In situ biogas upgrading: Method development of a potential activity assay

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Contents

Abstract

Excess wind power, from when production is high but demand low, could be stored as biogas by using the electricity to power hydrogen production. Through the in situ hydrogen (H_2) addition to the anaerobic digester, CO_2 can be biologically converted to methane. This aim of this study is to produce an assay to evaluate the upgrading potential of anaerobic digestion reactors. The assay should produce comparable results over time both within the reactor and between reactors. Anaerobic digesters at Viby WWTP, Åby WWTP and Bånlev Biogas were investigated. Methane production rates (MPRs) were measure from relatively small amounts of digester slurry incubated with a headspace of either H_2/CO_2 (80:20) or N_2 as control and compared with reactor MPR. When using diluted slurries, the MPRs were significantly higher with H_2/CO_2 headspace compared to control, but all lower than reactor MPR. Dilution series showed higher MPRs with undiluted slurry, which led to further investigations. Undiluted slurries were tested with pressurized (~1.5bar) headspace of H_2/CO_2 or N_2/CO_2 (80:20). With Bånlev slurry the MPRs were the highest achieved so far, with control samples matching reactor MPRs, but with Åby and Viby slurries the results were less clear. The rate should be independent of the slurry concentration but was not. Similar observations have been made in other studies examining biomethane potential experiments. It seems unlikely that oxygen contamination would be the cause of the low MPRs, but inhibition due to increasing pH values could in some cases. Experiments were conducted to evaluate upon the CO_2 viability, but not clear results were achieved. Two types of adaption experiments were tested with the potential activity assay, none of them showed any adaption, one due to too short adaption time and the other due to inhibition of increasing pH values. Visualization of the methanogens in a slurry sample with fluorescents in situ hybridization failed. Visualization though the auto fluorescent F_{420} were not possible due to the load of organic substrates in the sample.

Introduction

Since the industrial revolution the main sources of electrical power and fuel have been coal, oil and natural gas, all depletable fossil fuels. Furthermore, these sources emit pollutants and greenhouse gases, which affect the climate all around the world. Sustainable energy sources provide a much needed alternative to fossil fuels. Wind, hydro and solar power are growing industries and do provide some of the solutions. Last year (2015) 42% of the Danish energy demand was covered by wind generated electricity (Energinet Danmark), which makes Denmark the leading country of wind power utilization (REN21, Renewable Status Report 2016). Still 78% of the global energy consumption in 2014 was from fossil fuels and only 19% from renewable sources (REN21, Renewable Status Report 2016). Human caused global warming, from emission of greenhouse gases from fossil fuels, is a known fact, and a problem that will increase if action is not taken. World leaders agreed at Paris COP21 (The Sustainable Innovation Forum) that the global temperature should not increase above 2°C, and the world should strive to keep the increase at 1.5°C. If the temperature would rise just 2°C more, i.e. to 4°C, it would have severe consequences for the climate; sea-level would rise with critical implications for coastal zones and low elevation arears, which covers 2% of the world's land area but contains 10% of the world's population (McGranahan 2007). A temperature rise of 1.5°C would limit the rise of the sea-levels to below 2 m in year 2300, where with a 2°C temperature increase the levels can be expected to rise between 1.5 m and 4 m. To make the 2°C increase goal possible there need to be invested in renewable energy and fuel sources and the renewable sources need to be implemented so that fossil fuels can be phased out.

As mentioned, wind, hydro and solar powers do provide some of the solutions together with geothermal energy (heat and power) and energy form biomass. In Denmark, who cannot rely on solar or hydropower in the same extent as other countries, wind power is dominating. Some of the problem with wind power, and some of the other sustainable sources, is that the production does not always correlates with the demand. This results in periods of excessive wind energy production where electrical power is exported at low profit or in windmills standing still when they could generate electricity (Danmarks Vindmølleforening 2015, Energinet Danmark). This excess energy would be better utilized if there was a feasible way of storing it until the demand was higher. One way could be to transform and store the energy as biofuel. This could be done by upgrading biogas (CH_4 and CO_2) to a higher

CH_4 concentration by addition of H_2 to the anaerobic digester, where H_2 is produced by the energy demanding hydrolysis of water.

Processes in the biogas reactor

One of the oldest ways of treating organic waste is by anaerobic digestion. With the rise of energy prices in the 1970s the interest in the process grew (Angelidaki et al. 2003). The interest continued after the oil crisis due to the environmental aspects of the digestion.

Today the anaerobic digestion process is mainly utilized in four sectors: municipal waste water treatment, industrial waste water treatment, waste from livestock, and industrial anaerobic digestion of organic municipal waste (Angelidaki et al. 2003). Primary sludge, i.e. sludge captured from waste water that has not gone through biological treatment yet, and secondary sludge, i.e. sludge that has gone through biological treatment, are treated by anaerobic digestion at the waste water treatment plants (WWTP). As the primary sludge has not been biologically treated it contains more easily degradable material. Anaerobic digestion of industrial waste, e.g. from food-processing industries, can reduce the environmental impact by treating the waste before disposal into the environment or the sewage system. The volume of waste is reduced and so is the amount of pathogens, especially anaerobic digester operated at thermophilic temperature (45-60°C) (Weiland 2010). Treating waste from livestock farming can improve the fertilizer quality of manure and at the same time produce energy. Processing municipal organic waste on industrial scale by anaerobic digestion is a relative new application of anaerobic digestion, and is a way of reducing the amount of organic material being dumped in landfill or utilized in CHP plants (Combined Heat and Power) (Angelidaki et al. 2003). By digesting the organic municipal waste it is possible to return the nutrients back to agricultural sector as fertilizer.

The amount of CH_4 produced in the anaerobic digestion process varies between sources of organic material and reactors, as they contain different amounts of water, nutrients and biodegradable substrates. A way to normalize the CH_4 yield from different sources is by comparing it to amount of biodegradable substances in the material. This could be by measuring volatile solids (VS) in the reactor (e.g. $mL(CH_2)/g(VS)$) (Yu et al. 2010).

In the anaerobic digester organic material is degraded by different processes and the carbon chains are broken down into the smallest units, CH_4 and CO_2. CH_4 has a high combustion value and can be used as fuel.

The process begins with hydrolysis of the complex organic polymers (Ahring 2003). The polymers are broken down into monomers and oligomers, which include long chain fatty acids such as propionate and butyrate, sugars such as glycose and cellobiose, amino acids and glycerol (Adney et al. 1991; Yu et al. 2010). This initial breakdown is facilitated by a consortium of microorganisms. Common organisms found to be responsible are mostly strict anaerobes such as *Clostridia*, *Bacteriocides* and *Bifidobacteria* (Weiland 2010). Facultative anaerobes as *Streptococci* and *Enteribacteriaceae* also participate (Weiland 2010). The hydrolysis is catalysed by extracellular enzymes such as cellulases and lipases secreted by the bacteria. Polymers such as cellulose and xylan can slow down the hydrolytic process due to them being embedded in refractory molecules (e.g. lignin) and the general insolubility of the polymers. Often the hydrolytic process is the rate limiting step of the entire process in the anaerobic digester.

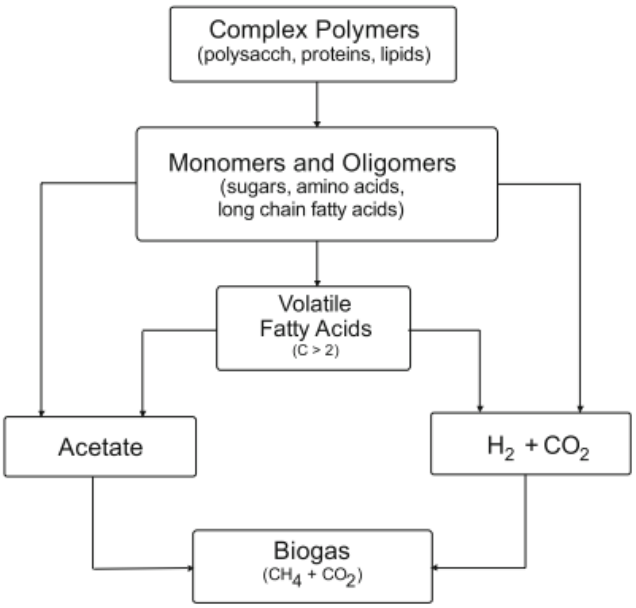

Figure 1: Schematic presentation of stages in the anaerobic digestion. (Weiland 2010)

Fermenting organisms use the mono- and oligomers and produce short chain fatty acids (volatile fatty acids, VFA), e.g. butyrate, propionate and acetate, CO_2, and H_2. This phase is called fermentative aci-

dogenesis. Butyrate and propionate are then converted to acetate, CO_2 and H_2 by specialized acetogenic bacteria (Wang et al. 1999; Ahring 2003; Weiland 2010). Small amounts of alcohols are also produced. The fermentative acidogenesis is often quite fast and the overall process in the reactor depends on an immediate utilization of the products to avoid inhibition of the processes.

A low partial pressure of H_2 is needed for the degradation of butyrate and propionate to avoid product inhibition of the process. In granules the process only occurs though syntrophic H_2 oxidizing methanogens thus keeping the H_2 partial pressure below inhibitory levels (Angelidaki et al. 2003)

Acetate, CO_2 and H_2 are converted into CH_4 and CO_2 by strict anaerobic methanogenic archaea (Kiener and Leisinger 1983). These are found in natural environments such as sediments, wetlands, rice fields, and in the rumen of ruminant animals. The methanogens in the anaerobic digester can be divided into two groups, hydrogenotrophic and acetotrophic methanogens (Bapteste et al. 2005). Hydrogenotrophic methanogens utilize H_2 to reduce CO_2, format, methanol, methylamines, or methylsulfide into CH_4. Except form CO_2, these compounds are only formed in small extents in anaerobic digesters. Acetate is converted into CH_4 and CO_2 by the acetotrophic methanogens. As acetate is a major product of the fermentation process, these methanogens produce approximately two-thirds of the CH_4 produced in an anaerobic digester (Liu and Whitman 2008). The utilization of acetate is fast and the concentration in the reactor is thereby kept low even though it is the dominating VFA produced (Gavala et al. 2003). Two genera of acetotrophic methanogens are identified: *Methanosaeta* and *Methanosarcina*, where the latter is both acetotrophic and hydrogenotrophic (Yu et al. 2010). *Methanosaeta* is important in anaerobic digesters as they help form granules (Demirel and Scherer 2008). Granules are multicellular structures of methanogenic archaea and bacteria and can provide protection from outer stressors (Ahring 2003). Most methanogens are able to utilize H_2 and CO_2, and hydrogenotrophs in the anaerobic digester are hence a more diverse group, e.g. including species of *Methanobacterium, Methanosprillum* and *Methanobrevibactor* (Demirel and Scherer 2008).

Even though the processes in anaerobic digesters are well known, only small parts of the organisms responsible are well characterized. Even less is known about the dynamics and interactions between the microbes (Weiland 2010).

Operating conditions in anaerobic digesters

Anaerobic digesters are in general operated at either mesophilic (35-42°C) or thermophilic (45-60°C) temperatures (Weiland 2010). Drawing on experience from investigated different plants the mesophilic reactors have been running close to 40°C. Keeping the temperature constant in the anaerobic digester is important for a steady biogas production (Weiland 2010). The microbial community in mesophilic reactors tolerates temperature fluctuations of +/- 3°C without significant effect on CH_4 production. Thermophilic reactors are more sensitive to changes in temperature as the microbial diversity can be lower in these reactors (Karakashev et al. 2005). Temperature changes in both meso- and thermophilic digesters can result in an efficiency drop, until an adaptation to the new temperature has occurred. Slurries from mesophilic anaerobic digesters were primarily used in experiments described in this rapport.

As ammonia (NH_3) toxicity is shown to increase with temperature, thermophilic reactors are in higher risk of inhibition. Despite the higher sensitivity, both regrading NH_3 and temperature fluctuations, a well-functioning thermophilic reactor can be more efficient. The growth rates of methanogens are higher at thermophilic temperatures, and a higher biogas production and CH_4 concentration is possible. The thermophilic reactor can be fed at a higher rate and operated at a lower hydraulic retention time (HRT), compared to mesophilic reactors (Weiland 2010). This is due to a faster digestion process with faster degradation of polymers and a more active methanogenic community (Angelidaki et al. 2003). HRT refers to the time a compound is retained in the reactor and can be between 10 and 30 days depending on the material and type of reactor (Angelidaki et al. 2003).

Methanogens

Phylogeny and taxonomy, metabolisms

Methanogenic archaea all belong to the phyla *Euryarchaeota* and are divided into five well established orders: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Mehanopyrales (Bapteste et al. 2005). These orders are based on phenotypic as well as phylogenetic analyses. Furthermore novel physiological groups have recently been revealed with culture independent studies. These organisms are not closely related to any defined order. The rice cluster I (RC-I), aerotolerant hydrogenotrophs, is an example (Erkel et al. 2006). Methanogens are obligate anaerobes, and most are mesophilic and nonhalophilic, even though extremophiles also occurs (Madigan el al. 2010).

Methanogens can utilize a limited number of substrates, the three major types being: CO_2, methyl-group containing compounds and acetate (Madigan et al. 2015). More complex substrates must be degraded by other organisms first. As mentioned most methanogens are able to use H_2 to reduce CO_2 to CH_4 with H_2 as electron donor. Formate is another electron donor many hydrogenotrophic methanogens can. In that case four format molecules are oxidized to CO_2, which then is reduced though the normal hydrogenotrophic methanogenesis (Bapteste et al. 2005; Liu and Whitman 2008).

In hydrogenotrophic methanogenesis CO_2 is reduced through the formyl, methylene and methyl levels to form CH_4, the most reduced compound (Figure 1A) (Liu and Whitman 2008). Special coenzymes, methanofuran, tetrahydromethanopterin (MFR-H_4MPT) and coenzyme M (CoM-SH) are responsible for carrying the C1 unit. Initially CO_2 is activated by methanofuran (MFR) and reduced to the formyl level, forming formyl-MFR, where H_2 reduced ferredoxin (Fd) is the immediate electron donor. The formyl group is then transferred to tetrahydromethanopterin (H_4MPT), forming formyl- H_4MPT. The formyl group is dehydrated to a methenyl group which is reduced to methylene-H_4MPT and then to methyl-H_4MPT. $F_{420\ red}$ is the direct electron donor in these reductions. The methyl group is transferred to CoM-SH, forming methyl-CoM-SH which then is reduced a last time to CH_4 by the key methanogenesis-enzyme methyl CoM reductase (Mcr). In this reaction, coenzyme B (CoB-SH) is the direct electron donor. The reduced CoB-SH forms a heterodisulfide complex with CoM (CoM-S-S-CoB), which is finally reduced by H_2 to regenerate thiols together with Fd. A new round hence is ready to begin (Bapteste et al. 2005; Liu and Whitman 2008; Madigan et al. 2010).

In the acetotrophic methanogenesis acetate is split, the carboxyl group is oxidized to CO_2, and the methyl group is oxidized to CH_4 (Liu and Whitman 2008) (Figure 1C). Acetate is activated in two different ways by the two methanogens, *Methanosarcina* with the acetate kinase (AK)-phosphortransacetylase (PTA) system, and *Methanosaeta* with acetyl-CoA syntheatase (Jetten et al. 1992; Singh-Wissmann and Ferry 1995). Acetyl-CoA syntheatase has a higher affinity for acetate compared to AK-PTA, which correlates with *Methanosaeta* being a superior acetate utilizer that can use acetate concentrations as low as 5-20µM (Jetten et al. 1992; Conklin et al. 2006).

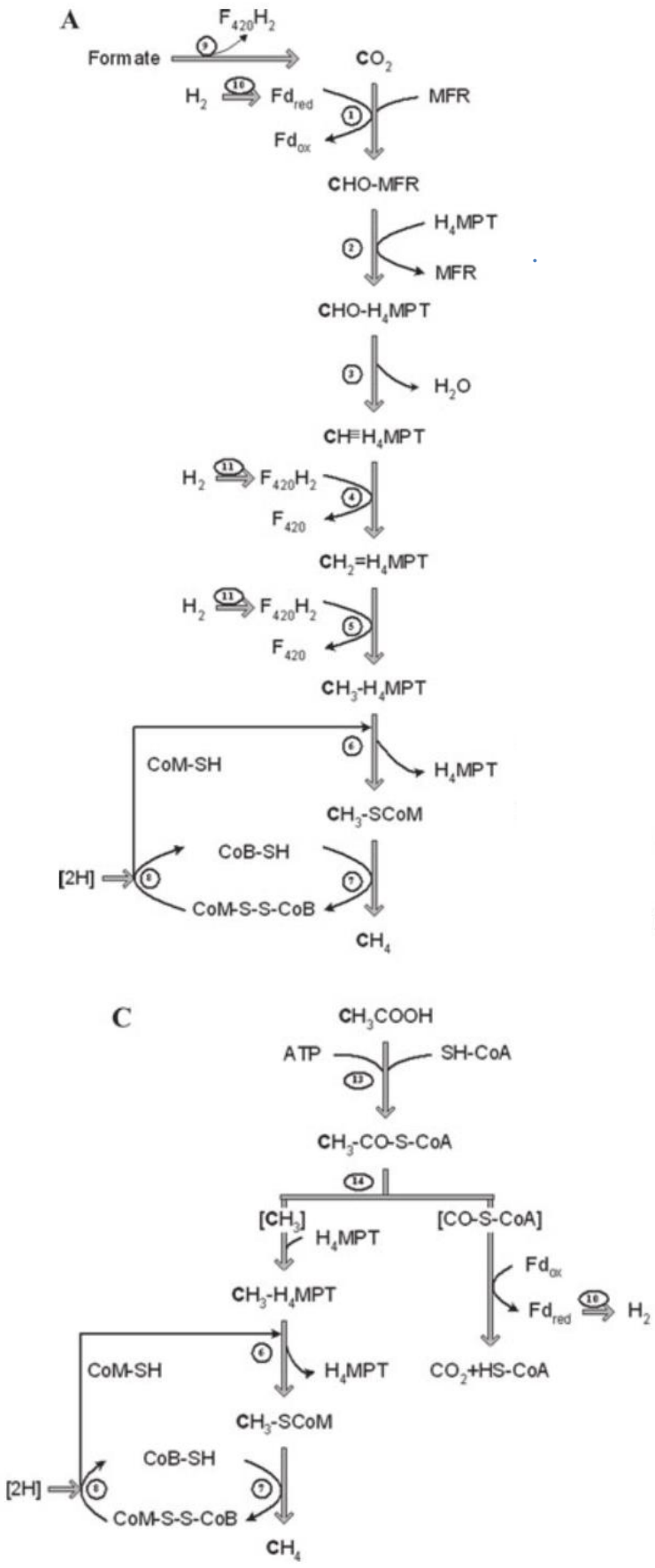

Figure 2:: Parthways of methanogenesis from (Liu and Whitman 2008). (A) Methanogenesis from H_2/CO_2 or formate. (B) Methanogenesis from methanol. (C) Methanogenesis from acetate. Abbreviations: Fd_{red} and Fd_{ox}, reduced and oxidized form of ferredoxin; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M. Enzymes: 1. formyl-MFR dehydrogenase; 2. formyl-MFR: H_4MPT formyltransferase (Ftr); 5. Methenyl- H_4MPT cyclohydrolase (Mch); 4. methylene-H_4MPT dehydrogenase (Hmd); 5. methylene-H_4MPT reductase (Mer); 6. methyl-H_4MPT:HS-CoM methyltransferase (Mtr); 7. methyl-CoM reductase (Mcr); 8. heterodisulfide reductase (Hdr); 9. Formate dehydrogenase (Fdh); 10. energy- conserving hydrogenase (Ech); 11. F_{420}-reducing hydrogenases; 12. methyltransferase ; 13. acetate kinase (AK)-phosphotransacetylase (PTA) system in Methanosarcina; AMP-forming acetyl-CoA synthetase in Methanosaeta; 14. CO dehydrogenase/acetyl-CoA synthase (CODH/ACS)

Lastly CH_4 production from substrates containing a methyl group is possible (**Fejl! Henvisningskilde ikke fundet.**B). This could be methanol, methylated amines, and methylated sulphides (Madigan et al. 2010). Methanol is metabolised by transferring a methyl group to CoM through a corrinoid enzyme (Burke and Krzycki 1997). CH_4 is formed from methyl-CoM in the same way as in CO_2 reduction. If H_2 is not available for the last step, some of the methanol will be oxidized to CO_2 by reversal of the steps in the methanogenesis which will yield electrons to perform the reduction (Bapteste et al. 2005; Liu and Whitman 2008). Three methyl groups are reduced to form one CO_2. This is a disproportionation as the electrons from oxidation of some of the substrate are used to reduce the rest of the reminder (Liu and Whitman 2008). Only methanogens from the order Methanosacinales are able to use methylated compounds for methanogenesis. There is one exception, *Methanosphaera* species, from the order of Methanobacteriales (Liu and Whitman 2008).

CO instead of CO_2 in the hydrogenotrophic methanogenesis pathway is observed in two species, *Methanothermobacter thermoautothrophicus* and *Methanosarcina barkeri* (Daniels et al. 1977; O'Brien et al. 1984). CO dehydrogenase (CODH) is used when oxidizing four CO to CO_2 in this metabolism, after which one molecule CO_2 is reduced to CH_4. CO is used in an unconventional and different way by *M. acetivorans*. The metabolism here distinct itself from that of *M. thermoautothrophicus* and *M. barkeri*, e.g. by not generating a H_2 intermediate, and not being able to grow on H_2/CO_2 (Liu and Whitman 2008).

F_{420} has been utilized as a way of visualizing methanogens due to its autofluorescence (Zeikus 1977). Furthermore, F_{420} works as a defence against reactive oxygen species and is oxidized to F_{390}. F_{390} needs to be reduced back to F_{420} to act in the methanogenesis. The oxidation of F_{420} results in the methanogenesis being inhibited by oxygen (Fetzer et al. 1993).

Methanogenesis is considered to be the rate limiting step in the anaerobic digestion, and high methanogen activity is essential for a functioning digestion. High concentrations of H_2 could inhibit the utilization of short chain fatty acids (VFA), which would accumulate resulting in a lower pH value in the reactor (Ahring and Westermann 1988).

Competition and inhibition of methanogens in anaerobic digestion

Sulphate reducing bacteria (SRB) are also found in anaerobic digesters (Chen et al. 2008). SRB are diverse in their metabolic pathways and can completely or partially degraded compounds such as long chain fatty acids, some alcohols, organic acids, and aromatic compounds (Elferink Stefanie et al. 1994). SRB prefer H_2 to other electron donors, but can use others, and is an effective competitor to hydrogenotrophic methanogens. H_2 is a highly potent reducing agent and can be used as electron donor in the reduction of sulphate to sulphide. SRB can also compete with acetogens or fermentative microorganisms, but competition between acetotrophic methanogens and SRB is not certain and has been reported to dependent on the ratio between organic material and SO_4^{2-} in the reactor (Chen et al. 2008). Besides the competition between organisms the product of sulphate reduction, hydrogen sulphide, can also cause problems in the anaerobic digester. H_2S or HS^- is toxic to the cell as it can diffuse through the membrane and interfere with coenzymes and denaturing proteins by binding to iron (Truong et al. 2006), e.g. iron molecules in cytochromes thus blocking the respiration. Sulphide from sulphate reduction ends up in the biogas making desulfurization necessary to prevent damage on the gas utilization units (Weiland 2010). Autotrophic acetogens, called homoacetogens, may also compete for H_2 in the reactor as they convert H_2 and CO_2 to acetate (Angelidaki et al. 2003; Madigan et al. 2015). Homoacetogens activity and ability to use H_2 is dependent on the terminal electron acceptor, but is said to be poor H_2 utilizers (Cord-Ruwisch et al. 1988; Angelidaki et al. 2003).

Another inhibitory factor in an anaerobic digester can be NH_3, which is a natural product of the degradation of proteins and urea. NH_3 (Free Ammonia, FA) and ammonium (NH_4^+) are the major forms of inorganic nitrogen in an anaerobic digester and both can have inhibitory effects. NH_3, the non-ionized form, is considered the most inhibitory as it is membrane permeable (Angelidaki et al. 2003; Chen et al. 2008). Mechanisms for inhibition have been proposed: changes in intracellular pH, increased required maintenance energy and inhibition of specific enzymatic processes (Angelidaki et al. 2003; Chen et al. 2008). The toxicity increases with higher pH value in the reactor, as more inorganic nitrogen would be in the form of FA. FA inhibits the utilization of fatty acids, which would accumulate and subsequently lower the pH value (Angelidaki and Ahring 1993). Inorganic nitrogen is a necessary nutrient for microorganisms, and hence a certain amount of NH_3 is tolerated. Different amounts are reported in the literature, Liu and Sung (2002) reported NH_3 concentrations below 200 mg/L beneficial (164 mg-N/L), where Angelidaki et al. (2003) referred to 800 mg-N/L being tolerated in an adapted reactor. Different

conditions and substrates can be responsible for the variety in reported inhibitory concentrations. Methanogens are most sensitive to toxic and inhibitory compounds in the anaerobic digester, and also to NH_3. Acetotrophic methanogens are generally more sensitive to NH_3 than hydrogenotrophic (Chen et al. 2008).

From the pH effect on NH_3 inhibition it is evident that pH plays an important factor in anaerobic digestion. An 'inhibited steady state' may arise from the interaction between pH, FA and VFAs, where the anaerobic digestion process is stable but with lower CH_4 yield (Angelidaki and Ahring 1993; Chen et al. 2008). The optimum pH value varies between organisms in the digester. Methanogens and acetogens have an optimum around pH 7, where acidogens prefer a pH value around 6 (Angelidaki et al. 2003). Methanogens grow very slowly at pH lower than 6.6. Most anaerobic digesters run at pH values between 6.0 and 8.5, and values outside this range can cause imbalance in the reactor. Instability in the reactor can lead to accumulation of VFAs, but the pH responds from this accumulation may be delayed due to the buffering capacity of the reactor material. When the pH change is observed the amount of VFAs would be very high, and the process may already have been damaged (Angelidaki et al. 2003). The pH and buffering capacity of a reactor is influenced by several factors. Organic acids and CO_2 lowers the pH, whereas NH_3 will increase the pH. Hydrogen sulphide and phosphates add to the buffering capacity of the reactor material but the main buffer is carbonate (Ahring 2003).

Upgrading biogas

Biogas normally consists of 50-75% CH_4 and 25-50% CO_2 and small amounts of other gasses (Weiland 2010). CH_4 has a high energy content, 35.3 MJ/m^3 (Börjesson and Mattiasson 2008), and the biogas can hence be used for heat, or be converted to electricity and added to the power grid. If the biogas could be upgraded to a higher CH_4 content (>90%), it would be possible to utilize the biogas as vehicle fuel or to add the gas directly into the natural gas grid (Deng and Hägg 2010). Four main chemical and mechanical ways of upgrading the biogas are water washing, chemical absorption, pressure swing adsorption, and membrane separation, where membrane separation is the cheapest (Deng and Hägg 2010). Luo et al. (2012) suggested to biologically upgrade biogas by adding H_2 to the reactor. By adding H_2 to the reactor the hydrogenotrophic methanogens are provided with the electron donor they need to reduce CO_2 to CH_4:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = 130.7\ kJ/mol$$

H_2 also have a high combustion value but CH_4 is at least three times cheaper to store, and hence has the advantage as a fuel. This is due to a higher boiling point and higher volumetric energy of gaseous CH_4 (Luo et al. 2012). As many countries already have a natural gas grid, upgraded biogas would be easy to supply to the consumers.

The in situ biogas upgrading was investigated further after the first results, e.g. by Luo and Angelidaki (2012) who showed successful conversion of CO_2 and H_2 to CH_4 (H_2 added to the reactor), increasing the amount CH_4 in the laboratory reactor. Further investigations were made with the perspective of utilizing H_2 gas from a coke gas oven (Wang et al. 2013) which illustrated in situ biogas upgrading once again. Recently Bassani et al. (2015) showed successful upgrading with a two stage system where H_2 were added to the second reactor.

To make H_2 addition to anaerobic digesters feasible, the H_2 should be made available at reasonable costs. H_2 can be produced by the energy costly electrolysis of water. Wind power is the most used sustainable energy source in Denmark (Energinet Danmark) and due to varying wind conditions and difference in electricity demands during the course of the day and year, excess wind power is exported at small profit or some windmills are not run at full capacity (Energinet Danmark). By using the cheap excess energy to produce H_2, and using the H_2 to convert CO_2 to CH_4, the energy can be utilized in a whole new way (Luo et al. 2012; Luo and Angelidaki 2012). It is essential that the energy for H_2 production comes from a sustainable source to make the whole process environmental friendly.

A concern with adding H_2 to an anaerobic digester is a possible inhibition of the utilization of VFAs, which are a product of protein degradation. The metabolism of acetogenic bacteria can be inhibited by relatively low H_2 pressure, gradual inhibition was reported from 0.1 kPa (Ahring and Westermann 1988), which would result in accumulation of VFAs and subsequently a decreasing pH value. The accumulated VFAs inhibit further degradation of proteins causing a negative upstream effect on the anaerobic digestion system (Angelidaki and Sanders 2004; Raposo et al. 2011). Preliminary studies where H_2 have been added in amounts corresponding to the CO_2 concentration have not shown any indications of VFA accumulation (Luo and Angelidaki 2013; Bassani et al. 2015). Studies where a large amount of H_2 were added together with vigorous stirring, more or less VFA accumulation was observed (Luo et al. 2012; Wang et al. 2013).

As H_2 has a low solubility one of the major problems with addition to a reactor is to make the H_2 available to the methanogens. The low solubility is evident from Henrys constant of H_2: $K_{H_2} = 7.9 \cdot 10^{-9} \frac{mol}{L \cdot Pa}$ (Pauss et al. 1990). Limitation due to gas-liquid mass transfer has been observed previously where it was treated by increasing the stirring (Luo and Angelidaki 2012). As CH_4 from methanogenesis forms bubbles in the reactor, there is a risk that H_2 will mix with these and be removed with the effluent gas if it is not consumed. Therefore it is at the utmost importance that the H_2 gas is added properly and in small bubbles. Different techniques from big scale fermenters already exist, but needs to be adapted for this particular problem. Development of a technique that would be attachable to existing reactors would be ideal.

As CO_2 is removed through methanogenesis when extra H_2 is added to the reactor the buffer capacity of the reactor material will decrease. CO_2 is in equilibrium with HCO_3^- and CO_3^{2-} and contributes to keeping the pH stable. By continuously removing CO_2 from the environment, the alkaline compounds dominate the reactor and thereby increase the pH value. High pH values will increase the amount of NH_3 in the reactor, as the equilibrium between NH_4^+ shifts to the deprotonated form, NH_3. NH_3 is as mentioned in general inhibitory for the anaerobic digestion, where methanogens are effected the most (Kayhanian 1994). It is hence important to add the right amount of H_2 allowing the CO_2 production to keep up with the consumption. Alternatively pH can be controlled by adding acidic or alkaline solutions to the slurry (Wang et al. 2013).

The methanogens in the anaerobic digester are adapted to a low partial pressure of H_2. It is expected that the hydrogenotrophic methanogens will utilize the H_2 as fast as it is provided, thereby keeping the partial pressure low, and preventing any inhibition of upstream processes (Luo et al. 2012). By adding H_2 at a rate that corresponds to the rate of CO_2 produced in the reactor, the pH should be kept stable and not impose any inhibitions. When H_2 is added in the right amount, and when the methanogens are not inhibited otherwise, it is possible to observe a shift in methanogenic community structure. In all previous studies where the community structure were investigated a shift towards hydrogenothrophic methanogens were observed (Luo and Angelidaki 2012; Luo and Angelidaki 2013; Wang et al. 2013; Bassani et al. 2015).

The ElectroGas project:

The ElectroGas project strives to provide a feasible solution to electricity storage. The approach is a biological production of methane (CH_4) from CO_2 in anaerobic digestion by supplying reducing equivalents ex situ or in situ. Reducing equivalents might be provided indirectly by the addition of H_2 gas, produced by electrolysis of water, or directly via cathodic supply of electrons to the microorganisms. ElectroGas use recent advances in fundamental microbial ecology, bioelectric synthesis, ion permeable membranes, gas-liquid mixing and anaerobic digester engineering to overcome microbial limitations and engineering constraints for biogenic energy conversion in operating anaerobic digesters. Two approaches will be tested in the ElectroGas project, firstly electrolysis of water to H_2 and subsequently adding this to anaerobic digesters, secondly microbial electrosynthesis using direct electron transfer to a biofilm on a biocathode. The technology of the first approach is more developed than the second approach. The project spans from basic research of biological questions to development of engineering solutions and cooperates with industrial partners to develop the needed technology.

This study is based on the first approach in the ElectroGas project where biogas is upgraded to a higher CH_4 content by adding H_2 directly to the anaerobic digester. It is previously shown to be possible to get a better CH_4 yield in laboratory scale reactors by applying H_2 in situ (Luo et al. 2012; Luo and Angelidaki 2012; Luo and Angelidaki 2013; Wang et al. 2013; Bassani et al. 2015) but further investigations are necessary. The aim of this study is to evaluate upon the upgrading potential of anaerobic digesters. We strive to develop an assay to estimate the upgrading potential of anaerobic digesters, i.e. how well does slurry from different anaerobic digesters react to H_2 addition, mentioned as the potential activity assay. This is done in small scale laboratory experiments by measuring the CH_4 production over time and comparison of methane production rates (MPRs). Different experimental settings are tested, e.g. different amounts of slurry and variations of the headspace composition is applied. The experimental MPR is then compared to the MPR of the anaerobic digester at the day of sampling. The assay should make it possible to compare upgrading potentials between reactors and the potential of the same reactor over time. Furthermore, the assay should be able to detect adaption of the microbial community, when this is subjected to higher H_2 concentrations over a period, by observing higher MPRs. Adaption of slurry to higher H_2 concentration is attempted and subsequent evaluated with the potential activity assay. Furthermore will this study try to evaluate the methanogenic community visually, which would lead to a better understanding of the community structure.

Materials and methods

Sample origin

The inoculum was sampled from anaerobic digesters at Bånlev Biogas, Viby wastewater treatment plant (WWTP) and Åby WWTP all located near Aarhus, Denmark. Bånlev Biogas is a private business that collects organic waste from farmers and other establishments to use as feed for the reactors. This could be waste from livestock farming, such as swine or poultry manure or straws used for bedding material.

The reactors at Bånlev Biogas functioned just below 40°C, making them mesophilic reactors. Some variations in temperature will occur, e.g. caused by the ambient temperature. For the trials in this report slurry was sampled from reactor 2, which has an active volume of 2900 m^3. The methane percent of the produced biogas was between 62% and 67% at the days of sampling.

Viby WWTP uses secondary (bio-sludge) and primary sludge as reactor feed. Secondary sludge has gone through the mechanical and biological treatment of the waste water treatment plant and is hence lower in organic material and nutrients, whereas primary sludge has not been treated; only the bulky parts have been removed, and therefore contain more energy. The amount of secondary sludge to primary-sludge varies, but an example of added sludge to the reactor from the 2^{th}-3^{th} Dec. 2015 is 8.8 m^3 secondary sludge with a total solids content of 4-6% and 17.4 m^3 primary-sludge with a total solids content of 7-9%. That means that 33.6% of the added sludge that day was secondary/bio-sludge and 66.4% was from the primary source. The reactors at Viby WWTP run around 38°C, making them mesophilic. For the experiments described in this rapport slurry was sampled from reactor 3 that has an active volume of 1600m^3, and a methane percentage that varies between 50% and 60% at the days of sampling.

Åby WWTP feeds the reactor with bio-sludge and has an active volume of 1200 m^3. During the first sampling from Åby WWTP the reactor ran thermophilic (49°C) but shifted to mesophilic (37°C) between then and the last sampling. All places have a hydraulic retention time of around 20 days. The biogas reactor slurry was collected the day of the experiments to ensure as high similarity of activity as compared to the activity in the reactor as possible.

Data about the amount of biogas produced and methane percentage from the anaerobic digester was collected at time of sampling and used as comparison for the results of the laboratory experiments. The gas flow measured at the anaerobic digester has an amount of uncertainty that is unknown to us, and hence may only give an approximation of the gas flow from the reactor.

Potential activity assay

Procedure. Slurry was tapped directly from the digester into a gas tight plastic bag made from 180µm-thick transparent laminated plastic (Amcor, Denmark), which is extremely gas impermeable (Hansen et al. 2000). The plastic bag was flushed on beforehand with Argon to minimize oxygen contamination and after tapping the slurry it was sealed with a clipper. The slurry was transported directly back to the laboratory (transport time: 15-30min). Serum bottles with a volume of 117mL were prepared and auto-claved with media or saline solution for dilution, empty if the slurry was tested undiluted, and with argon as headspace gas.

Figure 3: Slurry in plastic bag, sealed serum bottle with slurry

Slurry was transferred from the plastic bag into serum bottles with a 1 or 10mL syringe with a 10cm glass elongation. Both pure and diluted slurry was tested. The slurry was either diluted in media (Pennings et al. 1998) or in KCl (0.5%) saline water. Different dilution-factors were applied (1:10 and 5:5). A counter flow of N_2 was applied when transferring the slurry to the serum bottles and after transfer the headspace was flushed with N_2 to avoid oxygen contamination. The serum bottles were sealed with a butyl rubber stopper (Ochs laboratories) and capped with an aluminium crimp (Thermo Scientific).

The inoculated serum bottles were flushed with a gas mixture containing H_2/CO_2 in stoichiometric ratio 4:1 (80:20%), and control bottles were flushed with N_2. There were 3-4 replicas for each treatment. During trials with pure slurry a gas mixture of N_2/CO_2 in the ratio 80:20 was applied instead of pure

N_2. The gas ratio was controlled with a flow controller. Either a small overpressure was applied, obtained by removing the outlet needle before the inlet needle, or a large over pressure around 1.5 bar (0.5 bar above atmospheric pressure) was applied. In the experiments with a large overpressure the pressure was checked with a digital multimeter (Millarco, Denmark) with a pressure sensor attached.

0.5mL samples of the headspace were taken with a syringe equipped with a 0.4 mm hypodermic needle and transferred to a 6 mL nitrogen flushed Exetainer (Labro Limited) immediately after flushing to estimate the starting concentration of methane in the samples. The inoculated serum bottles were incubated at the temperature of the anaerobic digester at the time of sampling. The bottles were placed on a Stuart SB3 rotator (Fisher Scientific, UK) at 40rpm to limit any gas-liquid mass transfer limitations. Every 30 min a 0.5mL sample was sampled from the headspace using a syringe with a 0.4mm needle and transferred to a 6 mL nitrogen flushed Exetainer (Labro Limited) and stored for later gas chromatographic measurements.

Progression of the experiment. The first trials were with digester slurry diluted in media in a 1:10 ratio and only a slight overpressure of H_2/CO_2 or N_2 was applied. Slurry from the three different digesters was tested. After the experiments with dilutions using the mineral growth medium, KCl (0.5%) saline water dilutions were used, both 1:10 dilution and dilution series with undiluted, 1:1 and 1:10 dilutions were tested, and only a slight overpressure of H_2/CO_2 or N_2 was applied. This was followed by trials with undiluted slurry under ~1.5bar pressure; this procedure was tested with slurry from all the different digesters. The headspace was flushed with a pressure of ~1.5bar H_2/CO_2 or N_2/CO_2.

Gas Chromatography. The methane content was measured with a gas chromatograph from ©SIR Instruments equipped with a Flame Ionization Detector (FID). The column was a 3’ silica gel column (Sir Instruments Europe GmbH). Data were collected with the program PeakSimple (SIR industries Europe GmbH). CO_2 was measured with a Thermal Conductivity Detector (TCD) using a 3’ silica gel column (Sir Instruments Europe GmbH).

Media. A medium described by Pennings et al. (1998) was applied for dilution of the slurry. The medium contained KH_2PO_4 (6,8g/L), Na_2CO_3 (3,3g/L), NH_4Cl (2,1g/L) and cysteine·HCl·H_2O (0,6g/L) (Sigma-Aldrige). 10mL or the desired amount of medium for dilution was added to a serum bottle, sealed with butyl stopper (Ochs laboratories) and caped with an aluminium crimp (Thermo Scientific).

The medium was autoclaved with headspace of argon. After autoclaving trace element stock solution and Na_2S was added (0.6g/L) and pH was kept at 7.

Saline solution. A saline solution was also used to dilute digester slurry. 0.5 g KCl (Sigma-Aldrige) was diluted in 100mL MilliQ water resulting in a 0.5% KCl saline solution. A volume between 10mL and 5mL was transferred to serum bottles, depending of the desired dilution in the trial, sealed with a butyl stopper (Ochs laboratories) and caped with an aluminium crimp with a top hole (Thermo Scientific) and then autoclaved with argon as headspace gas.

Hydrogen Adaption in small scale reactor. In addition to sampling from large scale anaerobic digesters, samples were also taken from small scale laboratory reactors inoculated with slurry from the large scale digesters at Viby WWTP or Bånlev Biogas. Hydrogen had periodically been added to 3 reactors, corresponding to a 4:1 ratio to the daily produced CO_2 (~ 50ml). Hydrogen was added 3 times before sampling. At the day of sampling, 21-01-2016, 193 (±15) mL H_2 were added to the reactors and at the day of the second sampling, 05-02-2016, 206(±6) mL H_2 were added. The hydrogen was consumed within 5 hours in both cases. In 3 control reactors Argon was added to the headspace instead. The syringe for sampling was flushed with argon prior to sampling and was equipped with a two-way valve that was closed after sampling, allowing the syringe and slurry to be transported while minimizing oxygen contamination. A potential activity assay was conducted as described above: The slurry was diluted 1:10 in media or saline water, and flushed with a slight overpressure of H_2/CO_2 (80:20) or N_2 as control.

Serum bottle scale adaption experiment. A small scale adaption experiment was initiated (day 1) by a potential activity assay where the serum bottles inoculated with 10mL undiluted sludge were flushed with a overpressure of 1.5bar H_2/CO_2 or N_2/CO_2 in the ratio 80:20, 3 replicates of each. After the initial potential activity assay the bottles were flushed in the same way and incubated to the next morning. The next day (day 2) the bottles were flushed and a potential activity assay was performed. The same was conducted day 3. After day 3 pH of all the replicas were measured with a pH sensor connected to a pH-meter (Mettler Toledo International).

CO_2 series. To evaluate the effect of the amount of CO_2 available a potential activity assay was performed with N_2/CO_2 and varying amounts of CO_2 in the headspace. The N_2/CO_2 mixtures were made

beforehand in gas tight bags (Amcor, Denmark) wherefrom the CO_2 concentration was measured with GC-TCD as described previously. The concentrations were 0%, 1%, 12%, 27%, 66% and 100% CO_2, the rest being N_2. A potential activity assay was conducted with 10mL pure slurry from Bånlev Biogas, buffered to pH=7.8 with a potassium phosphate buffer (Sigma-Aldrige), and with the mentioned headspace concentration of N_2/CO_2. One replicate was made for each CO_2 concentration. Two samples of the headspace were taken every 30 minutes, one for methane measurements and one for CO_2 measurements. The potential activity assay with the different CO_2 headspace-concentrations was replicated, but with acetate (Sigma) (resulting concentration 0.02M) added to each replica. By adding acetate it would be certain that the acetotrophic methanogens were not substrate limited. Two samples were taken from the headspace every 30 minutes, one for methane measurements and one for CO_2 measurements. GC measurements were conducted as previously described. After the end experiments the pH value was measured in each replica with a pH-meter (Mettler Toledo International).

Volatile Solids measurements. A protocol from Foulum experimental station was followed (Foulum, 2012). Volatile solids were measured by drying a specific amount of reactor slurry to constant mass in an oven at 105°C for ~24h, and then igniting the dry matter in a muffle-oven at 525°C. Volatile solids were then calculated by $VS\% = \frac{A}{B} * 100$, where *A* is the mass of the ashes residue, *B* is the mass of the wet sample.

Visualization, fluorescent in situ hybridization (FISH)

By using FISH it is possible to visualize archaea in digester slurry. Most archaea in the digester slurry is methanogens. Digester slurry was fixated in a 4% paraformaldehyde-phosphate-buffered solution and kept at 4°C for minimum 1h. The fixed sample was then washed with Phosphate Buffered Saline (1xPBS) and resuspended in PBS-ethanol (1:1) solution, homogenized by ultrasonic treatment (Sonoplus HD2070, Bandelin) (low energy, 3x20s) and stored at -20°C. 100µL of the fixated sample was diluted in 880µL sodium pyrophosphate, preheated to 60°C and shaken in a thermoblock where after 20uL 1% agarose and the sample then immobilized on a gelatine coated glass slide with 10µL 1% agarose. When FISH is performed on a filter the filter is dipped into agarose in a petri dish and placed on a slide. After air drying the slide it was dehydrated in an ethanol series (50-80-100%). Two oligonucleotides probes, ARC15 and EUBmix (Biomers, Germany), were used for hybridization:

Table 1: Probes used for FISH visualization, their sequence, target site and fluorophores applied.

Probe	Label	Sequence	Target site	Target group	ref.
ARC915	Cyt3*	GTGCTCCCCCGCCAATTCCT	16S, 915-934	Domain *Archaea*	[1]
EUBmix:				Domain *Bacteria*	
EUB338	6-Fam**	GCTGCCTCCCGTAGGAGT	16S, 338-355	Domain *Bacteria*	[2]
EUB338-II	6-Fam**	GCAGCCTCCCGTAGGTGT	16S, 338-355	EUB-*Planctomycetes*	[3]
EBU338-III	6-Fam**	GCTGCCTCCCGTAGGTGT	16S, 338-355	EUB-*Verrucomicrobia*	[3]

*Ab_{max}554, Em_{max} 565 **Ab_{max}496, Em_{max} 525
[1](Stahl and Amann 1991), [2](Amann et al. 1990), [3](Daims et al. 1999)

EUBmix, a mixture of EUB338, EUB338-I and EUB338-II all labelled with 6-Fam, target almost all *Bacteria*. ARC915 target *Archaea* and is labelled with Cyt3. A hybridization buffer was prepared as described by Snaidr et al. (1997) but with 35% formamide for all probes. The probes diluted 1:10 in hybridization buffer was added and the slide was incubated for 1.5h at 46°C with the buffer. Following the slide was washed with a preheated 48°C washing buffer containing 20mM Tris-HCl, 70mM NaCl, 5mM EDTA (Ethylenediaminetetraacetic acid) and 0.01%SDS (sodium dodecyl sulphate). The slide was then incubated in the washing buffer for 15min at 48°C after which the slide air dried and one drop of DAPI (4',6-diamidino-2-phenylindole) (1 µg/mL) (Sigma-Aldrige) was added and a cover glass placed on top to visualize all microorganisms. The slide was examined with a fluorescence microscope with an x100 oil objective (AxioVert 200M, Zeiss).

Cell extraction and FISH on filter were also attempted. First the fixed sample was diluted 1:4 in 1xPBS-detergent mix (350µL PBS and 50 µL detergent) and sonicated at low energy for 3x30s (Sono-plus HD2070, Bandelin) after which it was inducted in a Genie 2 Vortex mixer and shaken for 1h. 500µL Nycondenz (Medinor) was added to the bottom of the sample and centrifuged at 6700 for 10min. The supernatant was transferred to 15mL falcon tube with 10mL sterile MiliQ water. The sample was then filtered through a 0.2µm filter. FISH was then performed on the filter as described.

Visualization, F_{420} autofluorescence

As all methanogens contain the autofluorescence coenzyme F_{420} it is possible to visualize these by examining a sample with a fluorescence microscope. The digester slurry was tested both diluted (1:1 in

1xPBS) or undiluted and sonicated at low energy (25%-30%) level for 3x20s and 30s pause in between (Sonoplus HD2070, Bandelin). The samples were then examined using AxioVert 200M fluorescence microscope with FITC filter.

Statistical analysis

The methane production was plotted as a function of time and the methane production rate was calculated from the slope of the linear regression of this. Comparison of the rates of the samples treated with hydrogen and the control samples was achieved by a Students t-test. The variance of the hydrogen treated and control samples were compared with an F-test to clarify if equal variance could be assumed. Statistical programs in Microsoft excel (2010) was used.

Results

Dilution experiments

Slurry from anaerobic digesters was diluted 1:10 with medium, to provide optimal conditions and avoid mass transfer limitations, and set up with a headspace of H_2/CO_2 (80:20) or (pure) N_2. Slurries from reactors at Viby WWTP, Bånlev Biogas, and Åby WWTP were tested.

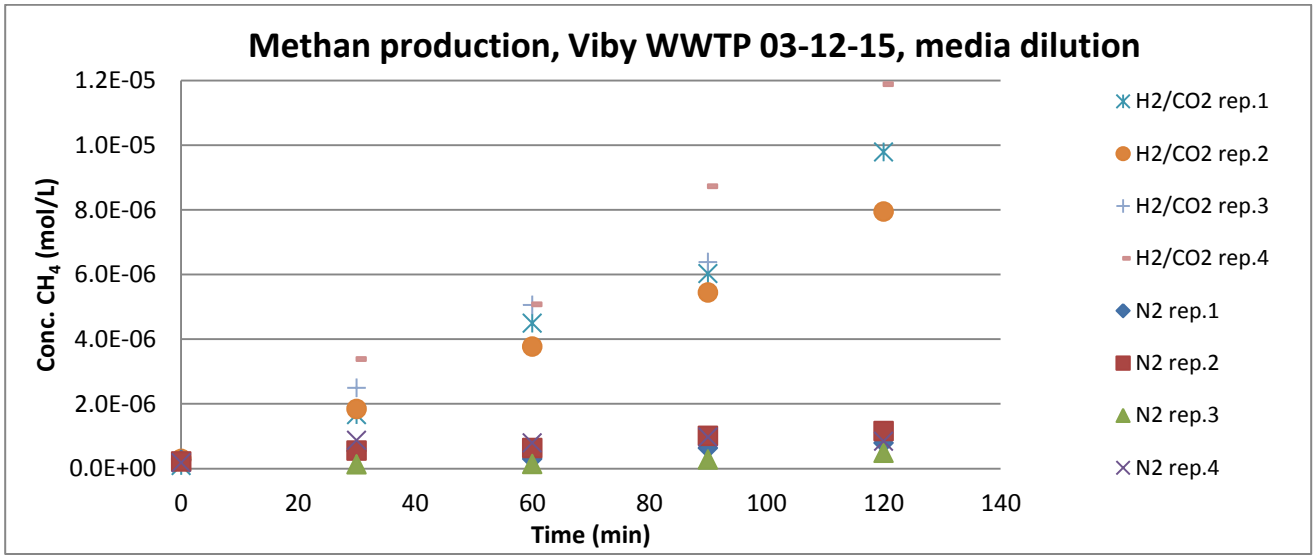

Figure 4: Methane production from reactor slurry from Viby WWTP, diluted 1:10 with media and with a headspace of either H_2/CO_2 in the ratio 80:20 or N_2. Linear regressions, H_2/CO_2 headspace samples: y(rep.1)= 7.92E-08x - 3.40E-07 (R^2=0.978), y(rep.2)= 6.30E-08x + 8.01E-08 (R^2=0.993), y(rep.3)= 7.03E-08x + 3.68E-07 (R^2=0.985), y(rep.4)= 9.76E-08x - 5.92E-08 (R^2=0.990). Linear regression, N_2 headspace samples: y(rep.1)= 3.75E-09x + 2.80E-07 (R^2=0.637), y(rep.2)= 7.76E-09x + 2.50E-07 (R^2=0.969), y(rep.3)= 3.74E-09x - 1.89E-08 (R^2=0.932), y(rep.4)=4.71E-09x+4.49E-07 (R^2=0.504).

The first experiment with media diluted slurry from Viby WWTP is shown in Figure 4. Headspace methane conc. in the diluted slurry from Viby increased in a linear fashion during the 2h experiment and the replicas with H_2/CO_2 headspace exhibited a much faster increase in CH_4 than replicates with a N_2 headspace (Figure 4). The methane production rates (MPRs) were calculated from the slope of the linear regressions for H_2/CO_2 ad N_2 addition, respectively (Table 2), and this applies for all experiments described in this rapport. The average MPR in samples with H_2/CO_2 headspace was 0.319 (±0.0532) $L_{CH_4}/L_{slu.}/day$ while the average MPR in samples with N_2 headspace was 0.0205 (±0.0068) $L_{CH_4}/L_{slu.}/day$ (Table 2), resulting in a significant difference between the two treatments (p=0.0024).

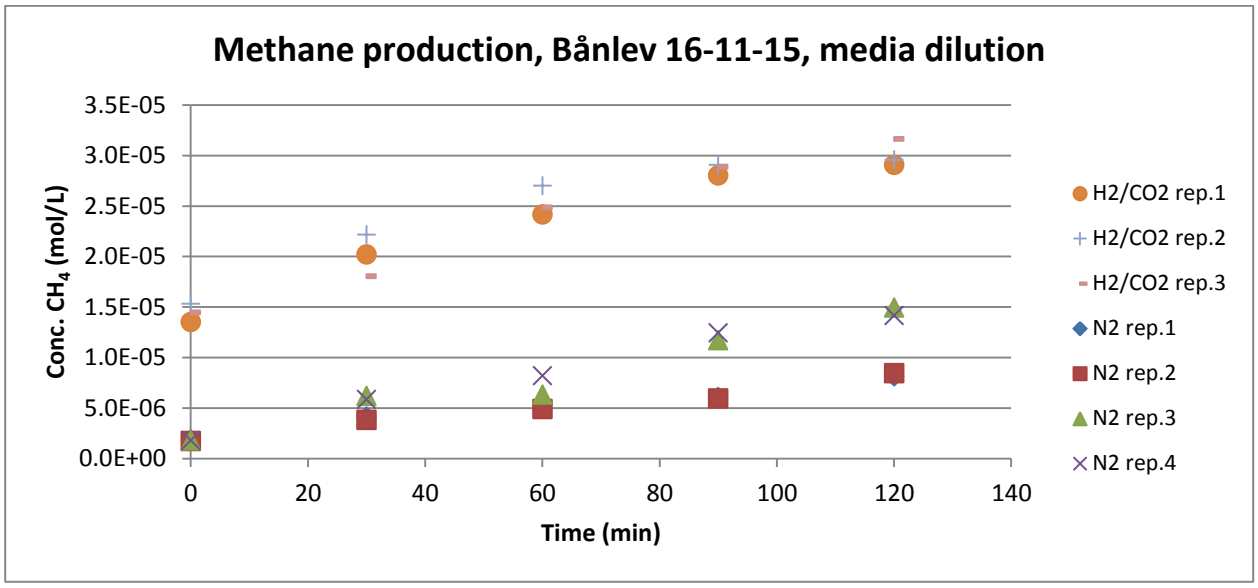

Figure 5: Methane production with slurry from Bånlev Biogas, diluted 1:10 with media and with a headspace of either H_2/CO_2 (80:20) or N_2. Linear regression, H_2/CO_2 headspace samples: y(rep.1)= 1.30E-07x + 1.52E-05 (R^2=0.940), y(rep.2)= 1.18E-07x + 1.75E-05 (R^2=0.879), y(rep.3)= 1.51E-07x + 1.45E-05 (R^2 = 0.980). Linear regression, N_2 headspace samples: y(rep.1)= 4.81E-08x + 2.20E-06 (R^2=0.969), y(rep.2)= 5.18E-08x + 1.85E-06 (R^2=0.973), y(rep.3)= 1.06E-07x + 1.82E-06 (R^2=0.952), y(rep.4)= 1.04E-07x + 2.22E-06 (R^2=0.984).

The data from Bånlev diluted slurry are shown in Figure 5. The headspace concentration of methane in replicates with diluted Bånlev slurry and H_2/CO_2 headspace increased faster than replicates with N_2 headspace, as observed with diluted slurry from Viby. All replicas increased in a linear fashion during the 2 hours, even though the initial methane concentration in the samples with H_2/CO_2 headspace was higher than usual (Figure 5). The Bånlev slurry seemed to be more active than Viby slurry as the average MPR with H_2/CO_2 headspace was 0.547 (±0.055) $L_{CH_4}/L_{slu.}/day$ and the average MPR with N_2 headspace was 0.319 (±0.113) $L_{CH_4}/L_{slu.}/day$ (Table 2). There was a significant difference between the two treatments (p=0.0309) (Figure 7).

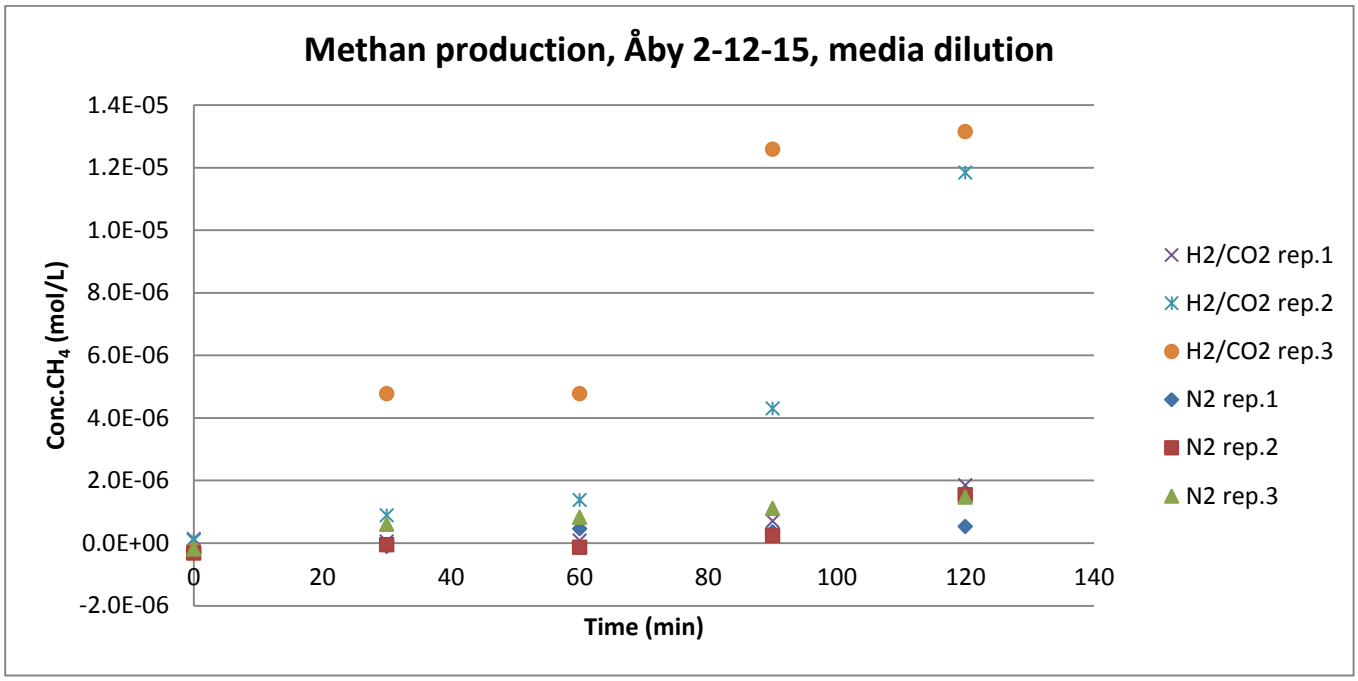

Figure 6: Methane production with slurry from Åby WWTP, diluted 1:10 with media and with a headspace of either H_2/CO_2 in the ratio 80:20 or N_2. Linear regression, H_2/CO_2 headspace samples: y(rep.1)= 1.34E-08x - 5.49E-07 (R^2 = 0.724), y(rep.2)= 8.97E-08x - 1.68E-06 (R^2 = 0.779), y(rep.3)= 1.15E-07x + 9.14E-08 (R^2 = 0.911). Linear regression, N_2 headspace samples: y(rep.1)= 7.22E-09x - 2.53E-07(R^2 = 0.831), y(rep.2)= 1.34E-08x - 5.49E-07 (R^2 = 7.24E-01), y(rep.3)= 1.28E-08x - 3.92E-09 (R^2 = 0.940).

The data from Åby diluted slurry are shown in Figure 6. Methane concentration in slurry from Åby increased with some fluctuations in a linear fashion during the 2h experiment (Figure 6). During the first hour of the experiment H_2/CO_2 rep.1 did not seem to be active, and only produced small amounts of methane in the last hour compared to the other H_2/CO_2 replica. These produced 10x more methane during the 2h. Therefore MPR of H_2/CO_2 rep.1 was excluded when calculating the average MPR. The average MPR for the two replicas with H_2/CO_2 headspace was 0.422 $L_{CH_4}/L_{slu.}/day$ and the average MPR with N_2 headspace was 0.0458 (±0.0114) $L_{CH_4}/L_{slu.}/day$ (Table 2). There were not enough replicas to determine if the two treatments were significantly different (Table 2).

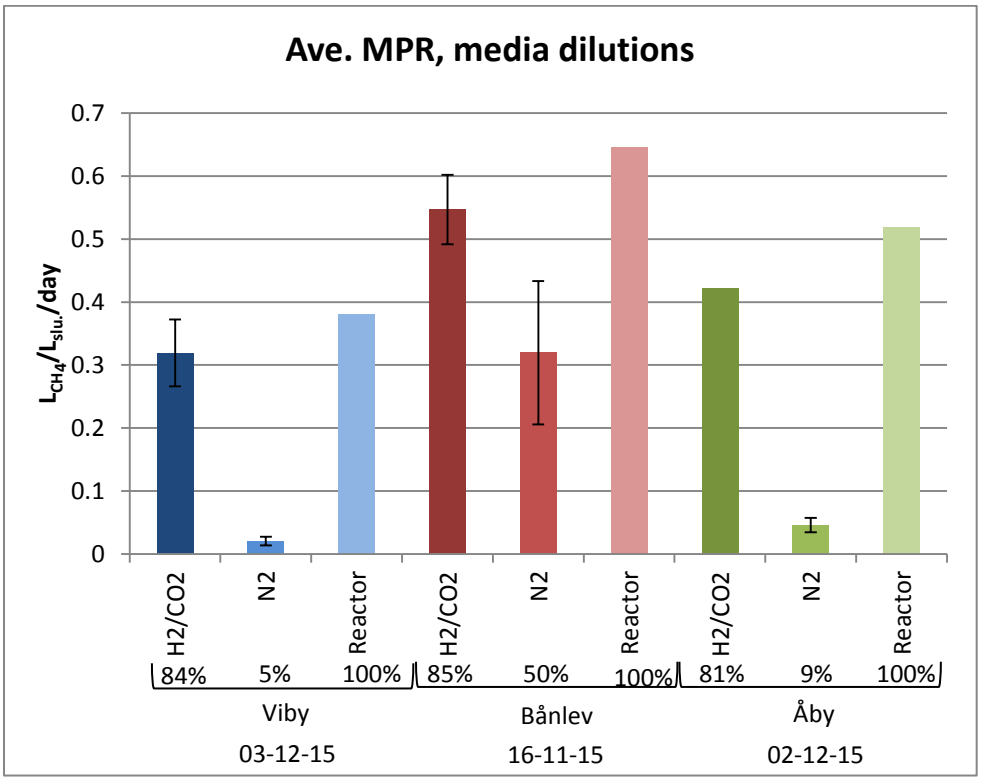

Figure 7: Average MPRs in experiments with medium diluted slurry. In all experiments reactor slurry was diluted 1:10 with media and the headspace was H_2/CO_2 (80:20) or N_2. Reactor production rate was calculated from reactor data collected at site. Experiments were conducted with slurry from anaerobic digesters at Viby WWTP, Bånlev Biogas and Åby WWTP. Ave. MPR in percentage of the reactor MPR is indicated below the columns. There was a significant difference between the H_2/CO_2 and N_2 headspace treatment with slurry from Viby WWTP (p=0.0024) and with slurry from Bånlev (p=0.0309), but too few replica to determine significant in Åby samples, tested with two-tailed Student t-test. There were not enough replicates to conduct statistical analysis of Åby WWTP. Standard deviations are indicated by error bars.

The average MPRs from the media dilution experiments (Table 2) were compared to the MPRs from the biogas reactors at the time and place of sampling. The replicas with N_2 headspace had ave. MPRs ranging from 5% to 50% of reactor MPRs. Ave. MPRs of replica with H_2/CO_2 headspace were less diverse and ranged from 81% to 85% of the reactor MPRs (Figure 7). Overall the two treatments, H_2/CO_2 or N_2 headspace, where significant different in the experiment with diluted Bånlev slurry (p=0.0031) and diluted Viby slurry (p=0.0024). There were too few replicas to determine a statistical difference of Åby WWTP slurry with H_2/CO_2 or N_2 headspace. In all cases ave. MPRs, from samples with both H_2/CO_2 and N_2 headspace, were lower than the reactor MPRs in all cases (Viby, Bånlev, and Åby) (Figure 7).

Table 2: Methane production rates, liter methane produced per liter reactor slurry per day, in replicas with either H_2/CO_2 or N_2 head-space. Slurry was from the anaerobic digester at Viby WWTP, Bånlev Biogas and Åby WWTP in a 1:10 media dilution. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment. *too few replicas for SD.

Methane production rate $L_{CH_4}/L_{slu.}/day$						
Location:	Viby		Bånlev		Åby	
Gas:	H_2/CO_2	N_2	H_2/CO_2	N_2	H_2/CO_2	N_2
Rep.1	0.326	0.016	0.535	0.198	-	0.030
Rep.2	0.259	0.032	0.486	0.213	0.369	0.055
Rep.3	0.290	0.015	0.620	0.436	0.474	0.053
Rep.4	0.402	0.019	-	0.430		
Ave. (±SD)	**0.319 (±0.053)**	**0.0205 (±0.007)**	**0.547 (±0.055)**	**0.319 (±0.114)**	**0.422 ***	**0.046 (±0.011)**
Reactor		0.381		0.645		0.518

As the MPRs from the media diluted slurries were not up to reactor rate, an experiment with 0.5% KCl saline water dilutions was conducted to elucidate the effect of the medium on MPRs. The experiment was performed with slurry from Bånlev Biogas (Figure 5 and Table 2).

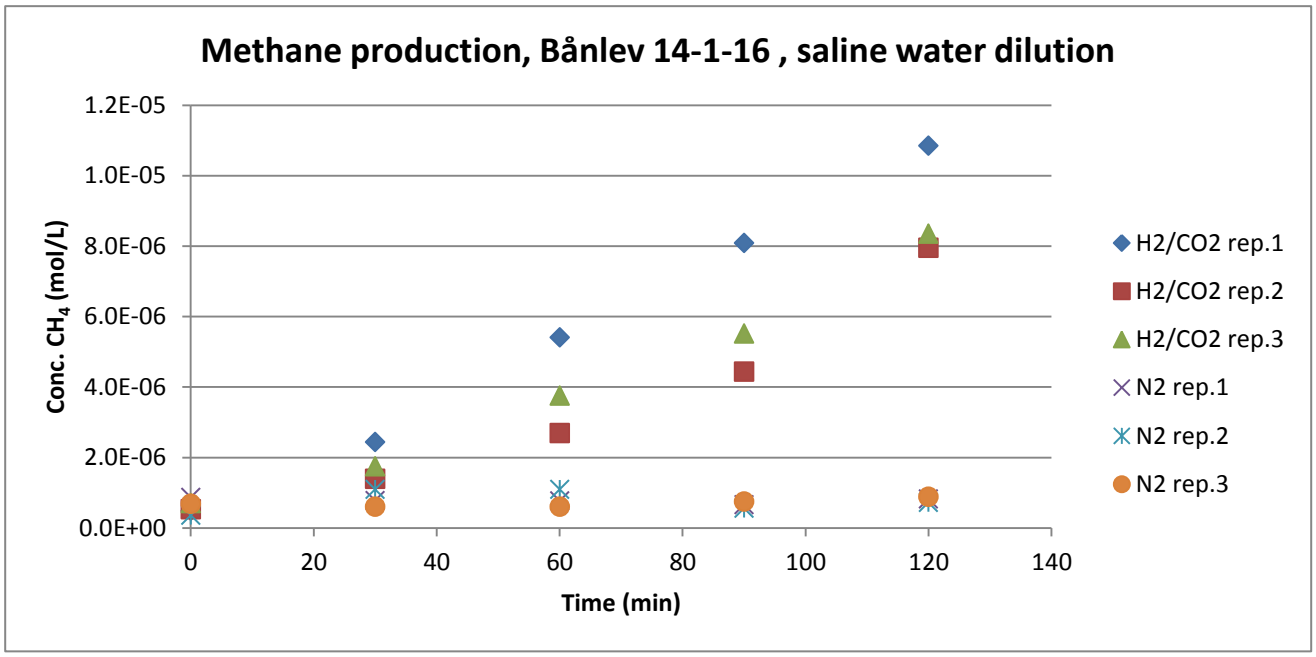

Figure 8: Methane production with slurry from Bånlev biogas, diluted 1:10 with saline water and with a headspace of either H_2/CO_2 in the ratio 80:20 or N_2. Linear regression, H_2/CO_2 headspace samples: y = 8.80E-08x + 1.76E-07 R^2 = 9.96E-01, y = 5.96E-08x - 1.78E-07 R^2 = 9.26E-01, y = 6.36E-08x + 2.00E-07 R^2 = 9.76E-01. Linear regression, N_2 headspace samples: y(rep.1) = -6.65E-10x + 8.18E-07 (R^2 = 0.162), y(rep.2) = 6.39E-10x + 7.27E-07 (R^2 = 0.846), y(rep.3) = 1.77E-09x + 5.99E-07 (R^2 = 0.531).

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2/CO_2	N_2
rep.1	0.362	
rep.2	0.246	0.0026
rep.3	0.262	0.0073
Ave. (±SD)	**0.289 (±0.052)**	**0.0050 -**

Table 3: Methane production rates, liter methane produced per liter reactor slurry per day, in replicas with either H_2/CO_2 or N_2 headspace. The slurry was from the anaerobic digester at Bånlev biogas in a 1:10 KCl (0.5%) saline water dilution. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment. No methane was produced in N_2 rep.1 and was excluded from the calculations.

The slurry diluted in saline water appeared to have lower MPRs than media diluted samples (Table 2 and (Table 3). During the experiment with water dilution, the methane concentrations increased in a linear fashion and, as previous observed, replica with H_2/CO_2 headspace increased faster than N_2 headspace replica. Ave. MPR in the samples with H_2/CO_2 headspace was 0.290 (±0.052) $\boldsymbol{L_{CH_4}/L_{slu.}/day}$ and 0.0050 (±0.0023) $\boldsymbol{L_{CH_4}/L_{slu.}/day}$ in samples with N_2 headspace (Figure 8). Even though the ave. MPRs were lower than the reactor MPR the difference between H_2/CO_2 and N_2 headspace was significant (p=0.009) (Figure 9).

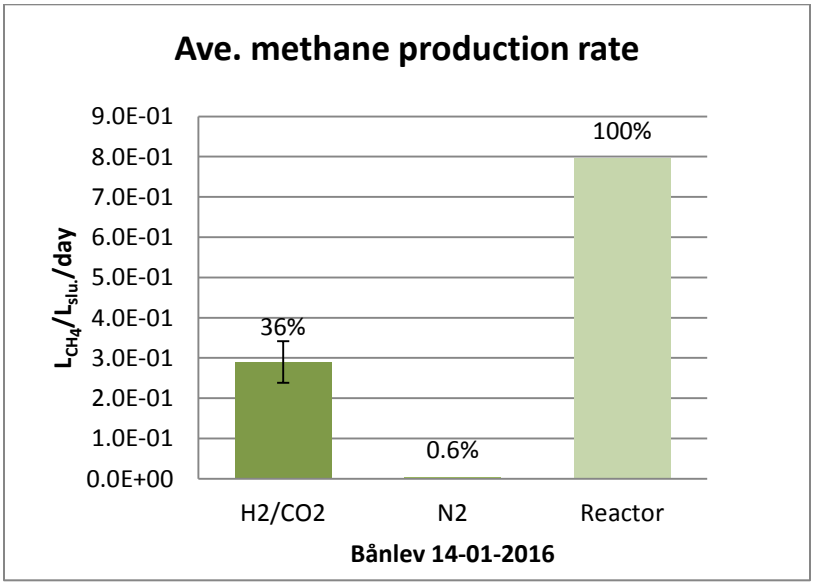

Figure 9: Methane production rate in liter methane produced per liter reactor slurry per day. In all experiments reactor slurry was diluted 1:10 with water and the headspace was H_2/CO_2 in ratio 80:20 or N_2. The reactor production rate was calculated from reactor data received from reactor monitoring programs. Experiments were conducted with slurry from anaerobic digesters at Bånlev Biogas. Ave. MPR in percentage of the reactor rate in indicated above the columns. There was a significant difference between H_2/CO_2 and N_2 headspace (p=0.0091) tested with a two-tailed Students t-test. Standard deviation is indicated by error bars.

As the MPRs did not match the reactor MPRs in experiments with diluted slurry, a dilution series were made to test the effect of the reactor slurry concentration on MPR. A comparison was conducted of

10mL undiluted slurry and saline water dilutions of slurry in 1:1 (5mL) and 1:10 dilution (1mL), all with H_2/CO_2 headspace (80:20). To test the methane production without H_2addition a 1:1 dilution was tested with N_2 headspace (Figure 10).

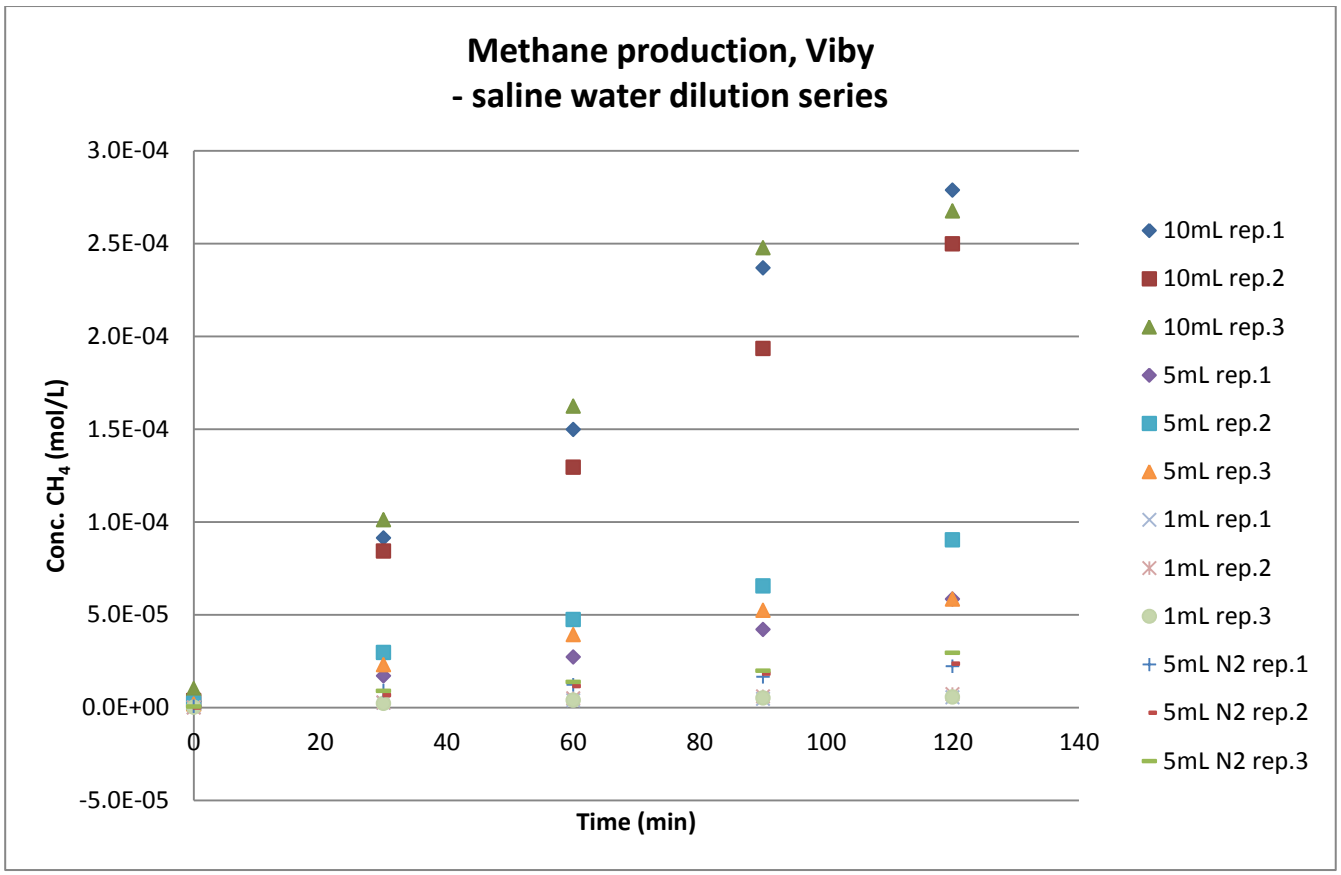

Figure 10: Methane production with slurry from Viby WWTP, 1:1 and 1:10 dilution in saline water or undiluted with headspace of either H_2/CO_2 (80:20) or N_2. Linear regression 10mL, H_2/CO_2 headspace samples: y(rep1) = 2.32E-06x + 1.26E-05 (R^2 = 0.989), y(rep.2 = 2.01E-06x + 1.18E-05 (R^2 = 0.994), y(rep.3)= 2.21E-06x + 2.54E-05 (R^2 =0.969). Linear regression 5mL H_2/CO_2 headspace samples: y(rep.1) = 4.68E-07x + 9.87E-07 (R^2 =0.995), y(rep.2) = 7.08E-07x + 4.49E-06 (R^2 =0.994), y(rep.3)= 4.74E-07x + 6.43E-06 (R^2=0.962). Linear regression 1mL H_2/CO_2: y(rep.1) = 4.33E-08x + 6.35E-07 (R^2 = 0.9409), y(rep.2)= 5.87E-08x + 5.92E-07 (R^2 = 0.955), y(rep.3)= 4.68E-08x + 4.75E-07 (R^2 = 0.949). Linear regression 5mL N_2 headspace samples: y(rep.1)= 1.96E-07x + 1.56E-07 (R^2 =0.998), y(rep.2) = 2.30E-07x + 6.07E-07 (R^2 = 0.987), y(rep.3)= 1.69E-07x + 1.92E-06 (R^2 =0.977).

In all samples methane concentration increased in a linear fashion during the 2h experiment. The highest methane concentration was obtained in the sample with undiluted slurry and a headspace of H_2/CO_2 (Figure 10). Ave. MPRs from the different dilutions are indicated below (Table 4 and Figure 11).

Worth noticing is the MPR in 10mL undiluted slurry (H_2/CO_2) which was 235% of the reactor MPR. In addition, ave. MPR of 5mL (1:1, H_2/CO_2) also exceeded reactor MPR. Ave. MPRs of 1mL (1:10,

H_2/CO_2) and 5mL (1:1, N_2) were both lower than reactor MPR. There was a significant difference between all samples with H_2/CO_2 headspace and between 5mL (H_2/CO_2) and 5mL (N_2) (Figure 11).

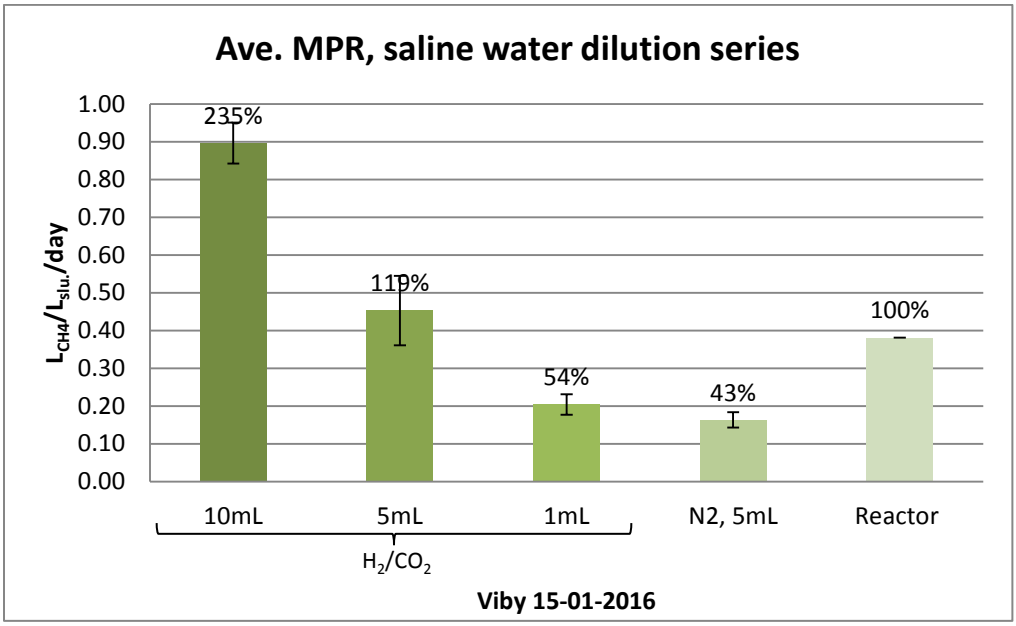

Figure 11: Methane production rate in liter methane produced per liter reactor slurry per day**.** 5mL 1:1 dilution was tested with N_2 and H_2/CO_2 headspace, 10mL undiluted and 1mL 1:10 dilution was tested with H_2/CO2 headspace. The reactor production rate was calculated from reactor data received from monitoring programs at site. Slurry was collected from Viby WWTP. Ave. MPR in percentage of the reactor rate in indicated above the column. Significant difference between 10mL and 5mL (p=0.0041), 10mL and 1mL (p=0.000084), 5mL and 1mL (p=0.021) and 5mL and N_2 5mL (p=0.0498), tested with Student t-test. Standard deviations are indicated by error bars.

Methane production rate $L_{CH_4}/L_{slu.}/day$				
	10mL (H_2/CO_2)	5mL (1:1) (H_2/CO_2)	1mL (1:10) (H_2/CO_2)	5mL (N_2)
rep.1	0.956	0.385	0.179	0.139
rep.2	0.826	0.583	0.242	0.161
rep.3	0.908	0.391	0.193	0.189
Ave. (±SD)	**0.897 (±0.054)**	**0.452 (±0.091)**	**0.204 (±0.027)**	**0.163 (±0.021)**

Table 4: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2 headspace. The slurry collected from the anaerobic digester at Viby WWTP was tested both undiluted and diluted in a 1:10 ratio and 1:1 ratio saline water solution. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

As the results in the experiment described above indicate a higher rate with undiluted slurry, a new trial was performed with the aim of making the difference between undiluted (10mL) and diluted (1:10 = 1mL) slurry more evident (Figure 12).

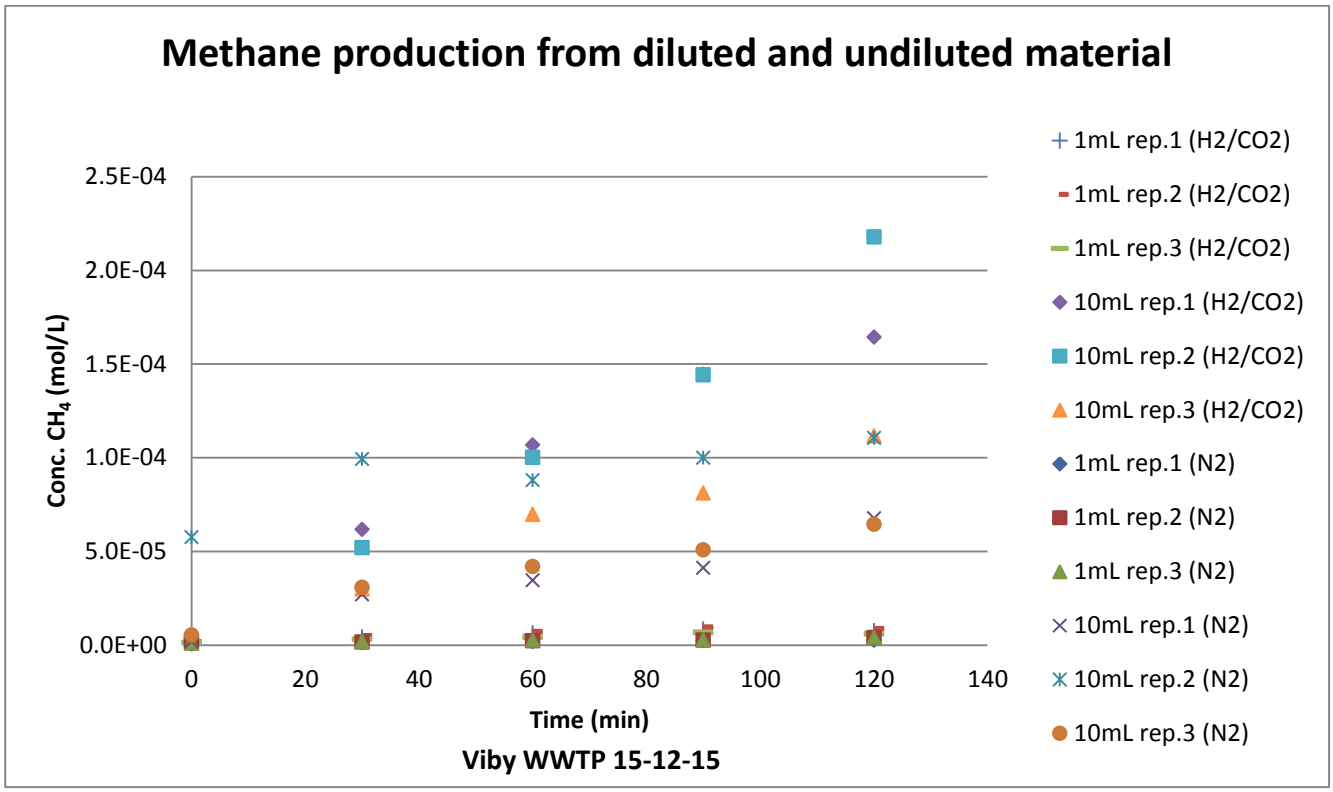

Figure 12: Methane production with slurry from Viby WWTP, 1:10 dilution in saline water or undiluted and with a headspace of either H_2/CO_2 in the ratio 80:20 or N_2. Linear regression 10mL, H_2/CO_2 headspace samples: y(rep.1) = 1.35E-06x + 1.49E-05 (R^2= 0.969), y(rep.2) = 1.74E-06x - 1.29E-06 (R^2= 0.991), y(rep.3) = 8.99E-07x + 4.96E-06 (R^2= 0.980). Linear regression 10mL, N_2 headspace samples: y(rep.1)= 4.86E-07x + 5.32E-06 (R^2= 0.938), y(rep.2) = 3.56E-07x + 6.97E-05 (R^2= 0.689), y(rep.3)= 4.61E-07x + 1.10E-05 (R^2 = 0.958). Linear regression 1mL, H_2/CO_2 headspace samples: y(rep.1)= 4.95E-08x + 1.94E-06 (R^2= 0.868), y(rep.2)= 6.12E-08x + 2.61E-06 (R^2= 0.864), y(rep.3)= 4.48E-08x + 1.58E-06 (R^2= 0.882). Linear regression 1mL, N_2 headspace samples: y(rep.1)= 1.33E-08x + 1.11E-06 (R^2= 0.982), y(rep.2)= 2.19E-08x + 9.92E-07 (R^2= 0.940), y(rep.3)= 2.24E-08x + 1.13E-06 (R^2= 0955).

The undiluted sample with H_2/CO_2 headspace exhibited the highest activity, but the difference between the undiluted and 1:10 diluted sample was not significant (p=0.082). Nevertheless, the methane concentration did increase in a linear fashion with some variations during the 2h trial (Figure 12).

It was only with diluted slurry that the difference between H_2/CO_2 and N_2 headspace was significant (p=0.0069) as the ave. MPRs were 0.2133 (±0.0284) $L_{CH_4}/L_{slu.}/day$ and 0.0356 (±0.0401) $L_{CH_4}/L_{slu.}/day$ respectively (Table 5, Figure 13). It was not possible to determine a significant difference between the two headspace treatment with 10mL undiluted slurry (p=0.0682) where the ave. MPR was 0.5482 (±0.1420) $L_{CH_4}/L_{slu.}/day$ with H_2/CO_2 headspace and 0.1788 (±0.0232) $L_{CH_4}/L_{slu.}/day$ with N_2 headspace.

The dilution series showed promising results for the potential activity assay undiluted slurry, even though the control samples with N_2 headspace were still slower than reactor MPR.

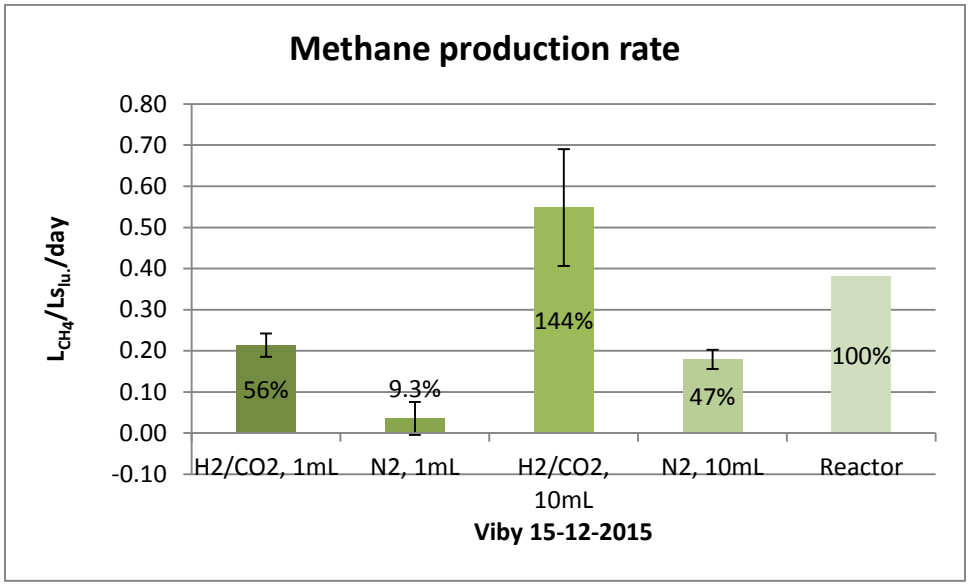

Figure 13: Methane production rate in liter methane per liter slurry per day, displaying the average rates from diluted slurry in the ratio 1:10 and undiluted slurry with H_2/CO_2 or N_2 headspace. Slurry was collected from Viby WWTP. The reactor rate was calculated with data from reactor monitoring programs at site. Ave. MPR in percentage of the reactor rate is indicated in the columns. There was a significant difference between 1mL (H_2/CO_2) and 1mL (N_2) (p=0.0069), but not between 10mL (H_2/CO_2) and 10mL (N_2) or between 1mL (H_2/CO_2) and 10mL (H_2/CO_2). Significance was tested with Student t-test.

Methane production rates $L_{CH_4}/L_{slu.}/day$				
	1mL (1:10) (H_2/CO_2)	1mL(1:10) (N_2)	10mL (N_2)	10mL (H_2/CO_2)
Rep.1	0.204	0.006	0.200	0.557
Rep.2	0.252	0.009	0.147	0.718
Rep.3	0.184	0.092	0.190	0.370
Ave. (±SD)	**0.213 (±0.028)**	**0.036 (±0.040)**	**0.179 (±0.023)**	**0.548 (±0.142)**

Table 5: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2 headspace**.** The slurry was from the anaerobic digester at Bånlev biogas and tested undiluted or in a 1:10 saline water dilution. The rates were calculated from the slope of the linear regression of the methane production over time**.**

Undiluted slurry and 1.5bar H_2/CO_2 or N_2/CO_2

The dilution series lead to investigations of 10mL undiluted slurry, this time from Bånlev biogas, and with headspaces of either H_2/CO_2 or N_2/CO_2 in the control, instead of pure N_2, both in the ratios 80:20. CO_2 was applied in the control to keep the pH stable. The pressure was raised to 1.5 bar to ensure that enough H_2 and CO_2 is present, and that no vacuum would evolve. The increased pressure also insures a better mass transfer as more H_2 is able to dilute (Henrys law).

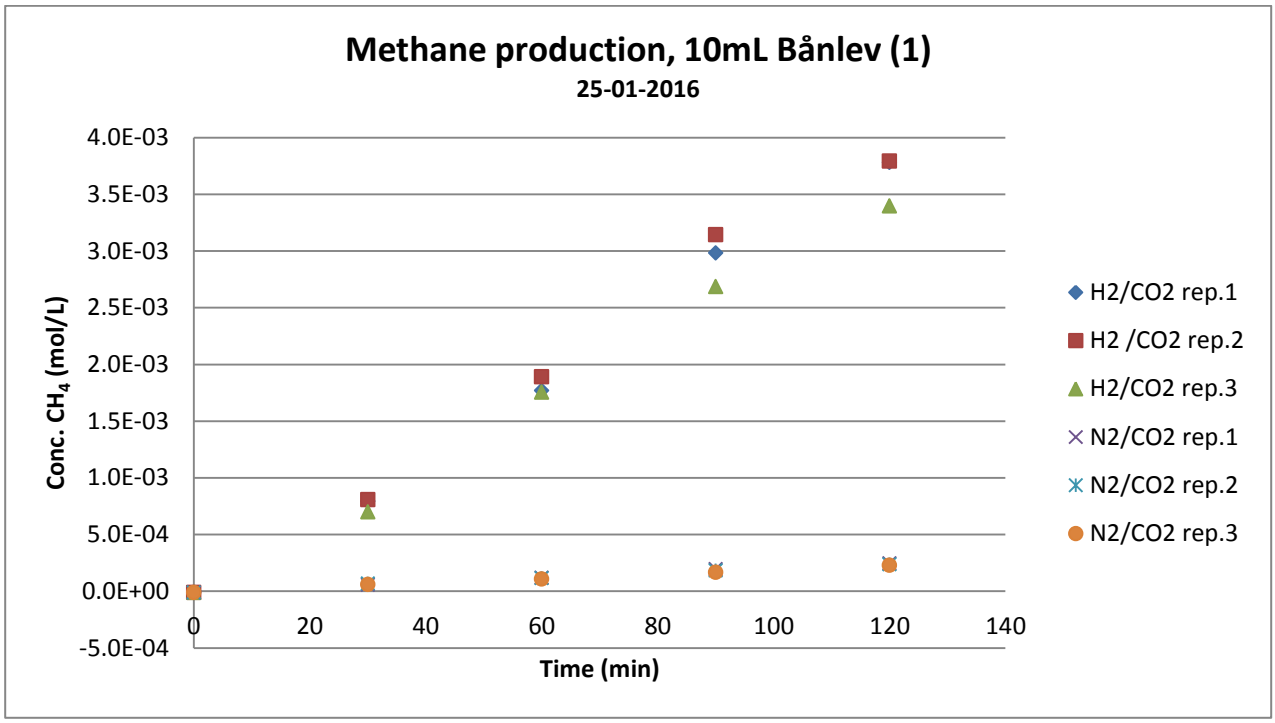

Figure 14: Methane production with slurry from Viby WWTP, 10mL undiluted slurry with a headspace of either H_2/CO_2 in the ratio 80:20 or N_2/CO_2. Linear regression 10mL, H_2/CO2 headspace samples: y(rep.1)= 3.26E-05x - 9.22E-05 (R^2= 0.995), y(rep.2)= 3.32E-05x - 6.59E-05 (R^2 = 0.992), y(rep.3) = 2.93E-05x - 5.56E-05 (R^2 = 0.996). Linear regression 10mL, N_2/CO_2 headspace samples: y(rep.1)= 2.18E-06x - 1.00E-05 (R^2 = 0.997), y(rep.2)= 2.04E-06x - 4.43E-06 (R^2 =0.995), y(rep.3)= 1.96E-06x - 7.72E-06 (R^2 =0.997).

The highest concentrations and fastest MPRs were achieved with 10mL undiluted slurry from Bånlev with 1.5 bar pressure (Figure 14). The methane concentration increased in a linear fashion during the 2h (Figure 14). With H_2/CO_2 headspace the ave. MPR was 13.05 $L_{CH_4}/L_{slu.}/day$ and with N_2/CO_2 headspace the ave. MPR was 0.8470 $L_{CH_4}/L_{slu.}/day$ which resembled reactor MPR (Table 6, Figure 15). There was a clear significant difference between H_2/CO_2 and N_2/CO_2 headspace (p=0.000015).

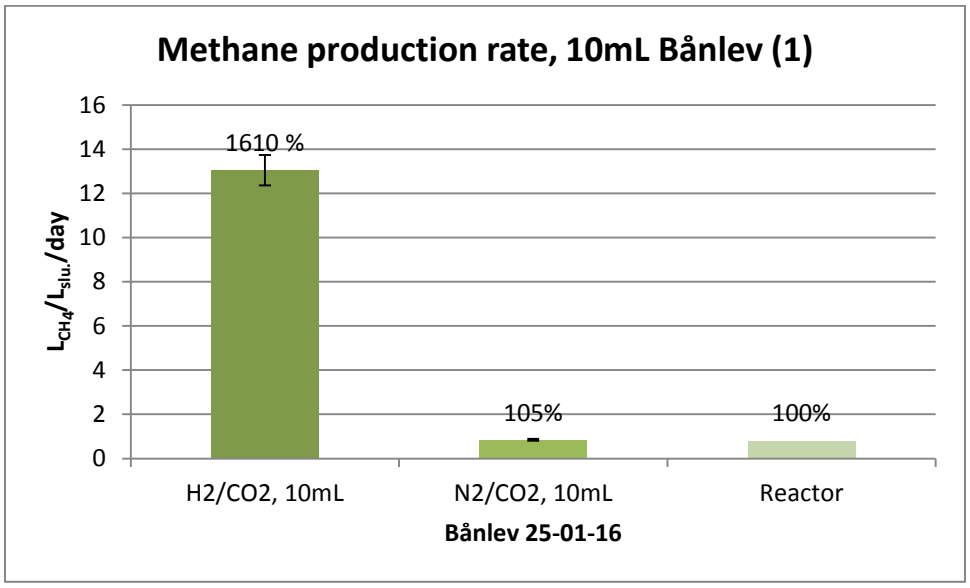

Figure 15: Methane production rate in liter methane produced per liter slurry per day**.** 10mL slurry from the anaerobic digester at Bånlev biogas was used with headspace of H_2/CO_2 or N_2/CO_2 and at 1.5 bar pressure. The reactor methane production rate at sampling time is shown. Ave. MPR in percentage of the reactor rate is indicated above the columns. There was a significant difference between the two treatments (p= 0.000015) tested with a Student t–test. SD is indicated by error bars.

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	10 mL H_2/CO_2	10mL N_2/CO_2
Rep.1	13.408	0.897
Rep.2	13.649	0.839
Rep.3	12.081	0.806
Ave. (±SD)	**13.046 (±0.689)**	**0.847 (±0.038)**
Reactor 25-01-16		0.810

Table 6: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2 headspace. The slurry was from Bånlev Biogas. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

Figure 16 summarizes some of the methane production rates of the control samples with N_2 or N_2/CO_2 headspace. The only control reaching reactor rate was from the 25-01-2016 using 10mL undiluted slurry from Bånlev biogas and 1.5 bar N_2/CO_2 headspace, which inspired further investigations.

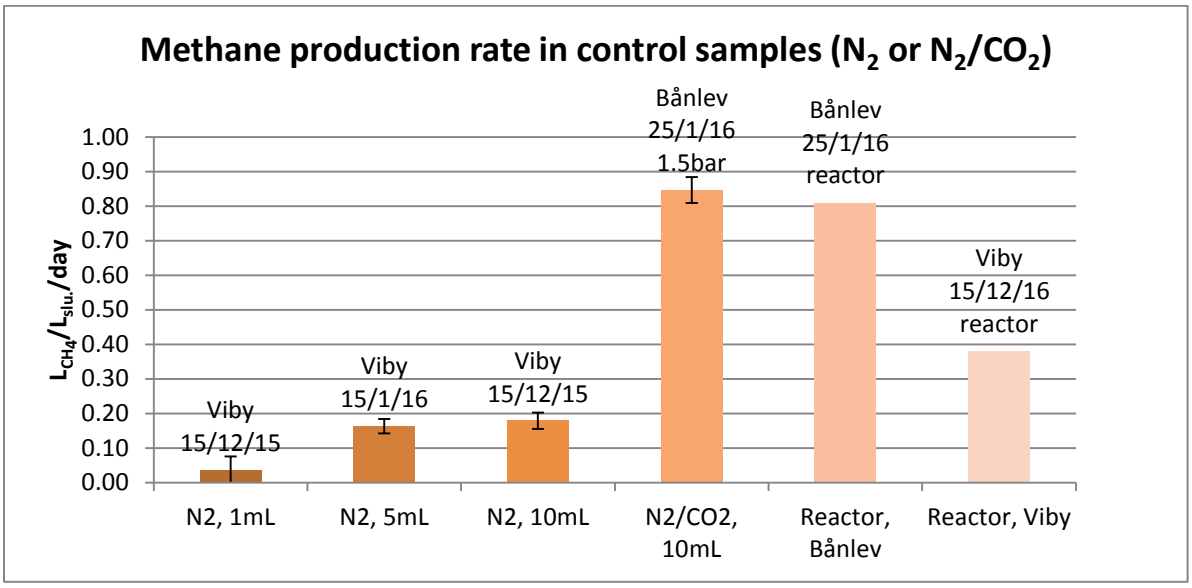

Figure 16: Methane production rate in liter methane per liter slurry per day, displaying the average rates of control samples. Slurry from Viby WWTP, 1mL (1:10 dilution) and 5mL (1:1 dilution), and undiluted (15/12/15) with N_2 headspace is shown. Undiluted slurry, 10mL, from Bånlev Biogas with N_2/CO_2 headspace is also shown. The reactor rates were calculated with data from reactor monitoring programs at site. Standard deviations are indicated by error bars.

With the aim of reproducing the results the experiment with 10mL undiluted slurry form Bånlev and H_2/CO_2 or N_2/CO_2 headspace at ~1.5bar pressure was repeated.

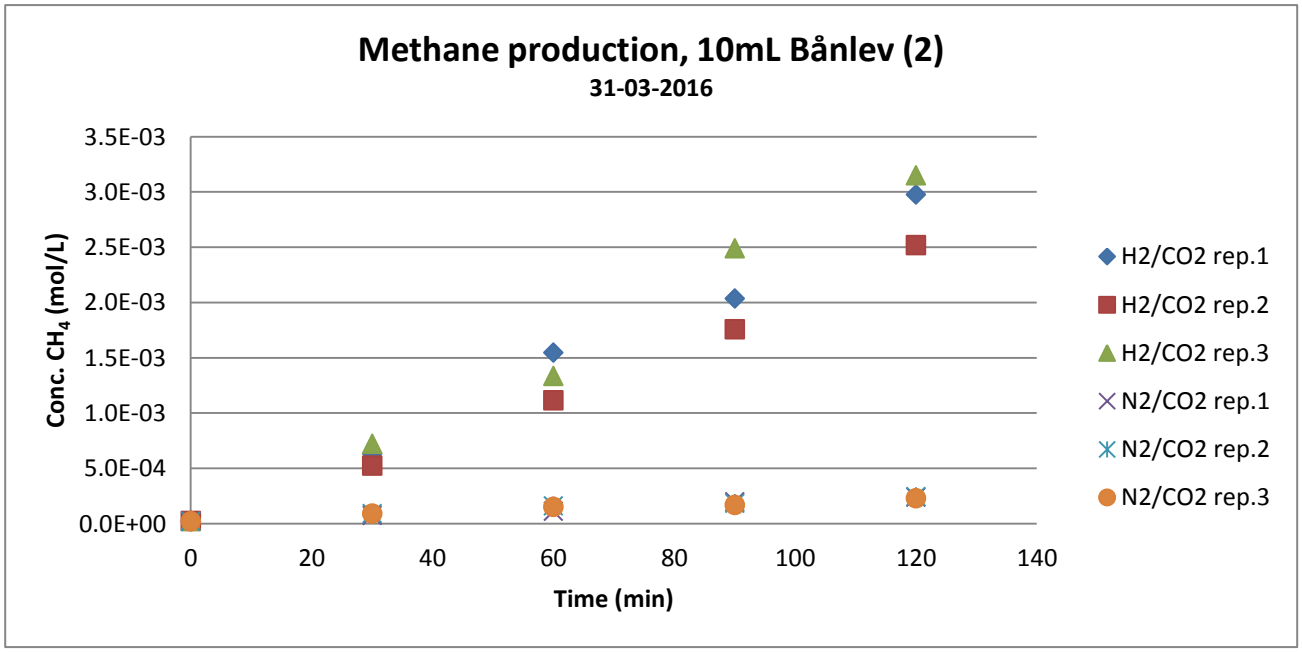

Figure 17: Methane production with slurry from Bånlev Biogas, 10mL undiluted slurry with a headspace of either H_2/CO_2 or N_2/CO_2 in the ratio 80:20. Linear regressions, H_2/CO_2 headspace samples: y(rep.1)= 2.44E-05x - 2.05E-05 (R^2 = 0.992), y(rep.2)=

2.08E-05x - 5.79E-05 (R^2 = 0.994), y(rep.3)= 2.67E-05x - 5.97E-05 (R^2 =0.989). Linear regression; N_2/CO_2 headspace samples: y(rep.1)= 1.87E-06x + 1.64E-05 (R^2 =0.984), y(rep.2)= 1.78E-06x + 3.35E-05 (R^2 =0.977), y(rep.3)= 1.65E-06x + 3.29E-05 (R^2 =0.971).

High MPRs were once again achieved with 10mL undiluted slurry from Bånlev. The methane concentration increased in a linear fashion during the 2h trial with a faster increase in H_2/CO_2 headspace samples (Figure 17). The ave. MPR in samples with H_2/CO_2 headspace was 9.863 (±1.010) $L_{CH_4}/L_{slu.}/day$ equivalents to 1035% reactor MPR. Even though the ave. MPR with N_2/CO_2 headspace was slower than in the previous experiment (Figure 15) the ave. MPR was 76% of the reactor MPR, 0.7259 (±0.0376) $L_{CH_4}/L_{slu.}/day$ (Table 8, Figure 18). The two treatments in this experiment were aging significantly different.

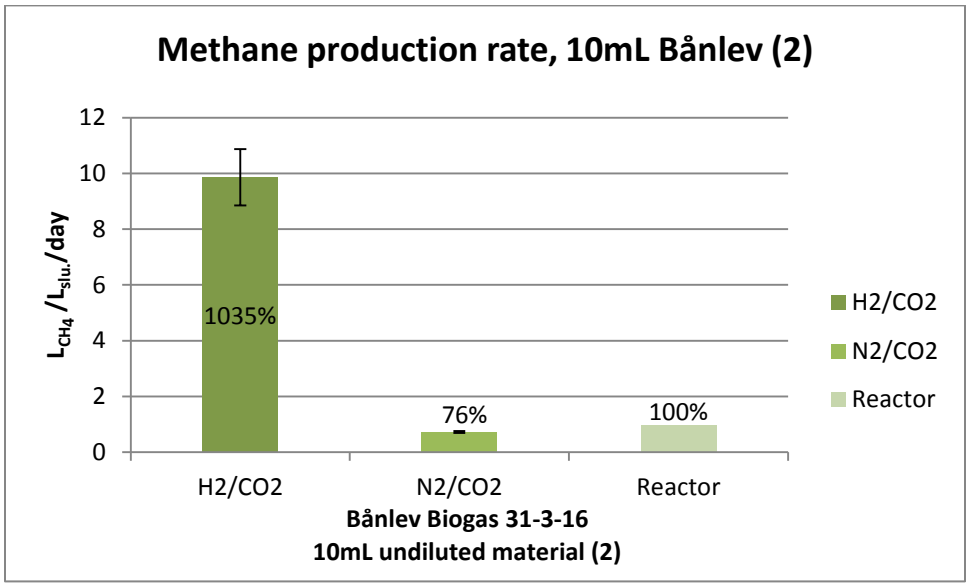

Figure 18: Methane production rate in liter methane produced per liter slurry per day. 10mL slurry from the anaerobic digester at Bånlev biogas was used with headspace of H_2/CO_2 or N_2/CO_2 at 1.5bar pressure. The methane production rate of the reactor at sampling time is shown. Ave. MPR in percentage of the reactor rate is indicated above the column. There was a significant difference between the two treatments (p= 0.0061) tested with a Student t–test. Standard deviation is indicated by error bars.

Table 7: Percentage of CO_2 in the gas mixture used to flush the serum bottles and the percentage of CO_2 in samples at the beginning and at the end of the experiment. The CO_2% in red are starting concentrations that are lower than end concentrations.

	CO2%			
	H_2/CO_2		N_2/CO_2	
Time(min):	0	120	0	120
Rep.1	5.3	9.26	2.99	6.9
Rep.2	4.93	7.67	3.3	17.98
Rep.3	12.94	11.5	4.87	15.66

CO_2 conc. was measured to control and monitor the concentration during the experiment. The start and end concentrations appeared to be lower than expected (Table 9). In all replicas, except for rep.3 (H_2/CO_2), the concentration was higher at the end (120min) and all had very low starting concentrations. This could indicate diffusion of CO_2 into the headspace, or that the Exetainer containing the sample were leaky. None of the CO_2 concentrations reached the 20% that was aimed for during flushing.

The pH value was measured at the end of the trial. All pH values increased during the experiment, but the largest increase was when treated with H_2 (Table 8).

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2/CO_2	N_2/CO_2
Rep.1	10.04	0.760
Rep.2	8.55	0.732
Rep.3	11.00	0.677
Ave. (±SD)	**9.863 (±1.010)**	**0.726 (±0.038)**
Reactor		0.953

Table 8: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2 headspace. The slurry was from Bånlev Biogas the 31-03-16. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

Table 9: pH values after end experiment in all replicas and in the reactor.

Gas:	H_2/CO_2			N_2/CO_2			Reactor
Rep.	1	2	3	1	2	3	
pH	8.72	8.66	8.70	8.68	8.41	8.40	7.89

The method used when achieving the results with Bånlev slurry needed to be tested on slurries from other digesters to evaluate if it was valid in general. Therefore, the same experiment was performed with slurries from Viby and Åby WWTP. In these cases the results were less clear compared to the results from Bånlev.

In the first attempt using slurry from Viby WWTP, only one of the replicas (H_2/CO_2 rep.1) had a linear increase in methane concentration during the 2h trial (Figure 19). The other replicas decreased in rate after the first 30min. It was not possible to distinguish samples with H_2/CO_2 headspace from those with N_2/CO_2 headspace (Figure 19).

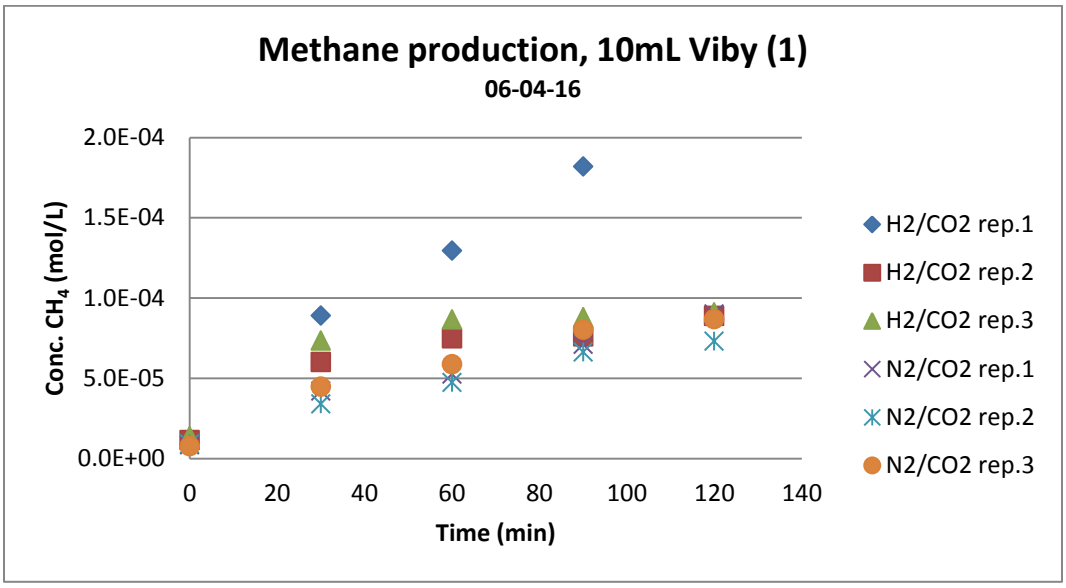

Figure 19: Methane production with slurry from Viby WWTP, 10mL undiluted slurry with a headspace of either H_2/CO_2 or N_2/CO_2 in the ratio 80:20.

Due to the unclear result from the first experiment the experiment with Viby slurry was repeated (Figure 20). Extra care was taken to minimize the risk of oxygen contamination.

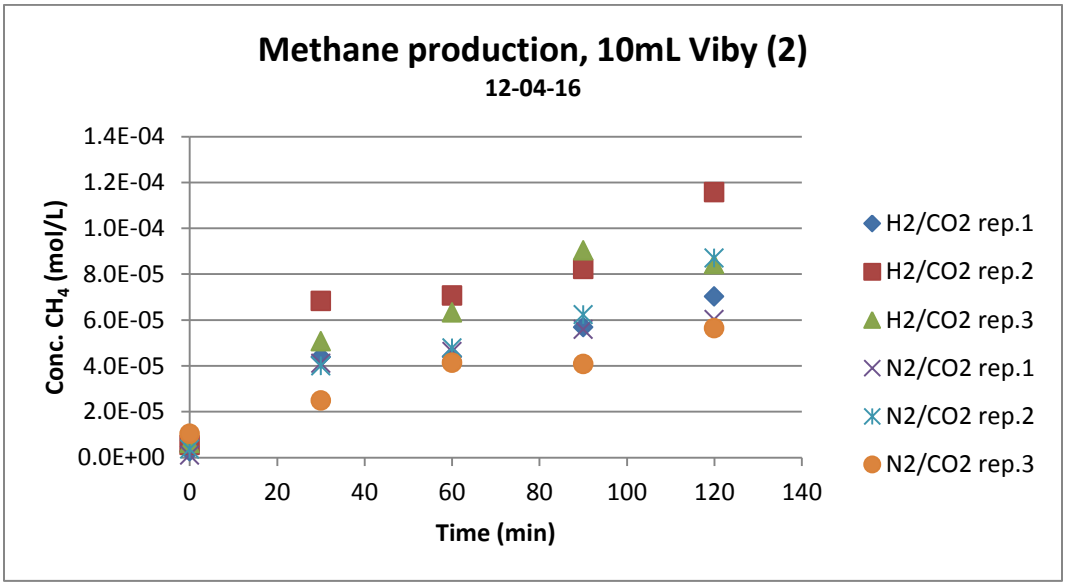

Figure 20: Methane production with slurry from Viby WWTP, 10mL undiluted slurry with a headspace of either H_2/CO_2 or N_2/CO_2 in the ratio 80:20. Linear regressions, H_2/CO_2 headspace samples: y(rep.1)= 4.95E-07x + 1.38E-05 (R^2=0.857), y(rep.2)= 7.82E-07x + 2.16E-05 (R^2 =0.860), y(rep.3)= 6.52E-07x + 1.98E-05 (R^2=0.853). Linear regression, N_2/CO_2 headspace samples: y(rep.1)= 4.45E-07x + 1.41E-05 (R^2 =0.801), y(rep.2)= 6.31E-07x + 1.03E-05 R^2 = 9.51E-01, y(rep.3)= 3.61E-07x + 1.31E-05 (R^2 = 0.939).

In this experiment the methane concentration increased in an approximately linear fashion during the 2h (Figure 20). MPRs as seen with Bånlev slurry were not observed here. With Viby slurry, the ave. MPR with H_2/CO_2 headspace was only 0.265 (±0.062) $L_{CH_4}/L_{slu.}/day$ and the rate with N_2/CO_2 headspace was 0.197 (±0.059) $L_{CH_4}/L_{slu.}/day$, which is 90% and 67% of the reactor MPR, respectively. The difference between ave. MPR with H_2/CO_2 and with N_2/CO_2 headspace were not significantly different (p=0.228) (Figure 21).

Table 10: Percentage of CO_2 in the gas mixture used to flush the serum bottles and the percentage of CO_2 in samples at the beginning and at the end of the experiment, Viby 10mL. The pH value was measured in all replicas at the end of the experiment.

	H_2/CO_2	**N_2/CO_2**
	CO_2 %	
Start	8.10	23.05
0min	10.80	24.75
120min	11.47	24.89
	pH	
Rep.1	8.25	7.58
Rep.2	8.30	7.59
Rep.3	8.28	7.57
Reactor pH:		7.53

This time CO_2 conc. was both measured at the start and end of the experiment, but also in the gas used to flush the bottles. Percentage of CO_2 was 23% in the N_2/CO_2 gas-mixture and 8% in the H_2/CO_2 gas-mixture (Table 10). The CO_2% were approximately the same at the beginning and at the end of the experiment, 11 % in the H_2/CO_2 headspace sample (rep.1) and 25% in the N_2/CO_2 headspace sample (rep.1). The N_2/CO_2 headspace exceeded the 20% aimed for where the concentrations in the H_2/CO_2 headspace were still lower than aimed for. In all samples pH increased compared to reactor pH, but the largest increase was observed in sample with H_2/CO_2 headspace.

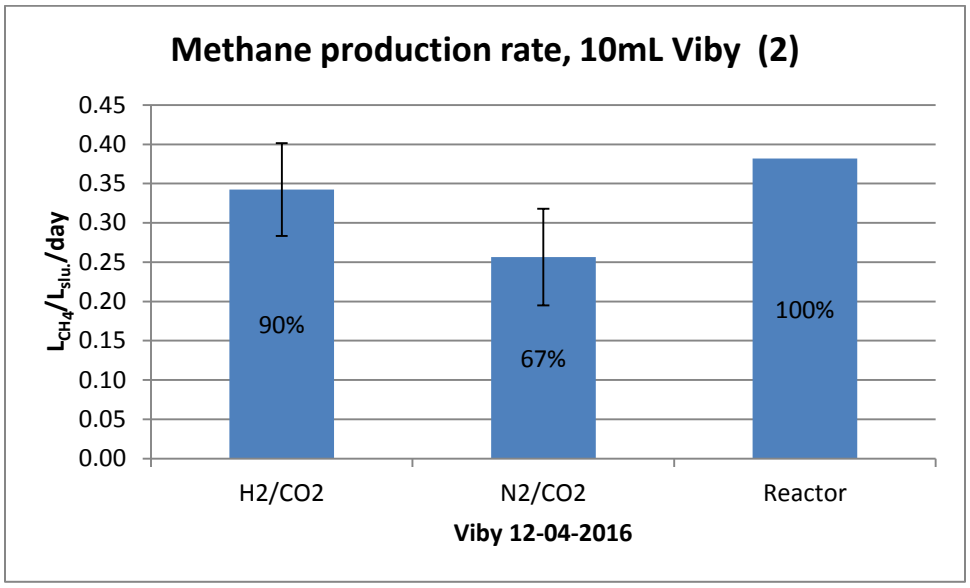

Figure 21: Methane production rate in liter methane produced per liter slurry per day. 10mL slurry from the anaerobic digester at Viby WWTP was used with headspace of H_2/CO_2 or N_2/CO_2 at 1.5bar pressure. The methane production rate of the reactor at sampling time is shown. Ave. MPR in percentage of the reactor rate indicated in the column. There was no significant difference between the two treatments (p= 0.227)) tested with a Student t–test. Standard deviation indicated with error bars.

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2/CO_2	N_2/CO_2
Rep.1	0.266	0.234
Rep.2	0.410	0.341
Rep.3	0.352	0.195
Ave. (±SD)	**0.343 (±0.059)**	**0.257 (±0.062)**
Reactor		0.382

Table 11: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2/CO_2 headspace at 1.5bar pressure. Undiluted slurry from Viby WWTP was tested. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

Besides Viby slurry, slurry from Åby WWTP was tested. Åby had been running thermophilic, which would have allowed us to evaluate the method on a different type of slurry, but had recently shifted to mesophilic operation. Data from the experiment with 10mL undiluted slurry from Åby WWTP with H_2/CO_2 or N_2/CO_2 headspace at 1.5bar pressure are shown in Figure 22.

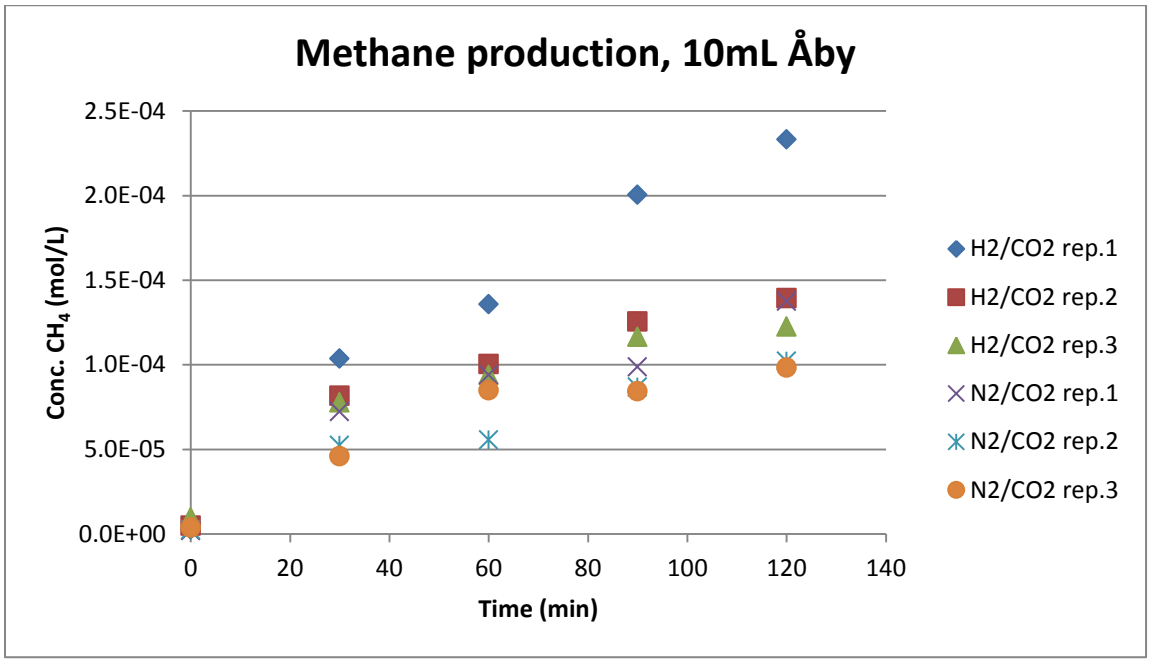

Figure 22: Methane production with slurry from Åby WWTP, 10mL slurry with a headspace of either H_2/CO_2 or N_2/CO_2 in the ratio 80:20. Linear regressions, H_2/CO_2 headspace samples: y(rep.1)= 1.86E-06x + 2.35E-05 (R^2 =0.960), y(rep.2)= 1.04E-06x + 2.78E-05 (R^2 =0.880), y(rep.3)= 8.80E-07x + 3.12E-05 (R^2 =0.853). Linear regressions, N_2/CO_2 headspace samples: y(rep.1)= 9.92E-07x + 2.13E-05 (R^2 =0.885), y(rep.2)= 7.79E-07x + 1.30E-05 (R^2 =0.929), y(rep.3)= 7.58E-07x + 1.79E-05 (R^2 =0.864).

During the 2h experiment the headspace methane concentrations increased in an approximately linear fashion, H_2/CO_2 rep.1 increasing to higher concentrations than the rest (Figure 22). Once again, the ave. MPRs did not reach the rates obtained from Bånlev slurry, ave. MPR with H_2/CO_2 headspace was 0.519 (±0.216) $L_{CH_4}/L_{slu.}/day$ and with N_2/CO_2 headspace it was 0.347 (±0.056) $L_{CH_4}/L_{slu.}/day$. It was not possible to detect a significant difference between the two treatments (p=0.259) (Figure 23, Table 14).

Once again did CO_2 measurements show that the aimed for concentrations was not reached. The starting CO_2 concentration (ave.) in the gas mixture was measured to be 17% in the N_2/CO_2 mixture and 7% in the H_2/CO_2 mixture (Table 12), both less than the 20% aimed for. CO_2% in the samples was

measured in the beginning and at the end of the experiment. Percentage of CO_2 in H_2/CO_2 headspace samples was between 10% and 12%, and in the N_2/CO_2 headspace samples, the CO_2 concentration varied between 14% and 22%. There was a pH increase compared to the reactor pH in all samples, but highest increase with H_2/CO_2 headspace (Table 13).

Sample:	Time (min):	CO_2%	
		H_2/CO_2	N_2/CO_2
Rep.1	0	11.15	19.42
Rep.1	120	11.72	19.62
Rep.2	0	10.82	17.88
Rep.2	120	11.69	19.20
Rep.3	0	11.20	14.41
Rep.3	120	11.80	22.37
Start 1		7.81	13.19
Start 2		6.61	20.66
Ave.		**7.21**	**16.93**

Table 12: Percentage of CO_2 in the gas mixture used to flush the serum bottles and the percentage of CO_2 in samples at the beginning and at the end of the experiment.

Table 13: pH values were measured in all replicas at the end of the experiment.

	Final pH	
	H_2/CO_2	N_2/CO_2
Rep.1	8.06	7.46
Rep.2	8.01	7.61
Rep.3	8.02	7.55
Reactor pH:		7.31

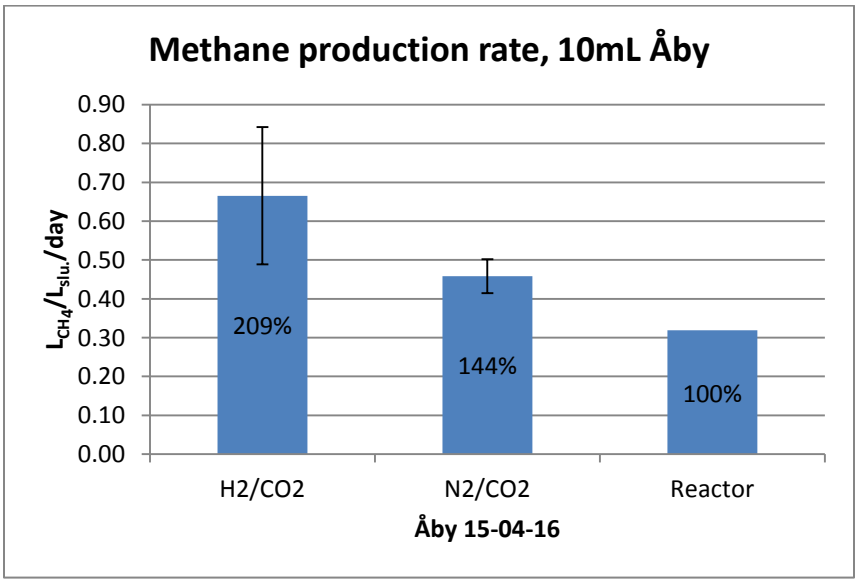

Figure 23: Methane production rate in liter methane produced per liter slurry per day. 10mL slurry from the anaerobic digester at Åby WWTP was used with a headspace of H_2/CO_2 or N_2/CO_2 at 1.5bar pressure. The methane production rate of the reactor at sampling time is shown. Ave. MPR in percentage of the reactor rate indicated above the column. There was no significant difference between the two treatments (p= 0.259) tested with a Student t–test. Standard deviation is indicated by error bars.

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2/CO_2	N_2/CO_2
Rep.1	0.766	0.408
Rep.2	0.429	0.321
Rep.3	0.362	0.312
Ave. (±SD)	**0.519 (±0.216)**	**0.347 (±0.056)**
Reactor		0.3188

Table 14: Methane production rates, liter methane produced per liter slurry per day, in replicas with either H_2/CO_2 or N_2/CO_2 headspace at 1.5bar. Undiluted slurry from Åby WWTP was tested. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

CO_2 series

To elucidate the effect of CO_2 on the methane production rate without added hydrogen, the methane productions were measured with a series of different headspace CO_2 concentrations.

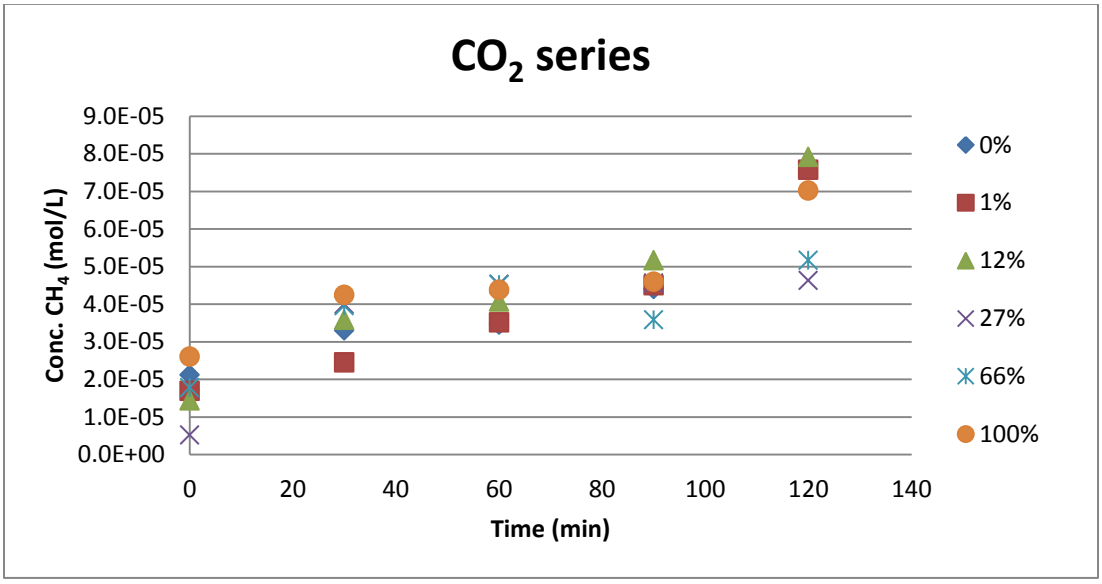

Figure 24: Methane production with slurry from Bånlev Biogas, 10mL undiluted slurry with a headspace of N_2/CO_2 where the CO_2 concentration differs between the serum bottles. Linear regression: y(0%) = 2.33E-07x + 2.27E-05 (R^2=0.931), y(1%) = 4.61E-07x + 1.18E-05 (R^2=0.910), y(12%) = 4.85E-07x + 1.53E-05 (R^2=0.940), y(27%) = 2.93E-07x + 1.89E-05 (R^2=0.618), y(66%) = 2.13E-07x + 2.52E-05 (R^2 =0.627), y(100%)= 3.06E-07x + 2.73E-05 (R^2 = 0.842).

During the 2h experiment the methane concentration increased in a similar linear fashion at all CO_2 concentrations (Figure 24). No clear pattern between the MPR and the headspace CO_2 concentration appeared (Figure 25).

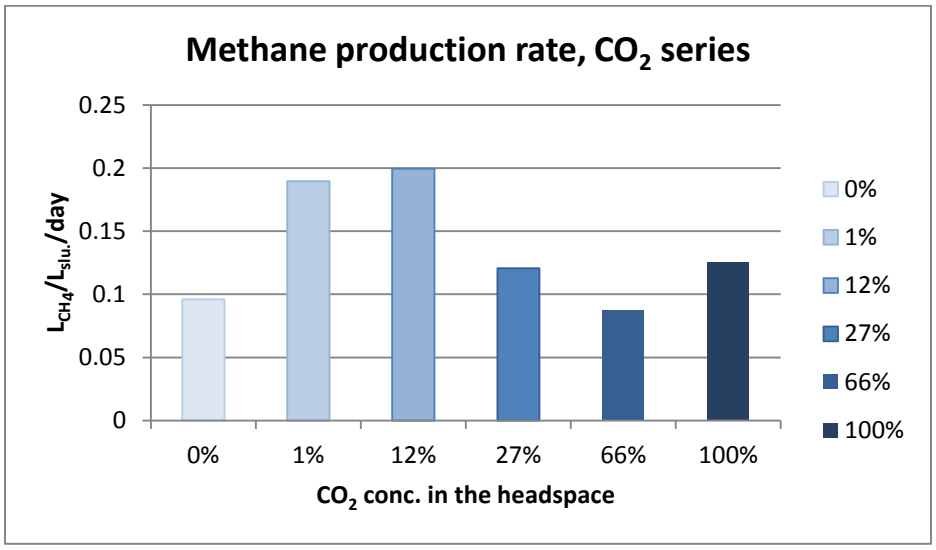

Figure 25: Methane production rate in liter methane produced per liter slurry per day. 10mL slurry from the anaerobic digester at Bånlev was used with headspace of N_2/CO_2 with different CO_2 concentration. MPR was calculated from the slope of the linear regression of methane production over time.

The methane production when acetotrophic methanogenesis was not limited was measured by adding 18mM acetate. The different headspace CO_2 concentrations were kept as in the previous experiment.

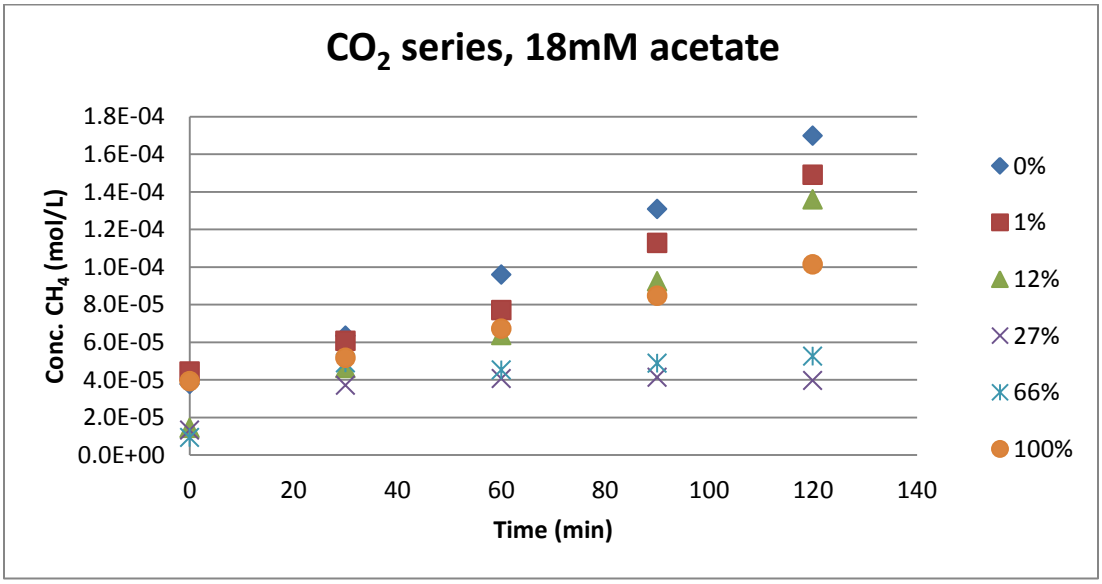

Figure 26: Methane production with slurry from Bånlev Biogas, 10mL undiluted slurry with 18mM acetate with a headspace of N_2/CO_2 where the CO_2 concentration differs between the serum bottles. Linear regression: y(1%)= 1.10E-06x + 3.33E-05 (R^2= 0.995), y(1%)= 8.72E-07x + 3.65E-05 (R^2=0.962), y(12%)= 9.65E-07x + 1.27E-05 (R^2=0.979), y(27%)= 1.91E-07x + 2.29E-05 (R^2 =0.571), y(66%)= 2.88E-07x + 2.36E-05 (R^2=0.582), y(100%)= 5.24E-07x + 3.74E-05 (R^2=0.996).

There was again no clear pattern between the CO_2 concentrations and MPRs, but in general MPRs were higher when acetate was added (Figure 27).The methane concentration increased in an approximately linear fashion during the 2h trial at all CO_2 concentrations (Figure 26).

All rates, both with and without added acetate, were below the MPR of the reactor (Figure 28, Table 15). There might be a vague tendency to higher rates with CO_2%≤ 12% when taking data from both series into account (Figure 28). The pH values in samples with CO_2%≤ 12% increased compared to reactor pH while CO2%≥ 27% decreased (Table 16).

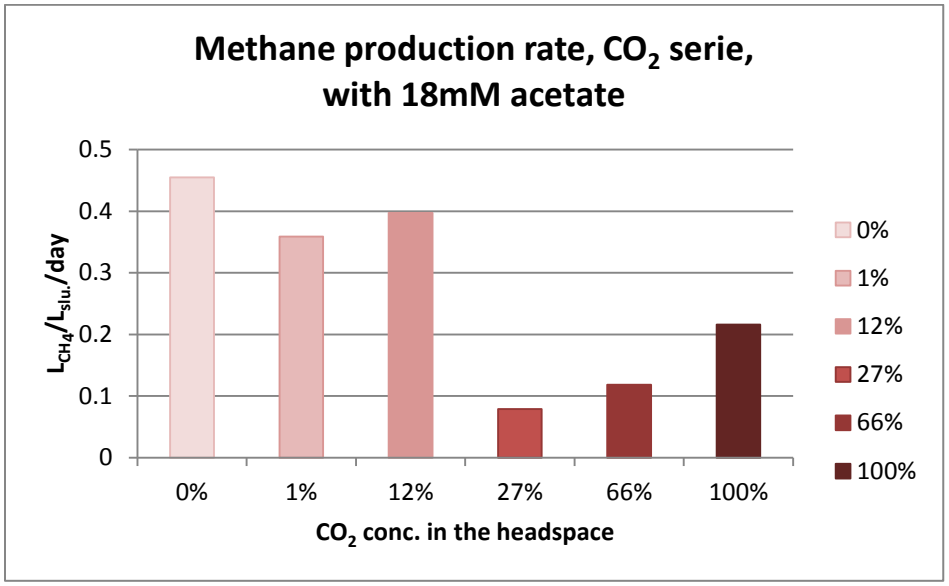

Figure 27: Methane production rate in liter methane produced per liter reactor slurry per day. The slurry was from Åby WWTP, 10mL slurry with 18mM added acetate and with a headspace of N_2/CO_2 with different CO_2 concentration.

Methane production rate $L_{CH_4}/L_{mat.}/day$		
%CO_2	-acetate	+acetate
0%	0.096	0.455
1%	0.180	0.359
12%	0.110	0.397
27%	0.121	0.079
66%	0.088	0.119
100%	0.126	0.216
Reactor rate:		0.847

Table 15: Methane production rates, liter methane produced per liter reactor slurry per day, in replicas with N_2/CO_2 headspace and different %CO_2. Slurry was from Bånlev Biogas. The rates were calculated from the slope of the linear regression of the methane production over time in the experiment.

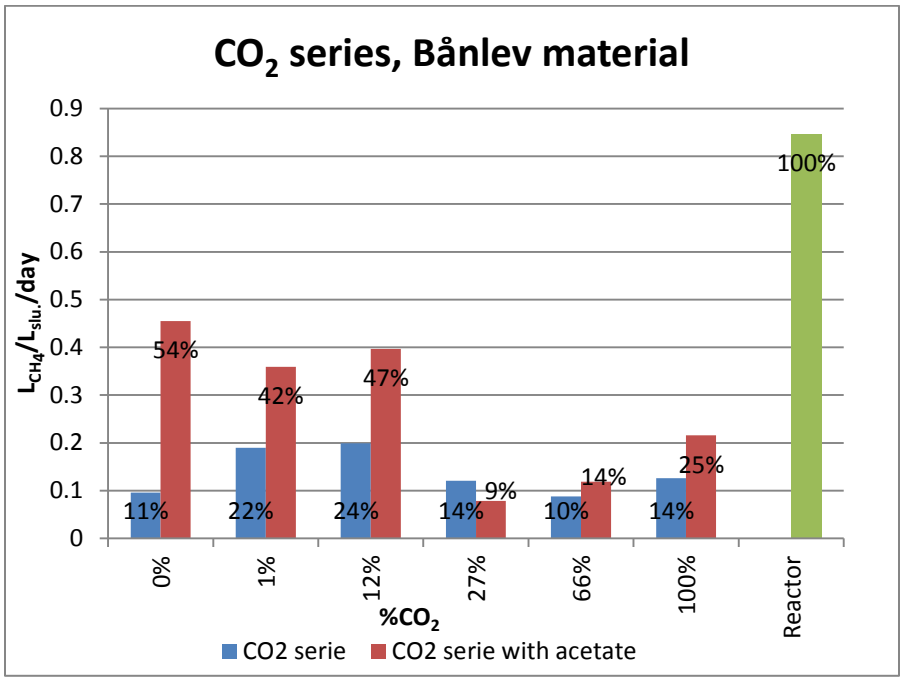

Figure 28: Methane production rate in liter methane produced per liter reactor slurry per day. 10mL slurry from the anaerobic digester at Bånlev Biogas was used with headspace N_2/CO_2 and different CO_2 concentration. Acetate was added in one of the series. The methane production rate of the reactor at sampling time is shown. The rate in percentage of the reactor rate is indicated.

Table 16: pH values were measured in all replicas at the end of the experiments, both with and without added acetate.

	%CO_2:	0%	1%	12%	27%	66%	100%	Reactor
pH values	- acetate	8.45	8.40	8.12	7.80	7.40	7.15	8.10
	+ acetate	8.55	8.42	8.20	7.80	7.40	7.20	

Adaption experiments

Adaption in laboratory scale reactor

By conducting the potential activity assay on slurry that previously had been exposed to high H_2 concentrations we expected to observe an adaption to hydrogen. The slurry was previously sampled from Bånlev Biogas. Control slurry had been exposed to argon instead (Figure 29).

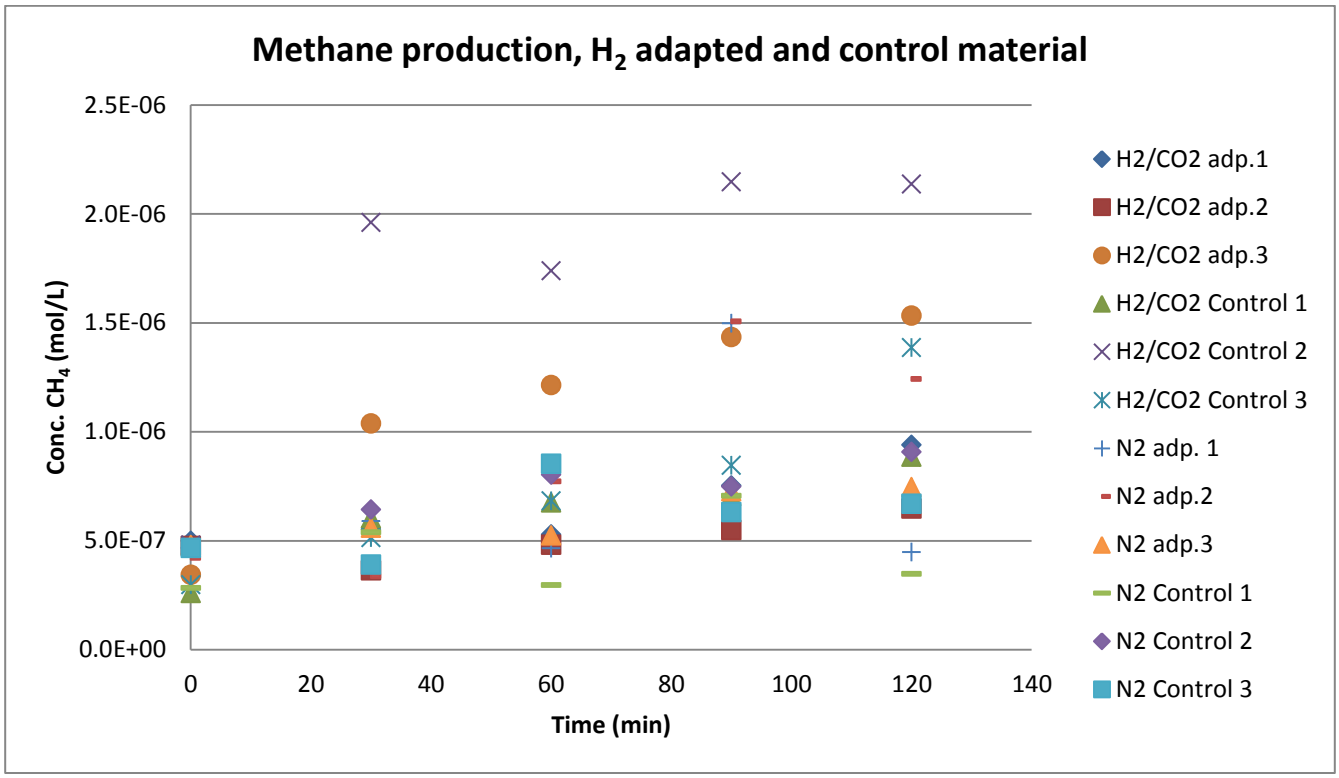

Figure 29: Methane production with slurry from H_2 adapted and control slurry, 1mL slurry diluted in 5mL saline water with a headspace of H_2/CO_2 (80:20) or N_2. Linear regression, *H_2 adapted*, H_2/CO_2 headspace: y(adp.1)= 3.61E-09x + 4.37E-07 (R^2=0.827), y(adp.2)= 1.76E-09x + 3.97E-07 (R^2=0.643), y(adp.3)= 9.26E-09x + 5.57E-07 (R^2=0.868). Linear regression, *H_2 adapted*, N_2 headspace: y(adp.1)= 2.92E-09x + 5.17E-07 (R^2 =0.0934), y(adp.2)= 9.38E-09x + 2.93E-07 (R^2 =0.762), y(adp.3)= 2.33E-09x + 4.67E-07 (R^2=0.828). Linear regression, *control*, H_2/CO_2 headspace: y(cont.1)= 4.51E-09x + 3.54E-07 (R^2 =0.871), y(cont.2)= 1.17E-08x + 9.92E-07 (R^2=0.629), y(cont.3)= 8.37E-09x + 2.42E-07 (R^2=0.927). Linear regression, *control*, N_2 headspace: y(cont.1)= 9.93E-10x + 3.75E-07 (R^2 = 0.0660), y(cont.2)= 3.29E-09x + 5.16E-07 (R^2= 0.865), y(conl.3)= 2.16E-09x + 4.72E-07 (R^2=0.319).

Even though some scatter was observed the methane concentration increased in a linear fashion in most samples (Figure 29). There was no pattern of H_2 adapted slurry having higher MPRs compared to controls (Figure 30). Ave. MPR from H_2 adapted slurry with H_2/CO_2 headspace was 0.020 (±0.013) $L_{CH_4}/L_{slu.}/day$ and 0.020 (±0.013) $L_{CH_4}/L_{slu.}/day$ with N_2 headspace (Figure 30, Table 17). Non-adapted control slurry with H_2/CO_2 headspace had as average MPR of 0.0337 (±0.0121) $L_{CH_4}/L_{slu.}/day$ while N_2 headspace replica had average MPR of 0.0089 (±0.00387) $L_{CH_4}/L_{slu.}/day$ (Figure 30 and Table 17). There were no significant differences between any of the samples.

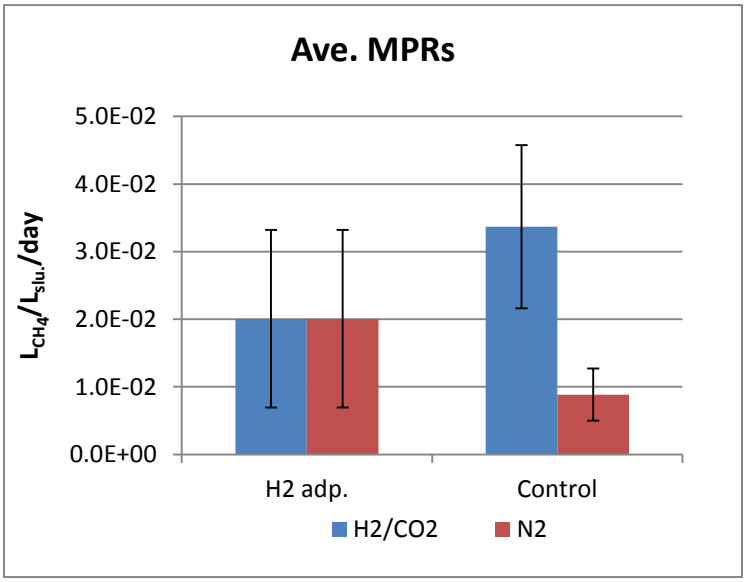

Figure 30: Methane production rate in liter methane produced per liter reactor slurry per day. The slurry from H_2 adapted and control laboratory scale reactors, 1mL slurry diluted in 5mL saline water with a headspace of H_2/CO_2 (80:20) or N_2. There was no significant difference between any of the samples, tested with a Students t-test. Standard deviations are indicated by error bars.

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2/CO_2	N_2
H_2 adp.1	0.015	0.012
H_2 adp.2	0.007	0.039
H_2 adp.3	0.038	0.010
Ave. (±SD)	**0.020 (±0.013)**	**0.020 (±0.013)**
Control 1	0.019	0.004
Control 2	0.048	0.014
Control 3	0.035	0.009
Ave. (±SD)	**0.034 (±0.012)**	**0.009 (±0.004)**

Table 17: Methane production rates, liter methane produced per liter reactor material per day, H_2 adapted and control replicas with H_2/CO_2 or N_2 headspace. The rates were calculated from the slope of the linear regression of the methane production over time.

As no adaption was observed in the first experiment another experiment were conducted to with new H_2 adapted and non-adapted slurries , this time only with H_2/CO_2 headspace. Originally the slurry was from sampled Bånlev Biogas.

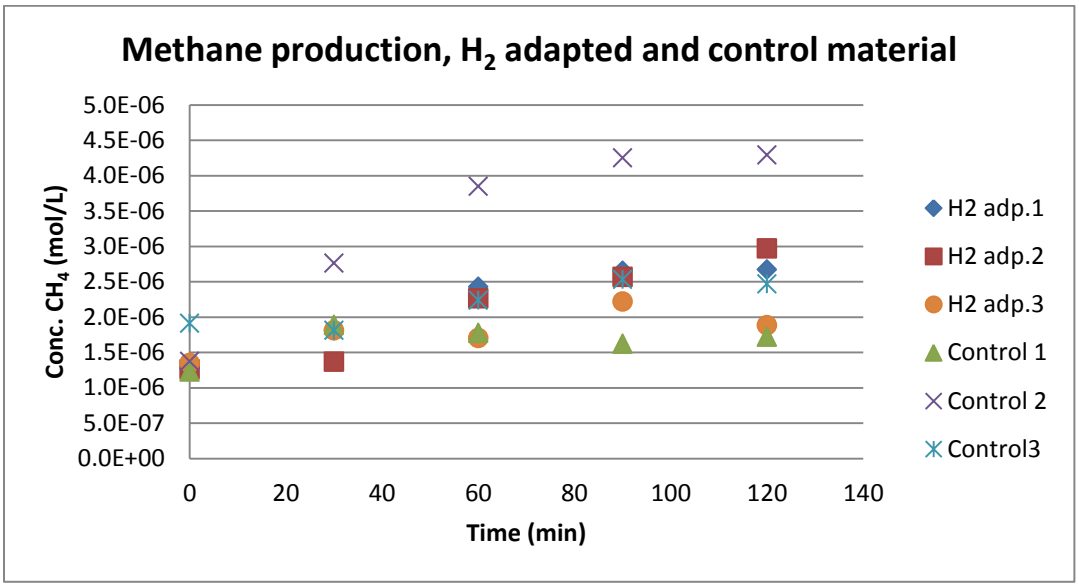

Figure 31: Methane production with slurry from H_2 adapted and control slurry, 1mL slurry diluted in 5mL saline water with a headspace of H_2/CO_2 (80:20). Linear regression, *H_2 adapted*, H_2/CO_2 headspace: y(adp.1)= 1.21E-08x + 1.44E-06 (R^2=0.893), y(adp.2)= 1.54E-08x + 1.16E-06 (R^2=0.951), y(adp.3)= 4.89E-09x + 1.50E-06 (R^2= 0.549). Linear regression, *control*, H_2/CO_2 headspace: y(cont.1)= 2.42E-09x + 1.50E-06 (R^2= 0.205), y(cont.2)= 2.44E-08x + 1.84E-06 (R^2=0.867), y(cont.3)= 6.10E-09x + 1.82E-06 (R^2 =0.807).

There was not apparent adaption of the slurry previously exposed to hydrogen. The methane concentration increased in an approximate linear fashion during the 2h (Figure 31). The ave. MPRs of adapted and non-adapted slurry was almost identical, and there were no significant difference between the two treatments (p=0.981). Ave. MPR for H_2 adapted slurry was 0.0444 (±0.0181) $\boldsymbol{L_{CH_4}/L_{slu.}/day}$ and for non-adapted control slurry it was 0.0452 (±0.0396) $\boldsymbol{L_{CH_4}/L_{slu.}/day}$ (Figure 32, Table 18).

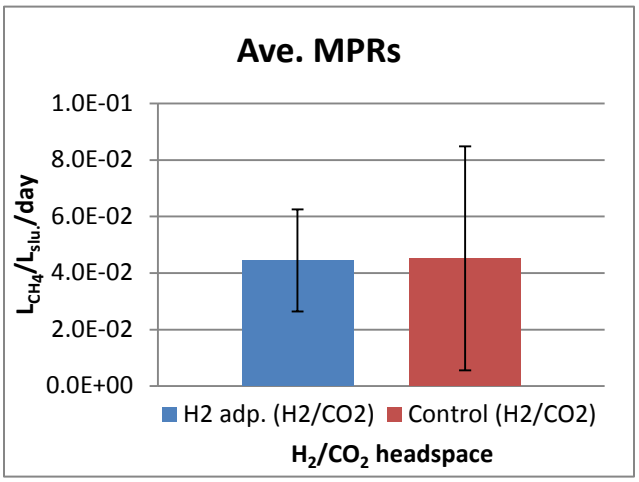

Table 18: Methane production rates, liter methane produced per liter reactor slurry per day, H_2 adapted and control replicas with H_2/CO_2. The rates were calculated from the slope of the linear regression of the methane production over time. Standard deviations are indicated by error bars

Methane production rate $L_{CH_4}/L_{slu.}/day$		
	H_2 adapted	Control
Rep.1	0.050	0.010
Rep.2	0.063	0.101
Rep.3	0.020	0.025
Ave. (±SD)	**0.044 (±0.018)**	**0.045 (±0.040)**

Figure 32: Methane production rate in liter methane produced per liter slurry per day. The slurry was from H_2 adapted and non-adapted control lab-scale reactors; 1mL slurry was diluted in 5mL saline water with a headspace of H_2/CO_2 (80:20). There was no significant difference between the treatments (p=0.981), tested with a Students t-test. Standard deviations are indicated by error bars.

Serum bottle adaption experiment (SBAE)

Adaption to higher H_2 concentrations was also attempted in smaller scale, in serum bottles with 10mL undiluted slurry from Bånlev. The headspace was either H_2/CO_2 or N_2/CO_2 at a pressure of 1.5bar, to ensure that no vacuum would evolve during the experiment. The results from the first day are shown above (Figure 14, Figure 15) and in the summery of the MPRs (Table 19). Day 1 Ave. MPR with H_2/CO_2 headspace was 1610% and N_2/CO_2 was 105% of the reactor rate. The second and third day the methane production was measured after flushing and replacing the headspace of the bottles.

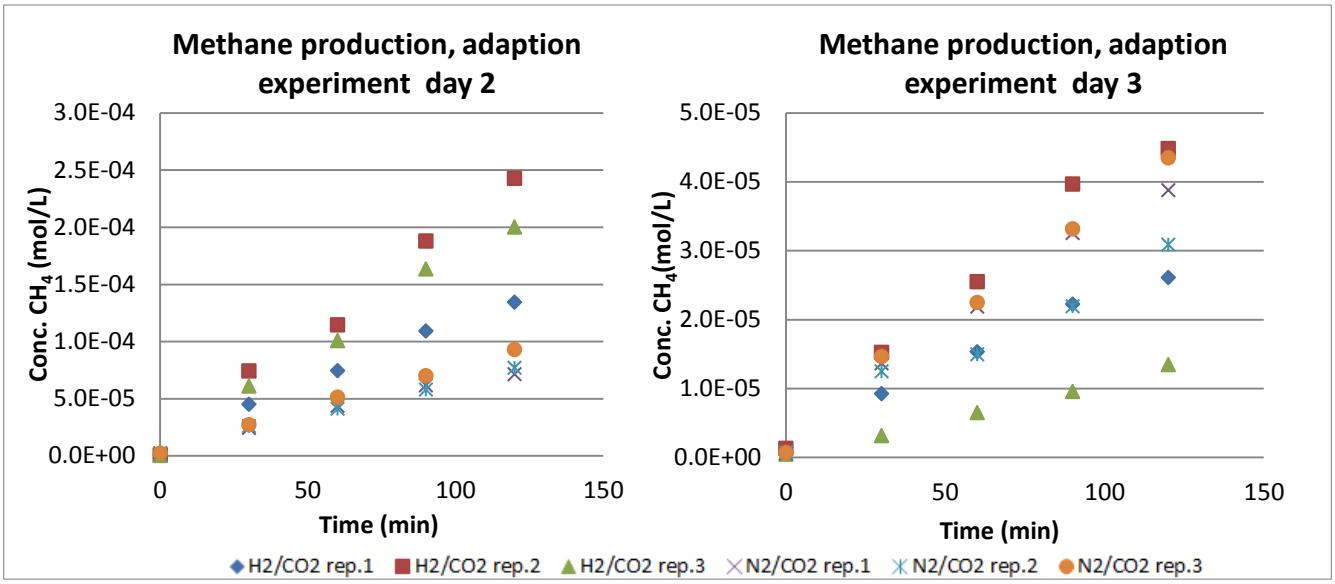

Figure 33: Methane production on the 2nd and 3tn day of the adaption experiment. 10mL slurry with a headspace of H_2/CO_2 or N_2/CO_2 (80:20). Day 2: Linear regression, H_2/CO_2 headspace samples: y(rep.1)= 1.11E-06x + 6.13E-06 (R^2=0.991), y(rep.2)= 1.99E-06x + 4.26E-06 (R^2 =0.994), y(rep.3)= 1.67E-06x + 4.67E-06 (R^2=0.993). Linear regression, N2/CO2 headspace samples: y(rep.1)= 5.85E-07x + 5.45E-06 (R^2=0.983), y(rep.2)= 6.07E-07x + 4.29E-06 (R^2=0.994), y(rep.3)= 7.47E-07x + 3.84E-06 (R^2=0.998). Day 3: Linear regression, H_2/CO_2 headspace: y(rep.1)= 2.12E-07x + 2.05E-06 (R^2=0.986), y(rep.2)= 3.72E-07x + 3.00E-06 (R^2=0.982), y(rep.3)= 1.08E-07x + 1.60E-07 (R^2=0.996). Linear regression, N2/CO2 headspace: y(rep.1)= 3.18E-07x + 2.43E-06 (R^2=0.987), y(rep.2)= 2.32E-07x + 2.27E-06 (R^2=0.967), y(rep.3)= 3.47E-07x + 2.06E-06 (R^2=0.994).

Both on the 2nd and 3th day there was a linear increase of headspace methane concentrations (Figure 33). On day 1 and 2 there were a clear difference between rates from H_2/CO_2 and N_2/CO_2 headspace samples, H_2/CO_2 samples producing methane at the higher rate, but not on the 3th day (Table 19). The pH values were measured the last day of the experiment and had all increased substantially compared to the reactor rate.

Methane production rate $L_{CH_4}/L_{slu.}/day$							Final pH	
Time:	Day 1		Day 2		Day 3		Day 3	
Gas:	H_2/CO_2	N_2/CO_2	H_2/CO_2	N_2/CO_2	H_2/CO_2	N_2/CO_2	H_2/CO_2	N_2/CO_2
Rep.1	13.408	0.897	0.684	0.362	0.131	0.196	9.50	8.93
Rep.2	13.649	0.839	1.232	0.375	0.229	0.143	9.56	8.93
Rep.3	12.081	0.806	1.034	0.462	0.067	0.214	9.44	9.04
Ave. (±SD)	**13.046 (±0.689)**	**0.847 (±0.038)**	**0.983 (±0.226)**	**0.399 (±0.044)**	**0.142 (±0.067)**	**0.185 (±0.030)**		
Reactor MPR:		**0.810**					**Reactor pH:**	**8.01**

Table 19: Methane production rates, liter methane produced per liter slurry per day from day 1,2 and 3 of the adaption experiment. The headspace was either H_2/CO_2 or N_2/CO_2. The rates were calculated from the slope of the linear regression of the methane production over time. The pH values of the slurry in the samples were measured after ending the experiment on day 3.

After the first day average MPR in samples with H_2/CO_2 headspace decreased from 1610% to 121% at day 2 and 18% day 3. The rate with N_2/CO_2 headspace decreased from 105% to 49% day 2 and 23% day 3 (Figure 34 and Table 19). pH values were measured at the end of the experiment (day 3) and appeared to have increased in all samples. The pH values had increased to above what normally is considered inhibitory (Costello et al. 1991), and might be the reason for the decreasing rates.

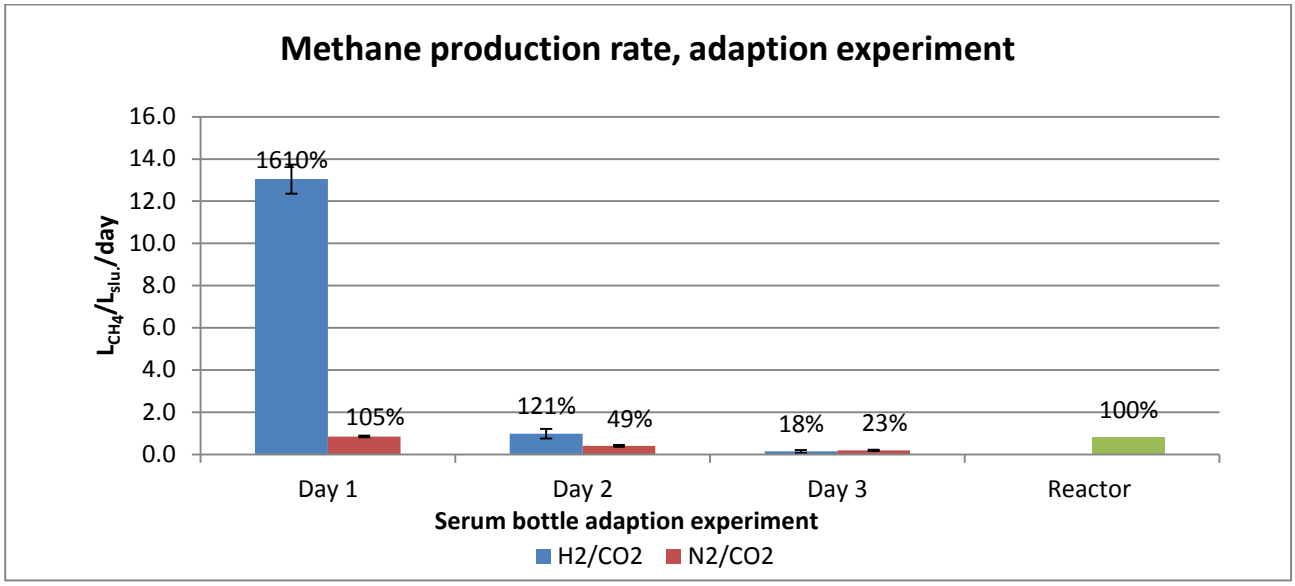

Figure 34: Methane production rate in liter methane produced per liter slurry per day. The same serum bottles and slurry was tested day 1, 2 and 3. The headspace was either H2/CO2 or N2/CO2 (80/20). Ave. MPR in percentage of the reactor rate indicated above the column. Standard deviations are indicated by error bars.

Volatile Solids

Volatile solids were measured to evaluate variations between anaerobic digesters. Low amounts of volatile solids (VS) were measured in different samples from all three digesters. Most samples had VS content between 1.50% and 1.71%, only one sample had a lower content of 1.08% (Bånlev 16-11-15) (Figure 35).

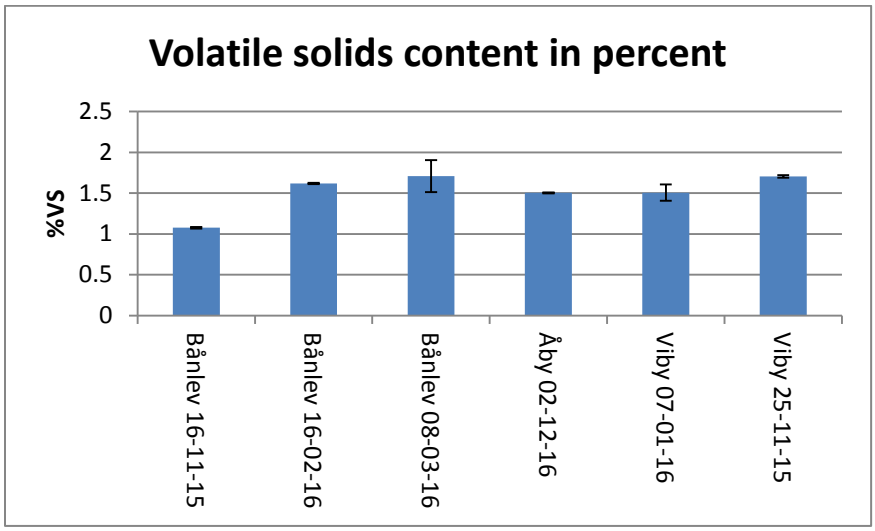

Figure 35: Volatile solids in slurry from Bånlev Biogas, Åby WWTP and Viby WWTP. Standard deviations are indicated by error bars.

Visualization

Visualization was attempted with fluorescent in situ hybridization (FISH) using a broad archaeal probe (Stahl and Amann 1991), and by fluorescent microscopy. Trough fluorescent microscopy it is possible to visualize methanogens as F_{420}, co-factor in methanogenesis, is autofluorescence (Amaral et al. 1991). No successful visualization with FISH was achieved and because of a large amount of fluorescents from the organic matter in the biogas slurry visualization with fluorescent microscopy was not possible (Figure 36). In Figure 36, a merge picture of DAPI, Cy3 and 6-Fam filters, bacterial and archaeal cells are visual through DAPI.

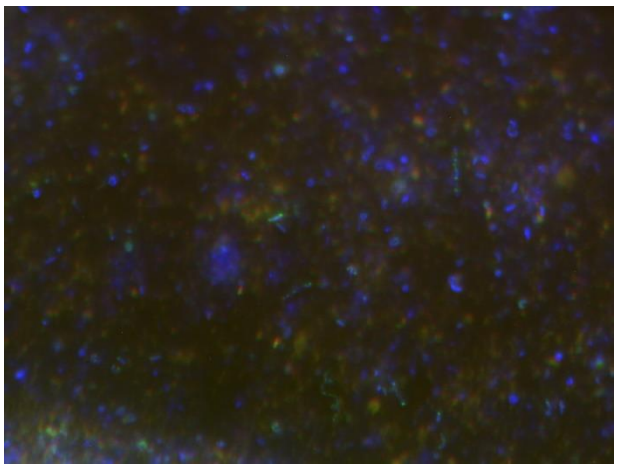

Figure 36: FISH of slurry from the anaerobic digester at Foulum. DAPI colors all cells blue, *Archaea* will appear red-ish (ARC915 with Cy3), and *Bacteria* will appear green (EUBmix with 6-Fam).

Discussion

Development of the potential activity assay

Exposure to oxygen

Methanogens are known to be strict anaerobes (Weiland 2010) so exposure to air would constrain the methane production process and reduce MPR. Oxygen contamination could hence be suspected to be involved in the low MPRs, compared to reactor MPR, observed in most experiments described in this report. When sampling the digester slurry some of the slurry could be subjected to oxygen from the air. Sampling in the air tight bag and fast incubation is hence essential and were aspired during the sampling. Exposure to oxygen inhibits the methanogens and decreases the methane production, especially when they have never been exposed to oxygen before, as you would expect in an anaerobic digester (Fetzer et al. 1993). With other setups, e.g. when testing the biomethane potential of a certain substrate, there would be an adaptation period, allowing the microbial community to adapt to the new conditions, until steady state is establish (Angelidaki et al. 2009). By allowing the microbial community an adaption period, the risk of oxygen contamination affecting the results will be reduced. There is no adaptation phase in this setup as the potential activity assay strives to estimate the existing upgrading potential of the reactor at the sampling time. As there most probably are facultative anaerobic bacteria present in the reactor (Ahring 2003; Weiland 2010) small amount of dissolved oxygen could be removed by these organisms and thereby reducing the risk of oxygen contamination and minimize inhibition of methanogens. It is therefore not likely that oxygen contamination during sampling and handling of the slurry was the cause of low MPRs compared to reactor MPR.

Oxygen contamination during the experiment, e.g. caused by a leaky stopper or an unflushed syringe, is most likely not the cause of the low MPRs observed. If an oxygen contamination happened during the experiment the slope of the production curve would decrease rapidly. Even though there were some deviations the measured headspace methane concentrations followed the linear regression.

In the experiments with 10mL undiluted slurry the samples were pressurized with 1.5bar H_2/CO_2 or N_2/CO_2. In these experiments the stoppers were tested for leaks after flushing the serum bottles (Figure 22, Figure 20, Figure 17), and the pressures were measured with a pressure sensor at the beginning and at the end of the experiments. All replicas still had an elevated pressure compared to ambient pressure

at the end of the experiments, indicating that the stoppers had not been punctured and hence the samples not oxygen contaminated from this source.

Nutrients

Having the required nutrients available is critical for microorganisms when assessing the potential activity. During experiments with media dilution of reactor slurry the media could be suspected of inhibiting the methane production if not suitable for this type of methanogens in the reactor. The media was designed for and tested on methanogens (Pennings et al. 1998) and includes trace element solution with essential nutrients for methanogens and methanogenesis. Angelidaki et al. (2009) suggested a similar medium for assessing biomethane potential (BMP), differing in added Mg and Ca. The medium or lack of nutrients is hence not expected to have caused the lower MPRs compared to reactor rate in the media dilution experiments.

To evaluate the effect of the medium and nutrients in the dilutions on MPR, the potential activity assay were performed with 0.5% KCl water dilutions, in the ratio 1:10 (Figure 8). By dissolving 0.5% KCl in the water the salinity should be comparable to that of the cells. Here the organisms only had the nutrients already available in the slurry. Both rates in the saline water were yet again lower than the reactor rate, and even more so than the media dilutions. Even though it is not expected that the methanogens grow significantly during the 2 hours, due to slower reported growth rate (Koster and Koomen 1988), it seems that the lack of medium do effect the MPR, but it is difficult to say if the nutrients is limiting, or if it is the lack of buffer that affected the rate.

Substrates

It is essential for the assay that the hydrogenotrophic methanogens have all the substrate that they need when assessing the potential activity. Furthermore is it necessary for acetotrophic methanogens to have the same possibilities as in the reactor to mimic the reactor MPR. When evaluating the potential activity H_2 and CO_2 was added to the headspace of the serum bottles in stoichiometric ratio (80:20). Keeping the H_2/CO_2 ratio in 80:20 ensures that the hydrogenotrophic methanogens is neither limited in electron donor or electron acceptor. Organic substrates required by the microorganisms would be provided by the reactor slurry, ensuring that the community has similar conditions as in the reactor. In N_2 headspace samples the hydrogenotrophic methanogens would only be able to utilize the H_2 and CO_2 produced by the digestion process, as when in the large scale anaerobic digester, and would therefore only

be expected to be responsible for maximum one-third of the methane production (Weiland 2010), the rest being produced by acetotrophic methanogens.

When the feeding stops (as when we sample) the first steps of the digestion process will be limited after a certain amount of time. In the media dilutions the medium provides nutrients, but is designed for methanogens, and does not contain all the needed substrates for the entire digestion process (Weiland 2010). In the anaerobic digester acetate is produced constantly, and removed in almost the same speed as it is produced resulting in low steady state concentrations (Gavala et al. 2003). If the reactor is not fed continuously the production of acetate and other substrates for the methanogens will decrease which would be reflected in the gas production. As it is impossible to start the experiment at the minute of sampling, some of the acetate will be utilized before starting the experiment. It seems possible that the acetate availability is limiting the MPR and might partly be responsible for the lower MPR compared to the reactor MPR. From the results of the acetate addition to the slurry in the experiments with different headspace CO_2 concentration, it is indicated that acetate is not the only limiting factor (Figure 26). The results showed higher MPRs when acetate were added, but did not reach reactor MPR. Lower amounts of available acetate could contribute to a lower MPR in the potential activity assay compared to reactor MPRs. Luo and Angelidaki (2012) added 20mM acetate to the reactor slurry to estimate the specific acetotrophic methanogenic activity, and had a headspace of H_2/CO_2 to estimate specific hydrogenotrophic methanogenic activity. Even though this provides insight in acetotrophic the community the goal in this experiment was to estimate the upgrading potential of the reactors at the time of sampling, adding acetate in a certain amount would distort the results. The assay strives to evaluate both the microbial community and the composition of the digester slurry at the time of sampling, with the amount of substrates present.

The experiments with different CO_2 concentrations were designed to elucidate the effect of CO_2 availability (Figure 24). It was essential that pH was kept stable to eliminate any pH effect, but this was not managed in this experiment. Despite of the changed pH, there was a tendency for higher rates with $CO_2\% \leq 12\%$. This is lower than the amount of CO_2 normally found in the headspace of anaerobic digesters (25-50% CO_2 (Weiland 2010)). It could be expected that the CO_2 utilizing organisms would be adapted to the concentration in the reactor, but this do not seen to be the case from this experiment.

pH

In the media dilutions a phosphate buffer was added to ensure stable pH values through the experiments. The hydrogenotrophic methanogens remove CO_2 and thereby some of the natural buffering capacity of the slurry, risking a pH increase with increased hydrogenotrophic activity. Methanogenesis is reported to have a pH optimum around pH 7 (Angelidaki et al. 2003) and is in risk of inhibition below or above this value (Chen et al. 2008). In the saline water dilutions no buffer was added which could affect the MPR and might be the reason for the lower MPRs in water dilutions compared to media dilutions. The buffering system of the slurry is largely dependent on the carbonate buffer system, the effect of which will be reduced when diluted (Ahring 2003). Furthermore diffusion of CO_2 in one direction from the liquid slurry into the headspace would occur, especially in the samples with pure N_2 headspace, as the headspace CO_2 conc. would not match that of the slurry. This will result in reduced buffer capacity. When the natural buffer capacity is reduced there is a risk of microbial activity changing the pH and a risk of CO_2 limitations of the hydrogenotrophic methanogens (Hori et al. 2006; Lin et al. 2013), both increasing the risk of reduced MPR. Adding CO_2 to the headspace of the samples ensures enough CO_2 for hydrogenotrophic methanogenesis and helps keep the pH stable (Angelidaki et al. 2009).

In the experiments with 10mL undiluted slurry a pH increase was observed in all replica, but only smaller increases (between 0.05 and 0.30) in replica with ~20% CO_2 (Table 8, 10, 11, 12). Normally a pH value below 6.0 or above 8.5 is expected to inhibit the process severely (Weiland 2010). In the experiment with undiluted slurry, pH values above 8.5 were only observed in replica with high activity. Smaller changes in pH could also affect the MPRs in lesser degree as the microbial community is adapted to a certain pH. This was showed in Luo et al. (2012), who observed a slight inhibition of the acetotrophic methanogenic community caused by a pH increase to 8.3, where the optimal pH was estimated to be between 7.0 and 7.5. A pH inhibition was probably what was observed in the SBAE (serum bottle adaption experiment) where the pH values on the 3th day all were above 8.5.

pH is not measured during the experiment so it is not known if pH value is increasing steadily throughout the experiment, or if is there is more sudden change as the buffer capacity is diminished. If the pH increased to inhibitory levels during the first period of the experiment, a decrease in the linearity, i.e. a decrease in the slope and MPR, could be expected. The majority of methane production curves pro-

duced in the experiments in this report increase in a linear or approximate linear fashion. Even though pH did not increase at much as previous observed, this could be suspected to cause the low rates in the experiments with 10mL undiluted slurry, as the rate during the first 30 min could be suspected of being faster than during the remaining 90 min. of the experiments (Figure 20, Figure 22). Only one sample was sampled at 30 min. and it is hence not possible to perform a regression. As the MPRs in both cases were not fast enough to deplete the slurry of CO_2 and thereby affect the pH during the first 30min., the apparent decrease in MPRs after the first 30min. are most likely not caused by pH changes.

The pH could be kept steadier by applying a buffer to the slurry in all experiments and not just the ones with media dilutions. A phosphate buffer was applied to the undiluted slurry in the CO_2 series, but the pH still changed during the experiment implying that the slurry were not buffered sufficiently. Even though phosphate is a natural part of the slurry and contribute to the buffering capacity of the slurry (Angelidaki et al. 2003), the phosphate buffer can have a negative effect on the MPR (Raposo et al. 2011). In that case adding extra carbonate buffer might be a better alternative.

Experimental conditions

The first potential activity assays were performed with diluted slurry. One of the arguments for diluting the slurry was to ensure sufficient amounts of H_2, so that the hydrogenotrophic methanogens would not compete for H_2 or be limited by the too small amounts dissolved H_2. H_2 has a low solubility, Henrys constant: $K_{H_2} = 7.9 \cdot 10^{-9} \frac{mol}{L \cdot Pa}$ (Pauss et al. 1990), and vigorous mixing is needed to ensure no gas-liquid mass-transfer limitations. This is achieved by incubation on a rotator (at 40rpm) in these experiments. There is no reason to suspect mass transfer limitations in the diluted slurry. If any limitation occurred, then production would decrease with higher concentrations. In fact the opposite was the case, as more methane was produced with higher concentrations of slurry.

As the diluted slurries did show lower MPRs than the reactor MPRs a dilution series were conducted to estimate the effect of slurry concentration. It was clear that the undiluted slurry reached the highest headspace methane concentrations and fastest MPR. It was expected that the headspace methane concentration would increase in proportion to the concentration of slurry. The MPR, which is normalized with the volume of the slurry, was expected to be the same with different dilutions until the H_2 consumption would exceed the H_2 gas-liquid mass transfer. The dilutions series indicated that 10mL undi-

luted slurry produced the highest rates, and when pressure was applied together with N_2/CO_2 headspace the MPR reached reactor MPR.

Overall, none of the samples with N_2 headspace is equivalent to reactor MPR. Control samples with N_2 headspace had small variations between 1:1 dilution and undiluted slurry (43% and 47% of reactor MPR respectively). 1:10 dilutions had larger dissimilarities from the 1:1 and undiluted slurry; saline water dilution 1:10 was 9.3% of reactor rate, medium dilutions 1:10 was 5%, 9% and 50% of the reactor rate. When CO_2 was added to the headspace and pressure applied (1.5bar) rates were achieved that were close to the reactor MPR (105% and 76%) with slurry from Bånlev. The results with slurry form Viby WWTP and Åby WWTP were more unclear. Increasing the pressure increases the amount of H_2 possible to dissolve, which helps to avoid gas-liquid mass transfer limitations increasing the MPRs in H_2/CO_2 samples. Increased rates were also observed in control replica with a pressure of 1.5bar. This is also observed in previous studies (Martin et al. 2013). The pressures in experiments with pressurized headspace were measured before incubation and at the end of the experiment. This was taken into account when calculating the headspace methane concentration.

In the experiments with 10mL undiluted slurry the CO_2 concentration in the headspace were measured to ensure that CO_2 concentrations were sufficient throughout the experiment. This showed that the aimed for concentrations were not reached with the flow controller used to mix the gas. Angelidaki et al. (2009) recommended a headspace with 20% CO_2 when assessing biomethane potential of solid organic wastes to ensure a stable pH value and a stable digester process. As the CO_2 were only measured in the last experiments with undiluted slurry, and in the CO_2 series, it is not possible to say if the concentrations were too low.

At the end of the experiments with 10mL undiluted slurry from Åby and Viby the pH had increased, but not as much as in experiments with slurry from Bånlev. This correlates with the MPRs in Åby and Viby experiments being lower, i.e. less CO_2 consumed. The previous observations of significant difference between treatments, where highest MPRs were obtained with H_2/CO_2 headspace, were not observed in experiments with Åby and Viby slurry (10mL undiluted). There were not detected any significant differences between the two treatments with slurry from either place in these experiments, even though there still were significant differences in experiments made with Bånlev slurry.

The potential activity assay

There have not yet been described assays with the same settings or aims as the potential activity assay described in this report. Evaluation of specific methanogenic potential described by Angelidaki et al. (2009) in connection to BMP assessments of organic substrates have some similarities, but have a different purpose. The potential activity assay strives to assess the upgrading potential of the reactor as it is. That includes the microbial community and the composition of the digester slurry at the time of sampling. It is therefore crucial to minimize influences of external factors, e.g. avoiding oxygen contamination and aspire for a fast incubation after sampling.

In the experiments conducted in this study there were significant differences between H_2/CO_2 and N2 or N_2/CO_2 headspace in almost all experiments. Except for the last experiments with 10mL undiluted slurry from Viby and Åby WWTP that did not react as expected or as observed previously. This inspires further investigations of the potential activity assay with 10mL undiluted slurry.

The MPRs in the experiments with diluted slurry were all lower than the reactor MPR. Oxygen, pH, nutrients, substrate availability (discussed above) could affect the MPRs, but there is no definite reason for the lower rates. Higher rates were achieved with higher concentrations of slurry and highest with undiluted slurry. If the assay should be used again it should be with undiluted slurry.

In previous BMP studies a inoculum substrate ratio or inoculum concentration is discussed (Angelidaki et al. 2009; Raposo et al. 2011; Raposo et al. 2012). Raposo et al. (2012) argue that according to the theory the methane yield should be independent of the inoculum-substrate ratio. But from experience the ratio both effect the extent and rate of the anaerobic digestion (Raposo et al. 2012). In the experiments conducted here the dissolved H_2 is the substrate that needs to be converted. It could appear the observation from BMP also applies to the slurry concentration in the potential activity assay.

From the results from the CO_2 series, headspace concentrations of CO_2 in the headspace did not appear to affect the MPR. In BMP experiments Raposo et al. (2011) reported that most experiments applied a headspace with pure N_2 and without CO_2 addition. This indicates that added CO_2 is not necessary for the methane production in control samples. However it could affect the pH as bicarbonate would diffuse into the headspace, especially in the potential activity assay as where gas-liquid mass transfer is

optimized. If any further experiments should be performed it would be with the potential activity assay, a headspace of H_2/N_2 and CO_2 should be applied.

By pressurizing the headspace of the samples with ~1.5bar an increase in MPR were observed with both types of headspace. In another study an over pressure of 1 atm were applied when assessing the methanogenic activity (Angelidaki et al. 2009) and a study by Martin et al. (2013) considered the effect of increased pressure on biogas upgrading and proposed further investigations. In the potential activity assay the effect of elevated pressure should also be investigated further.

Adaptation to high H_2 concentrations

Adaptation experiments in laboratory scale reactors

MPRs from slurry which were previously exposed to high concentrations of H_2 were not different from MPRs from control slurry, and it was hence not possible to observe any adaption to high H_2 concentrations.

MPRs were evaluated from slurry sampled from laboratory digester, where it had been exposed to high concentrations of H_2. Control slurry had been exposed to argon in the laboratory reactors. By adding H_2 to laboratory scale digesters in a rate corresponding to the CO_2 production the hydrogenotrophic methanogenic community should be able to adapt to the high concentrations, and a faster MPR would be expected. When adding H_2 in an amount corresponding to CO2 production, the increased MPR is not expected to deplete the digester slurry from CO_2. Furthermore, changes in the slurry composition are minimized and the pH is kept stable, allowing us to investigate the adaptation to high H_2 concentration. Initially the hydrogenotrophic methanogens would be expected to upregulate enzymes coupled to methanogenesis such as F_{420} as observed in other studies (Luo and Angelidaki 2013). A community shift to hydrogenotrophic methanogens would be anticipated later (Bassani et al. 2015). The increased methanogenesis and community shift would result in the increased MPR when adding H_2. In the experiment conducted here there were no significant difference and no pattern between H_2 adapted slurry and control slurry. When comparing to the MPR from newly sampled slurry, measured in the laboratory, the MPR in the adaptation experiment was far lower (Figure 29, Figure 31).

The lack of observed adaption to higher H_2 concentrations could have several causes. The first experiment had 3 periods of H_2 addition before slurries were sampled and MPRs measured. Methanogens

grow at different rates, maximum growth rates of different methanogens from anaerobic digesters were reported to vary between 0.029-0.144 h^{-1} (Koster and Koomen 1988). In Koster and Koomen (1988) they determined the maximum growth rate of the mixed community of biomass from a wastewater digester to be 0.126 h^{-1}, equivalent of a generation time(t) of 5.5h ($ln2 = \mu t$). The growth rate in the laboratory scale reactor used could be expected to be lower than in the large anaerobic digester, as the conditions have changed. It could be expected that 3 periods with higher levels of H_2 would give the hydrogenotrophic methanogens an advantage compared to the rest of the methanogenic community and allow it to adapt as previously observed (Luo and Angelidaki 2013; Bassani et al. 2015). In previous conducted experiments a higher amount of the enzyme F_{420} was observed (Luo and Angelidaki 2013), which indicated a community shift in the favour of the hydrogenotrophic methanogen. In the mentioned experiment H_2 was added continuously, giving the community more time to adapt. The 3 periods of H_2 addition in the adaptation experiment conducted here might not have been long enough for an adaptation to occur, or at least pronounced enough to be detected through the potential activity assay. Bassani et al. (2015) also observed a shift in the methanogenic community, but again with a different setup, indicating that longer H_2 exposure could be necessary to detect adaptation via the potential activity assay.

Additional trial in the laboratory reactor did indicate an adaptation to the higher H_2 concentrations by utilizing the H_2 faster (Agneessens, unpublished 2016).

The laboratory scale reactors were fed with slurry sampled at the same time as the inoculum slurry (stored cold until used). As a result the feed was already partly digested, and contained lower amounts of substrates compared to e.g. fresh manure than an anaerobic digester would normally be fed with (Eastman and Ferguson 1981). If the microorganisms were substrate limited, adaptation would occur at reduced rate, as cell growth would be inhibited.

The adaptation experiments were conducted with diluted slurry (1:10 or 1:5) as only small amounts of slurry could be removed from the laboratory reactor to keep the HRT at 20 days. Higher rates might have been achieved with undiluted slurry, as observed in described experiments, but a different pattern between adapted and non-adapted slurry would not be expected. If there had been a difference between

treatments, it would have shown even with dilutions factors of 1:10 or 1:5, as a difference between treatments with diluted slurry have been observed previously.

Sampling from the laboratory scale reactors was more difficult than sampling from large scale reactors, but the argon flushed syringes used to sample the slurry should not allow any oxygen to contaminate the slurry.

Serum bottle adaptation experiment (SBAE)

In the SBAE high MPRs were achieved the first day of the experiment, but decreased on day 2 and even further on day 3. By measuring the pH in the bottles after end experiment it was discovered that the pH had increased to high values, above what would normally be considered inhibitory.

By applying a headspace of H_2/CO_2 or N_2/CO_2 at 1.5bar to digester slurry in serum bottles it was hypothesized that an adaption to the high H_2 concentrations would occur. Slurry was sampled from Bånlev. The increased pressure was to ensure that a vacuum would not evolve during incubation, and high pressure also increases the mass transfer (cf. Henrys law) (Martin et al. 2013). The first day of the experiment the MPRs were the highest achieved so far, but decreased on day 2 and decreased even further on day 3 (Figure 31). At the end of the experiment pH values were measured and showed high values in all samples. In samples with H_2/CO_2 headspace pH increased 1.43 to 1.55 units, and in samples with N_2/CO_2 headspace pH increased 0.92 to 1.03 units. The large increases in all samples could indicate that CO_2 was consumed and thereby disturbing the natural buffer capacity of the slurry. It was expected that the 20% CO_2 in the headspace and the dissolved bicarbonate/CO_2 in the slurry would have kept the pH stable. Later it was discovered that the flow controller might be less precise than expected and the headspace concentrations could therefore have been lower than originally aimed for. This and high H_2 and CO_2 consumption rates and MPR could have caused the pH increase. This experiment could possibly be replicated with success if pH could be kept stable, i.e. by adding a buffer and by keeping the headspace CO_2 concentration around 20%. With a large concentration of H_2 there is a risk of inhibition of VFA (volatile fatty acid) degradation and thereby accumulation of VFAs such as propionate and butyrate (Ahring and Westermann 1988). Previous work with H_2 addition only experienced VFA accumulation during high mixing rates (Luo et al. 2012) or high mixing and together with a high H_2 addition (Wang et al. 2013). Other studies of H_2 addition did not experience any accumulation (Luo and Angelidaki 2013; Bassani et al. 2015) as the addition here were set to match the CO_2 produc-

tion. As VFAs were not measured in the SBAE it is not possible to rule out an accumulation. VFA accumulation might be a concern in the SBAE as small amounts of digester slurry were exposed to large amount of H_2, whereby the concentration of diluted H_2 could excess the inhibitory levels (Ahring and Westermann 1988). In previous studies of H_2 addition, H_2 was added in ratios corresponding to CO_2 production (Luo and Angelidaki 2012; Luo and Angelidaki 2013; Bassani et al. 2015), as was the case in H_2 adaptation experiment in laboratory scale reactors. Accumulation of VFAs could lower the pH of the slurry even though the natural buffer capacity will prevent it to some extent (Weiland 2010). As H_2 addition can increase the pH value by removing CO_2 from the slurry the pH is in risk of increasing, which would mask the VFA accumulation for a period. When attempting to adapt the methanogenic community it is therefore reasonable to monitor the VFA concentrations.

FISH and fluorescent microscopy

There were no successful hybridization and therefore no results of the FISH. It was not possible to see any methanogens through fluorescent microscopy as the background autofluorescent was too high in this environment.

Fluorescent in situ hybridization (FISH) was attempted on slurry from the anaerobic digester at Foulum experimental station. Hybridization was not achieved in this experiment, although FISH have been successfully preformed on anaerobic digester slurry before (Sekiguchi et al. 1999; Stabnikova et al. 2006). Performing FISH would have provided a visual estimation of the distribution of methanogens in the digester slurry, but for quantification of methanogens qPCR would have been suitable. qPCR would have allowed us to evaluate the MPRs in comparison to the density of the methanogenic community. FISH might have allowed us to evaluate upon the spatial location of the methanogens, and the proximity to other microorganisms. If a successful adaptation had occurred qPCR would have been a useful method of evaluating the community shift (Traversi et al. 2012).

Volatile Solids (VS)

VS were attempted measured by drying and igniting the organic material in the slurries. As VS were not measured right after sampling the resulting VS values might not be precise. The slurries were kept at 4°C from sampling to VS measurement, a time that varied from a week to months. It is hence not possible to conclude anything from these measurements. VS have been used as a way of characterize digester slurry, and is used when evaluating the biomethane protential (Raposo et al. 2012). VS is con-

sidered a useful indicator of possible methane yield, but varies with the source of organic material (Raposo et al. 2012).

Perspective

The experiments described in this report support that there is a possibility for H_2 upgrading of biogas, as has been observed previously (Luo et al. 2012; Luo and Angelidaki 2012; Wang et al. 2013; Bassani et al. 2015). It is still not certain that it possible to applie to a large scale reactor. Higher MPRs were achieved when H_2 was added to the headspace in laboratory experiment, but here mass transfer limitations were avoided by vigorous mixing of the slurry. Mixing of large scale reactors is expensive and the technology must be developed before H_2 upgrading can be relevant. The majority of anaerobic digesters are continuously stirred reactors (Weiland 2010). The stirring is set to mix the slurry and keep the tank homogeneous. A way of increasing the H_2 mass transfer in the large scale reactor could be by optimizing the H_2 addition to the reactor. Bassani et al. (2015) investigated the possibility of upfraing in a two stage set up, where H_2 was added to the second reactor. This could be an approach to hydrogen addition, but demands a second reactor. This could be a problem for smaller anaerobic digestion plants might only have one reactor. If H_2 could be added directly to the existing reactor, by some kind of add-on H_2 infuser, that would be ideal. Furthermore, in a second reactor the retention time of H_2 would be shorter, and re reactor would need to be fed continuously to avoid underfeeding the methanogens. H_2 addition would be more plausible if H_2 could be added periodically when excess energy would allow it. Possible VFA accumulation is not completely ruled out in the large scale reactor, despite of the good results from some of the laboratory experiments (Luo and Angelidaki 2013; Bassani et al. 2015). Further experimenting is needed to evaluate (how to avoid) VFA accumulation, pH increase and determine the desired amount of H_2 to be added. Additionally large scale experiments need to be conducted to evaluate these effects in working reactors.

Conclusion

H_2 addition to anaerobic digester slurry increased MPR compared to no H_2 addition. This was evident with slurry from Bånlev Biogas, Viby WWTP and Åby WWTP. Diluting the digester slurry did not produce as high MPR as undiluted slurry. Undiluted slurry from Bånlev Biogas with N_2/CO_2 headspace produced rates that matched the reactor MPR, and MPR over 1000% of reactor MPR with H_2/CO_2

headspace. This could not be replicated with slurry from Åby WWTP and Viby WWTP. Some of the decrease in MPR compared to reactor rate could be due to a lower amount of available acetate in the slurry. No adaptation to higher H_2 concentration was successfully illustrated by the potential activity assay. There was no difference between slurry submitted to H_2 and control slurry. Attempts for adaptation in serum bottles did not succeed as the methanogens were inhibited by increased pH value in the slurry. The effect of CO_2 concentration and acetate addition was investigated, and a tendency of higher MPRs at $CO_2\% \leq 12\%$ despite of decreasing pH values occurred, but no clear pattern was observed. Higher rates with added acetate were also evident.

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