervals of 0.05V. And, in some cases, chronoamperometry analyses were also performed applying voltages from -0.3V to 0.3V. Voltages were applied to two adjacent electrodes. During the application of each voltage a lapse of time was set for the system to reach steady state and to allow the decay of the transient ionic current in the gap (5 seconds for the Small/Pt Probe and 200 seconds for the Large/Au Probe). The measurements were performed with a potentiostat (Vertex, IVIUM technologies, Eindhoven, the Netherlands) and controlled with the software IviumSoft.

5.2.3 Biomass

Granular biomass was obtained from four different locations: *Eerbeek* (Eerbeek, The Netherlands), from a full-scale UASB reactor treating paper-mill wastewater. *FrieslandCampina* (Ede, The Netherlands), from a soft drinks wastewater reactor. *Bavaria* (Lieshout, The Netherlands), from three brewery wastewater treatment reactors. Finally, ethanol degrading granules were also collected from *Royal Nedalco* (Bergen op Zoom, The Netherlands). Samples were stored at 4^{o}C before measurement.

5.2.4 Procedure

The electrochemical tests were performed after carefully positioning the biomass on the probe so that both electrodes in which voltage was applied were completely covered. For the Small/Pt Probe two adjacent electrodes were connected. Biomass was taken directly from containers kept at 4^{o}C. As control, a conductive material (graphite felt, Coidan graphite products, York, UK) and the centrifuged mediums were tested.

Conductance is defined as the slope of the current vs voltage graph (I vs V graph). In the cases in which the conductivity of the granules was estimated, this was done using the Schwarz-Christoffel transformation (Malvankar *et a*l., 2011) after subtracting the conductance of the medium. This equation calculates biomass conductivity from the measured conductance (G) while correcting for the non-uniformities in current distribution and voltage gradient which are occurring in this measurement setup in the cases in which a<g<<b. This condition applies for all measurements with the Large/Au Probe, but not to all biomass types for the Small/Pt Probe. The formula is:

$$\vartheta = G\frac{\pi}{L}\Big/ln\left(\frac{8g}{\pi a}\right)$$

Equation 1. Biomass conductivity. G=conductance, L=electrode length, g=biomass thickness (1 mm), 2a=width of the non conductive gap.

5.3 RESULTS:

5.3.1 Measurements of graphite felt

Correct chronoamperometry results to determine granule conductivity must meet certain criteria: First, the measurements must be reproducible. Second, the sign of the current measured must be consistent with the sign of the voltage applied. Third, the obtained currents must be symmetric around the X axis of the I vs V graph. Fourth, a stable current must be reached after applying each voltage. Fifth, no signal must be measured from the medium alone.

Chronoamperometry of a conductive material (graphite felt) was performed in order to check the measurement probes when applying different voltages. The results of the Small/Pt Probe for two separate probes are presented in Figure 5.2. In can be observed how both probes gave the same response (Figure 5.2-A). From the slope of the I vs V curve (Figure 5.2-B) a conductance of 694 µS was calculated. Which is in accordance to the probe characteristics. This is because, due to its construction, this probe has an internal series resistance of about 1400 Ohms. This resistance is in the form of a small patterned wire connecting the electrodes. As a consequence, in the case a good conducting material is measured, this series resistance will be measured instead of the conductivity of the material. However, this will have no effect when conductances much lower than this value are measured. Thus, this probe is only suited for the measurement of low conductive materials. Because of this, and the results observed on Figure 5.2-A, it is consider the response of the probe is valid and reproducible. Similar results, but at higher currents (≈±55mA), were obtained for the Large/Au Probe (Figure 5.2-C).

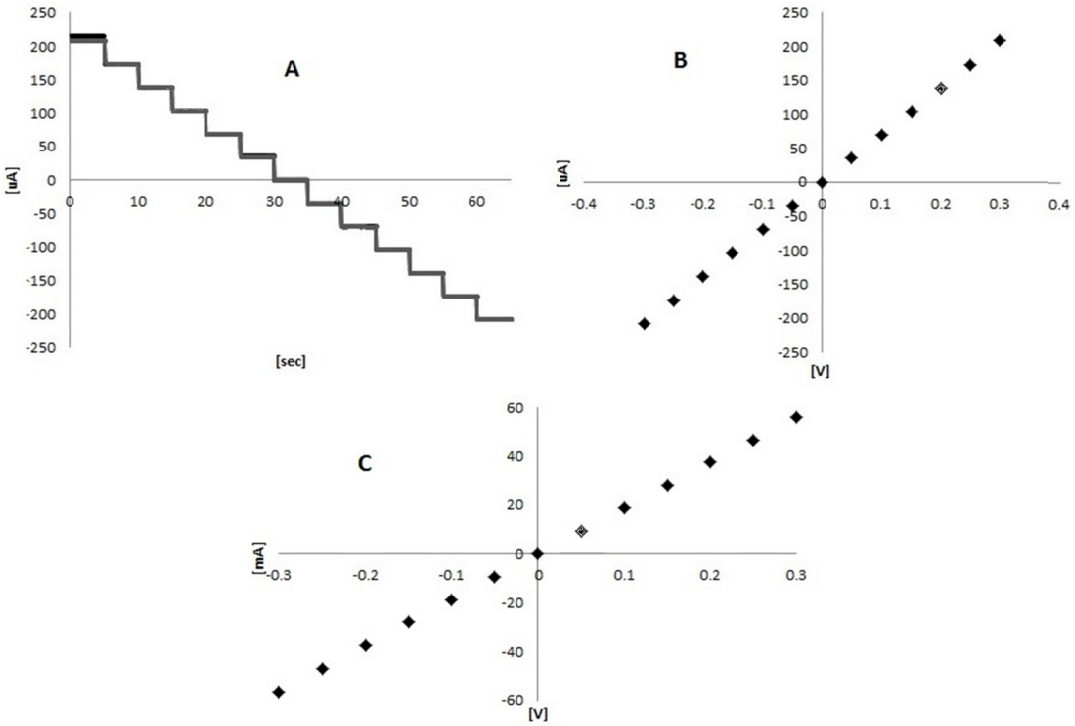

Figure 5.2. Results of the of graphite felt measurement. A. Chronoamperometry the Small/Pt Probe (from +0.3V to -0.3V). **B. I vs V curve for the chronoamperometry in the Small/Pt Probe. C. I vs V curve for the chronoamperometry in the Large/Au Probe.**

5.3.2 Measurements of granules and medium with the Small/Pt Probe.

Figure 5.3-A presents four examples of the measurements performed on the methanogenic granules from Campina with the Small/Pt Probe. This specific set of measurements met the criteria expected in good chronoamperometry measurements that were explained above. However, the values of the measured current were in the range of nanoamperes, which is 1000 times lower than those previously reported (Summers *et al.,* 2010, Morita *et al*., 2011).

However, and as can be observed in Figure 5.3-B, not all measurements presented these characteristics. Some of the measured granules did not present conductance higher than the medium but neither were all the graphs symmetric, nor was the current always above zero under positive voltages or below zero under negative voltages. This means that although the probe seemed to work fine in case of a conductive material (like graphite felt), the response with these granules (Campina) is not reproducible.

Finally, as can be observed in Figure 5.3-C, 50 measurements were done on the Campina granules. Each measurement was performed on a different granule. It can be seen how the responses were very different and granules gave sometimes even lower signal than the medium. Similar results were also obtained for the other granule types. Thus, it can be concluded that neither reproducible results nor conclusions on the conductivity of the Campina granules could be obtained using this probe.

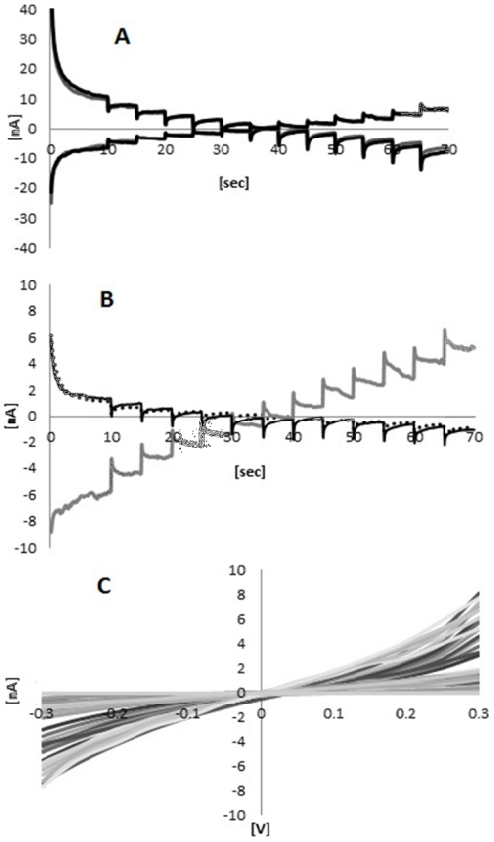

Figure 5.3. Results of measurements performed on granules from Campina with the Small/Pt Probe. A. Measurements that meet the proper chronoamperometry criteria. Measured from +0.3V to -0.3V and from -0.3V to -0.3V. **B. Measurements that do not meet the proper chronoamperometry criteria.** The black line corresponds to a measurement that shows current values significantly lower than those in presented in Figure 5.3A and are very similar to those of the medium alone (dotted line). The grey line is a measurement that is more unsteady than the others. Measurements with these characteristics were also continuously obtained; showing that not all the granules presented the same conductance characteristics and/or the method is not steady enough. **C. I vs. V curve for 50 measurements.** Results show that chronoamperometry results are not replicable.

5.3.3 Measurements with the Large/Au Probe

Measurements performed with Large/Au Probe showed to be, unlike those with the Small/Pt probe, reproducible (Figure 5.4). However, not all the measured biomass types were conductive. One example of this is presented in Figure 5.4-A. This Figure shows two measurements of granules from Eerbeek and two measurements of its medium. It can be observed how the medium had the same conductance as the biomass. On the other hand, Figure 5.4-B shows one example of granules from Nedalco which not only presented reproducible results but also showed higher current than their medium. Analysing Figure 5.4-B, it can be observed that the I vs V graph is not completely symmetrical around the X axis and a current different than zero ampere was measured when zero volts were applied. These two characteristics could be attributed to electric conductance caused by the presence of ions. However, the measurements were reproducible; the current through the medium was negligible; and the sign of the measured current corresponded to the sign of the applied voltage. Furthermore in addition to having reproducible results, and unlike the measurements of the granules performed with the Small/Pt Probe, the current was in the range of microamperes; which is in in correspondence with the results of Summers *et al.*, (2010) and Morita *et al.*, (2011).

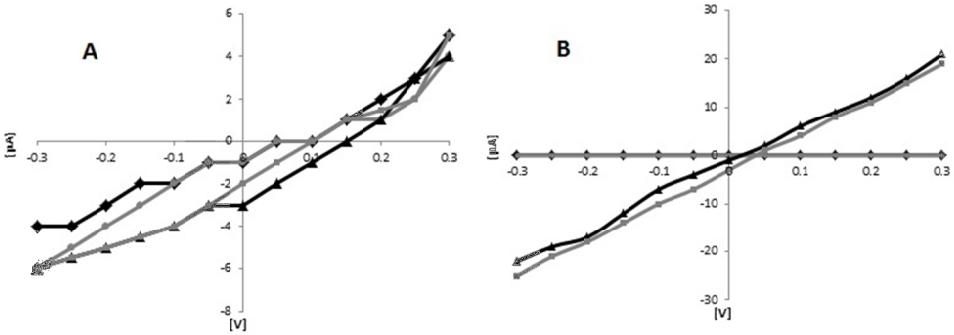

Figure 5.4. Results of measurements performed with the Large/Au Probe. A. I vs. V curve for measurements for granular biomass from Eerbeek **(▲, ■)** and its medium (•,♦). **B.** I vs. V curve for measurements for granular biomass **(▲, ■)** from Nedalco and its medium (•,♦).

Finally, Table 5.1 presents a summary of the measurements for the different granule types used in this study. The results presented in this table indicate that there is a link between the kind of granular biomass and the result of the tests performed. It can be observed that only methanogenic granules that use ethanol as electron donor seemed to be conductive while the other types of granular biomass gave no signal.

Table 5.1. Conductivity for different granules. Conductance and conductivity values of zero were assigned to those granules which measured the same conductance as their respective mediums. Carbon dioxide acted as electron acceptor in all the systems

Granules	Biomass type	Electron donor	Conductance [µA/V]	Conductivity [µS/cm]
Bavaria R1	Methanogenic, treating brewery waste	Ethanol	66.7	16.5
Bavaria R2	Methanogenic, treating brewery waste	Ethanol	152.7	40.6
Bavaria R3	Methanogenic, treating brewery waste	Ethanol	28.3	7.6
Nedalco	Methanogenic	Ethanol	72.5	19.4
Eerbeek	Methanogenic, treating paper-mill wastewater	Complex organics	0	0
Campina	Methanogenic, converts carbohydrates from soft drinks	Carbohydrates	0	0

These results show that the Large/Au Probe can be used to obtain reproducible measurements, while the Small/Pt Probe can not. In order to obtain a further understanding of why the Large/Au Probe had such a different behaviour to the Small/Pt Probe, one more test was performed. This test consisted on locating one single granule in the non-conductive gap of both electrodes. It would be expected that if both probes perform similar, then comparable currents should be obtained during a chronoamperometry. Results of this test indicated that at least 100 times higher current values were always measured with the Large/Au probe as compared to the smaller probe. This points to the possibility that the difference in the performance of both probes might be due to the difference of materials from which the electrodes were built (further discussed in section 5.4.2).

5.4 DISCUSSION

5.4.1 Conductivity of granular biomass.

As the results from the Small/Pt Probe were not reproducible, it is not adequate to calculate a conductivity value from them. However, in order to compare with other studies, a rough estimation using Schwarz-Christoffel transformation (Equation 1), with the probe dimensions and slope from Figure 5.3A, indicates a conductivity lower than 0.1 $\mu S \cdot cm^{-1}$. As can be observed in Table 5.2; this value is more than 10 order of magnitude lower than values reported in previous studies in which a similar measurement methodology was used. Furthermore, the maximum current values obtained with this probe were approximately ±10 nA whereas the values reported for other types of granular biomass were in the order of ±3µA for aggregates of *Geobacter* (Summers *et al*., 2010) or even ±6 µA granular biomass from a bioreactor treating waste water from a brewery (Morita *et al.,* 2011). The conductivity values obtained with the Large/Au Probe (7.6 -40.6 $\mu S.cm^{-1}$, Table 5.1) are well within the range of those presented in in previous studies (Table 5.2). This agreement with previous results suggests that the measurement methodology and probe construction are adequate.

Table 5.2. Conductivity of biomass. Measured with a probe similar to the Large/Au Probe and calculated using the Schwarz-Christoffel transformation.

Source	Conductivity [$\mu S.cm^{-1}$]	Reference
Granules from a bioreactor treating brewery waste water.	6.1	Morita *et al*.,(2011)
Granules propagated in laboratory. Original source were aggregates from a bioreactor treating brewery waste.	7.2	Morita *et a*l.,(2011)
Biofilm grown in anode of microbial fuel cell	18	Malvankar *et al.,* 2011
Granules from four different brewery waste treating bioreactor	0.8-36.7	Shrestha *et al.,* 2014

5.4.2 Dimensions and materials of the probes

Comparing the probes used in this study yields two obvious differences: dimensions and electrode materials (see Figure 5.1). Electrode length (L) and width (b) are respectively ≈ 63 and 42 times larger in the Large/Au Probe when compared to the Small/Pt Probe.

Probes with different contact areas will result in different currents for the same applied voltage and measured granule. In other words, probes with different sizes will result in different conductance values. However, the same granule should present the same conductivity with different probes. This conversion between conductance and conductivity is done by using a *cell constant*, which is a function of the contact area and the geometry of the probe. This explains partly why lower currents are obtained with the Small/Pt Probe. However, in order to have similar conductivity values to those presented in Table 5.2; current at least 100 times higher than measured would be expected with the Small/Pt Probe (Equation 1).

A second factor that could have influenced on the measurements is the material of the electrodes (Weis *et a*l., 2010; Richards and Sirringhaus, 2007). It is possible that gold has better contact characteristics with the granular biomass than platinum; thus explaining why higher currents and more reproducible results were obtained when using the Large/Au Probe.

In addition to dimensions and materials other factors could have played a role in the measurements. For example, both electrodes from the Small/Pt Probe could be completely covered by one single granule. However, a more stable signal and a more reproducible measurement for each kind of granule was obtained with the Large/Au probe. This could be due to its bigger size which allows locating more granules in the surface of the probe and therefore obtaining an average measurement for each biomass type. This was in contrast to the Small/Pt Probe where each measurement was dependant to the contact of one single granule.

To summarize, the differences in probe dimensions and specially in materials seem to be the main reasons that the measurements were not reproducible with the Small/Pt Probe; while on the other hand, a proper size and gold electrodes allowed the measurements with the Large/Au Probe to be reproducible.

5.4.3 Biomass types

The study of Summers *et al*., (2010) described the existence of a coculture of ethanol metabolising *Geobacter* microorganisms that show conductance when located between gold electrodes. In that report, it is hypothesized that the microorganisms use conductive appendages (nanowires, pili) or redox proteins (cytochromes) for electron transfer purposes. Furthermore, Morita *et al*., (2011) revealed that microbial aggregates originated in an ethanol converting methanogenic bioreactor also had conductive characteristics. Finally, Shrestha *et al*., (2014), further evaluates samples from different methanogenic bioreactors treating brewery waste and show that all of them have conductive characteristics.

Our results suggest that from the evaluated biomass types, only ethanol oxidizing methanogenic granules are conductive. Like previous studies (Morita *et al*, 2011 and Shrestha *et al*., 2014), we report that biomass from brewery waste treating bioreactors (Bavaria) has conductive properties. Furthermore, the methanogenic ethanol degrading granules from Nedalco did not originate from a brewery waste treatment plant but also showed to be conductive; further confirming that ethanol plays an essential role. On the other hand, methanogenic granules that did not use ethanol as electron donor (Campina and Eerbeck) did not show conductance. This suggests that direct electron transfer mechanisms are not spread through all granular biomass but is a characteristic exclusive to those biomass types. Previous studies have shown the presence of *Geobacter* microorganisms in the granules with conductive characteristics (Summers *et al*, 2010, Malvankar *et al*., 2011, Morita *et al.,* 2011). Furthermore, Shrestha *et al.,* (2014) found a positive correlation between the conductivity of granular biomass and the abundance of *Geobacter* species in the granules. Thus, suggesting that they contribute to this phenomenon. Consequently, it would be interesting to analyse the different types of biomass measured in this study in order to determine if *Geobacter* species were part of their microbial composition and if its abundance correlates with the reported conductivity.

5.5 CONCLUSIONS

Two main conclusions were obtained in this study. The first one is that using a probe with proper size and material characteristics is a crucial factor for obtaining reproducible measurements of the conductivity of granular biomass. The second conclusion is that only ethanol oxidizing methanogenic granules were conductive and therefore, could rely on the use of direct interspecies electron transfer mechanisms.

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CHAPTER 6

General discussion

Anaerobic oxidation of methane (AOM) occurs in marine sediments in the sulphate methane transition zone (SMTZ) where relatively high concentrations of methane and sulphate are available (Hinrichs and Boetius, 2002; Meulepas *et al*., 2010). Due to the fairly high availability and low prices of methane, AOM could be used in industrial processes for conversion of sulphate to hydrogen sulphide.

AOM coupled to sulphate reduction is thought to be performed by a community of microorganisms coexisting in a syntrophic relationship (Zehnder and Brock, 1980). However, their slow growth (which leads to low conversion rates) is one of the reasons why this process is not industrially feasible yet. Thermodynamic calculations indicate that more energy can be gained using electron acceptors such as sulphur or thiosulphate. Furthermore, previous studies have shown that higher AOM rates can be obtained using thiosulphate instead of sulphate as electron acceptor. Therefore, in Chapter 2, it was tested if higher conversion rates could be obtained using not only elemental sulphur but also thiosulphate as alternative sulphur sources.

The sulphate reduction rates observed in marine sediments are higher than the measured methane oxidation rates. This difference has been reported to be due to reduction of sulphate with not only methane but also other alkanes such as ethane, propane or butane (Jaekel *et al*., 2013; Kniemeyer *et al*., 2007; Savage *et al*., 2010). Consequently, it was also tested if sediments from Eckernförde Bay and Aarhus Bay were capable of oxidizing ethane and propane with the different sulphur electron acceptors (Chapter 2). The results of these incubations showed that no matter which alkane was added, the sulphide production rate was always enhanced when thiosulphate was present. However, as shown by the different ratios of thiosulphate disproportionation, the mechanisms of conversion were probably different for each alkane. These results, along with the carbon dioxide production measured in the thiosulphate incubations, revealed that the alkanes were oxidized while thiosulphate was reduced and/or disproportionated.

The conversion rates obtained with thiosulphate (between 86 and 108 $\mu mol \cdot L^{-1} \cdot day^{-1}$) were up to 50 times higher compared to those of sulphate. A previous study (Meulepas *et al*., 2009), in which the incubations were performed in similar conditions, obtained comparable rates when thiosulphate and methane were fed to sediment from Eckernförde Bay. However, thiosulphate and sulphate reduction rates were similar in that study. This might mean that our results are not ubiquitous. In despite of this, our results do show that for the mix of microorganisms used in our study and under the evaluated conditions, thiosulphate is the preferred electron acceptor. This indicates that higher growth rates are achieved with thiosulphate and therefore it could be used for faster enrichment of the microbial consortia performing AOM. This could even represent a start-

up strategy for industrial scale bioreactors treating sulphate wastewaters. After successful start-up with added thiosulphate treatment of sulphate-containing effluents could follow.

In order to test the use of thiosulphate as start-up strategy; a 5 litre membrane bioreactor fed with methane, sulphate and thiosulphate was operated during 454 days (Chapter 3). Furthermore, the reactor was also fed with acetate, which has been reported to selectively enrich for ANME microorganisms when thiosulphate was also present (Jagersma *et al*., 2012). However, and even though the reactor was operated under conditions thought to be capable of allowing thiosulphate reduction coupled to the AOM, the obtained reaction rates indicated that thiosulphate disproportionation was the dominant process. Molecular analysis showed that the most abundant microorganisms in the bioreactor were bacteria belonging to the genus *Desulfocapsa*, which are known thiosulphate disproportionating bacteria and green sulphur bacteria (GSB) belonging to the family *Chlorobiaceae* acting in a mutualistic relationship. The latter oxidized the sulphide produced by the former to elemental sulphur.

The main differences that allowed for the enrichment of these microbial communities in the bioreactor system used in this study when compared to others in which AOM took place were that light was not completely filtered and that sulphide was not completely stripped after its formation. As thiosulphate was disproportionated into sulphate and sulphide by the *Desulfocapsa*; the presence of sulphide and light allowed for the GSB to grow. And at the same time, the removal of sulphide performed by the GSB created energetically favourable conditions for the *Desulfucapsa*.

Even though the enrichment of the microorganisms capable of performing AOM was not achieved in the bioreactor, a process in which thiosulphate is removed was obtained. Thiosulphate containing wastewaters are common in pulp, bleaching and photographic fixing processes and its presence in effluent streams might cause sulphuric acid generation through its oxidation with oxygen. Therefore, a process in which thiosulphate is removed and sulphur recovered can be achieved in a single operation is interesting from the industrial point of view. This microbiological association developed in the membrane bioreactor might allow the reduction of investment and operational costs by simplifying in a single tank an otherwise three-step operation (thiosulphate disproportionation or reduction, sulphide oxidation and sulphur recovery). A process which allows elemental sulphur formation using light and its recovery through sedimentation might allow further reduction of the operational costs when compared to the current sulphide oxidizing technologies based on oxygen sparging. The reactor used in this study was covered with screens that blocked > 93% of the light, and was not designed in a way that allowed proper light dispersion (e.g. photo bioreactors). Therefore, by using technologies that allow for better light dispersion in the reactor, the conversion efficiency of the green

sulphur bacteria that was enriched in the system might by greatly enhanced and such process could become a cheaper alternative to current elemental sulphur recovery technologies.

A reactor with low operational and construction costs capable of using light instead of oxygen in order to transform sulphide into elemental sulphur at high conversion rates would have to meet two important requirements. 1. It would have to allow proper light dispersion in order to support the growth of the green (or purple) sulphur bacteria. This could be particularly difficult as biomass and elemental sulphur itself would be present in the media and therefore would avoid the light transfer throughout the whole reactor. 2. In order to maintain high conversion rates, the reactor must offer the possibility to remove the elemental sulphur while keeping (most of) the biomass. Such separation could be done by taking advantage of their different sedimentation velocities.

Current AOM-studies and the enrichment of the microorganisms that perform this conversion have been focused on sea water ecosystems. Chapter 4 shows the presence of that AOM coupled to sulphate reduction under freshwater conditions in samples originated from a location that captures natural gas from groundwater pockets with a 30 m long pipe. Furthermore, it was presented how anaerobic methanotrophic (ANME) microorganisms and sulphate reducing bacteria were actively growing in the incubations that contained methane and sulphate. Moreover, by measuring both, methane consumption and production, it was shown that the production of carbon dioxide in these incubations was not due to trace methane oxidation.

These results have large repercussions from the microbiological perspective because the association of ANME archaea and sulphate reducers in such environment while performing net methane oxidation has been proven to be somehow elusive. This is also interesting for the technological point of view; as a lower amount of salt is required for the conversion to occur, the potential biotechnological process that could be derived from this conversion would be more environmentally friendly.

Research into the AOM is difficult due to the slow growth rate of the microorganisms. Therefore, it would reasonable to focus on raising their growth rates. Enrichment of microorganisms has been showed in membrane and high pressure bioreactors (Meulepas *et al*., 2009b; Deusner *et al*., 2009). Using alternative substrates or metabolism intermediaries could also help to avoid rate limiting steps. And, understanding how the ANME microorganisms transfer the electrons to the sulphate reducers would give insight into the mechanisms used by these microorganisms. Research in this area has been focused on finding the interspecies electron transfer compound (Meulepas *et al*., 2010 and reference therein; Stams and Plugge, 2010 and reference therein).

In Chapter 5 a methodology for measurement of electrical conductance of granular biomass was evaluated. This methodology could potentially be used to study systems in which AOM takes place and determine if direct electron transfer occurs between the microorganisms that perform the involved conversions. This chapter, however, is focused on the methodology evaluation and measurement of different types of granular biomass. It was shown that from the measured granule types, only ethanol oxidizing methanogenic biomass had conductive characteristics. This means that theoretically they would be the only ones capable of performing direct interspecies electron transfer. Further research in this line should not only asses if other kinds of granules also have conductive behaviour but also if operational and environmental conditions affect the measured conductivity. This would allow to obtain a better understanding of electron transfer mechanisms which could be used in the development and optimization of waste water treatment processes.

A further development of this methodology would be beneficial for the understanding of the mechanisms used by (granular) biomass. However, the obtained results, which indicate that only ethanol oxidizing methanogenic granules are conductive, raise doubts about the possibility that direct electron transfer is used by the microorganisms involved in the AOM.

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Summary

Microorganisms capable of performing anaerobic oxidation of methane (AOM) coupled to sulphate reduction have high doubling times which make their enrichment difficult. However, due to higher energy gain, they might be rapidly enriched using alternative electron acceptors. In chapter 2, it was shown that up to 50 times higher conversion rates were obtained with thiosulphate when compared to sulphate. However, it was also presented that thiosulphate was not be exclusively used by microorganisms that reduce it, but that it was also disproportionated into sulphate and sulphide (Chapter 2).

In Chapter 3, a 5 litre membrane bioreactor was fed not only with methane and sulphate but also with acetate and thiosulphate. As previous experiments using these additional substrates had allowed to obtain either faster conversion rates or enrichment of methanotrophic microorganisms; it was expected that AOM rates in the reactor would increase relatively fast. However, the microorganisms that were enriched were not AOM related. They microbial community that showed the highest activity rates in the reactor was comprised by thiosulphate disproportionated bacteria and green sulphur bacteria. The former disproportionated thiosulphate into sulphate and sulphide while the latter converted the sulphide into elemental sulphur.

Chapter 4, unlike the previous chapters, focused on studying the occurrence of AOM in a fresh water ecosystem. Such system was located next to a natural gas source which captured methane for domestic purposes. It was presented how, with the different electron acceptors added, AOM and trace methane oxidation occurred. However, net AOM was only measured in the presence of sulphate as electron acceptor. Furthermore, the microorganisms that were enriched in the presence of methane and sulphate were also detected.

There are several hypotheses which attempted to explain the AOM coupled to sulphate reduction. One of them indicates that it is a process that involves two microorganisms working in a syntrophic relationship. The first microorganism would convert the methane into carbon dioxide and pass the electrons to the second one. Consequently, the second microorganism would convert the sulphate into hydrogen sulphide. In such a structure, the way that electrons are released by the conversion performed by the first microorganism is unknown. It is possible, that electrons are not transfer via electron shuttles or chemical compounds, but that they are transferred directly from one microorganism to the other. A methodology which could be used to determine if the AOM consortia uses direct electron transfer mechanisms was evaluated in Chapter 5. Different kinds of granular biomass were used for this evaluation and, the granule types that would potentially be capable of using direct electron transfer mechanisms were detected.

Resumen

Existen diferentes alternativas para el tratamiento del sulfato que se encuentra comúnmente en aguas residuales provenientes de algunos procesos industriales. Una de ellas es el tratamiento que usa metano como sustrato. El metano es menos costoso que otros sustratos usados tradicionalmente, pero los microorganismos capaces de realizar esta conversión tienen tiempos de duplicación muy altos, lo que hace muy difícil su cultivo. Debido a esto, diferentes condiciones de cultivo son evaluadas en el capítulo 2, donde se explica cómo distintos compuestos son usados para tratar de aumentar la velocidad de reproducción de estos microorganismos y se obtiene como conclusión principal que tasas de conversión hasta 50 veces mayores fueron obtenidas cuando se usó tiosulfato en vez de sulfato. Esto sugiere que se podría usar tiosulfato para cultivar estos microorganismos rápidamente, y una vez enriquecidos, usarlos para tratar aguas con sulfato.

Debido a estos resultados, un biorreactor con membranas internas fue alimentado no solo con metano y sulfato sino también con acetato y tiosulfato (capítulo 3). Esto dos últimos se adicionaron debido a que experimentos previos en los que se usaron estos sustratos permitieron obtener altas tasas de conversión y/o un rápido enriquecimiento de los organismos responsables de las conversiones. Era esperado que las tasas de conversión aumentaran rápidamente, pero debido a algunas condiciones de operación del biorreactor, diferentes microorganismos fueron enriquecidos. Estos microorganismos podrían ser usados para realizar otras interesantes conversiones.

La oxidación anaeróbica de metano (OAM) en ecosistemas de agua dulce fue estudiada en el capítulo 4. Específicamente el ecosistema seleccionado estaba ubicado al lado de una fuente de gas natural que capturaba metano para uso doméstico. En este capítulo fue demostrado que los microorganismos provenientes de dicho ecosistema eran capaces de realizar OAM acoplada a reducción de sulfato, y adicionalmente estos microorganismos fueron identificados.

Existen diferentes hipótesis que explican la OAM acoplada a la reducción de sulfato. Una de estas indica que este proceso se lleva a cabo a través de la relación sintrófica de dos microorganismos. El primero convertiría el metano en dióxido de carbono mientras el segundo tomaría los electrones derivados de la conversión del metano para usarlos en la conversión del sulfato a sulfuro. Una de las principales incógnitas de esta hipótesis es el mecanismo a través del cual los electrones son transferidos. Es posible que esto ocurra a través de estructuras que permitan el paso de los electrones directamente, sin necesidad de usar compuestos intermedios. Una metodología que podría ser usada para determinar si los electrones son transferidos directamente es evaluada en el capítulo 5. Para esto, diferentes tipos de biomasa granular fueron usados y aquellos tipos de gránulos que potencialmente serían capaces de utilizar mecanismos para transferir electrones directamente fueron identificados.

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About the author

Diego Andres Suarez Zuluaga was born on in the early hours of March 2nd 1984 in the beautiful city of Medellin (Colombia). In the year 2000, he graduated from high school (Instituto San Carlos) in Medellin. After this he studied Process Engineering with specialization in biotechnology at the EAFIT University. During his time in this university we was constantly involved in different biotechnological projects. In order to follow his passion in process engineering and biotechnology, he moved to the Netherlands to study at Delft University of Technology in 2008. In this university he obtained the Professional doctorate in engineering (PDEng) degree as Designer in bioprocess engineering. Finally, in 2010 he started his PhD research at the Sub-department of Environmental Technology at Wageningen University.

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Diego A. Suarez-Zuluaga, Peer H.A. Timmers, Caroline M. Plugge, Alfons J.M., Stams, Cees J.N. Buisman, Jan Weijma. “Thiosulphate conversion in a methane and acetate fed membrane bioreactor”. *Environmental Science and Pollution Research. Accepted for publication.*

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- Anaerobic Wastewater Treatment (2014)

Other PhD and Advanced MSc Courses

- PhD competence assessment, Wageningen University (2010)
- Supervising MSc thesis students, Wageningen University (2012)
- Interpersonal communication for PhD students, Wageningen University (2012)
- Voice Matters - Voice and Presentation Skills Training, Wageningen University (2012)
- Effective behaviour in your professional surroundings, Wageningen University (2012)
- OSPT Fundamentals and practice of process intensification, Delft University of Technology (2013)
- Presentation Skills, Wageningen University (2013)
- Career Perspectives, Wageningen University (2014)

Management and Didactic Skills Training

- Supervision two MSc students with thesis entitled 'Anaerobic Oxidation of Short Chain Alkanes for Biological Sulphate, Thiosulphate, Sulphite and Sulphur Reductions' (2012) and 'Electrical conductivity in granular biomass. Standardization and evaluation' (2014)

Oral Presentations

- *Sulphur formation during thiosulphate disproportionation.* The 7th International Young Water Professionals Conference, 7-11 December 2014, Taipei, Taiwan

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