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Biogas production at high ammonia levels

The importance of temperature and trace element supplementation on microbial communities

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Abstract

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In the pursuit for a fossil-free society, biogas production has recently attracted considerable interest. One reason for this is of course the clean and environmental friendly emissions this fuel leaves behind. Apart from this, the production of biogas acts as a way to treat our organic waste products and allows reusing the residues as fertilizer for farming. Therefore, it completes the recycling of nutrients. The methodology to produce biogas has been improved over time but many problems still remain.
Many biogas plants around the world are using high fat and protein rich substrates to achieve high quality biofuel, as well as, nutrient dense residues. In the meantime, they struggle with high ammonia levels which can cause instability of the biogas process. This thesis work has been focused on this specific issue.
Explicitly, different temperatures were investigated together with trace element additives to find optimal conditions for both methane production and process stability in lab-scale reactors operating at high ammonia levels.
A temperature shift from thermophilic (52°C) to mesophilic (37°C) process resulted in an ammonia drop from 0.80g/L to 0.07g/L which is a drop from potential toxic levels of ammonia to non-inhibitory levels. Such a change ensures a more reliable and stable process. However, the lower temperature caused the average daily biogas production to decrease by 8% in the same time as the substrate was decomposed to a lesser extent. Furthermore, at mesophilic temperature the process was more sensitive to overload compared to the thermo-philic process.
In the attempt to increase the abundance of syntrophic acetate oxidising bacteria (shown to be a key organism at high ammonia levels), the operating temperature was set to where they have their growth optimum. This resulted in a 20-fold higher abundance whereas no improvement of the gas production or ammonia content was achieved. The addition of trace metals on the other hand had a positive impact on the process performance. Both methane production and process stability were enhanced. Methane production was increased up to 30% when trace elements were added as well as disturbances by volatile fatty acid accumulations was lowered. One clue to this improvement was found from the quantification of methane producing archaea where the order of Methanomicrobiales was more abundant in reactors given trace elements.
In conclusion, adjusting temperature together with supplementing trace elements can help improve biogas production processes, but large-scale trials are needed.

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Populärvetenskaplig sammanfattning

Biogas är en blandning av, i huvudsak, metan och koldioxid vilka bildas genom anaerob (syrefri) mikrobiell nedbrytning av organiskt material. Denna process förekommer spontant i naturen, i bottensediment och risfält bland annat, men har utnyttjats av människan i kontrollerade former i ungefär 150 år. Biogastillverkningen har under de senaste årtionden växt till en av de mognaste förnyelsebara energiteknologierna och ses nu på allvar som ett viktigt alternativ till de fossila bränslena.

Sverige är ett land som arbetar flitigt för att bli oberoende av fossila bränslen. En av ambitionerna är till exempel att befria transportsektorn helt från fossila bränslen till år 2030 där biogasen ses som ett viktigt verktyg för att nå detta mål. Jämför man Sverige med andra europeiska länder så är Sverige en relativt liten producent av biogas än så länge. Däremot visar Biobränslebarometern 2013 gjord av EurObserv'ER att Sverige är det land i Europa som använder biogas som fordonsbränsle i störst utsträckning.

I biogasprocessen tas organiskt avfall om hand, såsom matavfall, boskapsgödsel och andra restprodukter från lantbruk, och bildar på så sätt en helt förnyelsebar energikälla som i slutändan kan användas till fordonsbränsle eller produktion av elektricitet och värme. Dessutom har biprodukten från biogasprocessen, s.k. rötrest, ett högt näringsvärde och kan därför med fördel återföras till lantbruket som biogödsel. På så sätt skapas också en hållbar näringscykel.

Själva nedbrytningsprocessen av organiskt material till biogas är mycket komplex och involverar hundratals olika arter av mikroorganismer som interagerar med varandra som i ett mikrobiellt samhälle. Varje typ av mikroorganism har sin unika roll i systemet och ställer sina egna krav på omgivande livsbetingelser. Allt ifrån pH och temperatur till krav på essentiella näringsämnen eller frånvaro av vissa inhibitoriska ämnen skiljer sig mellan de olika typerna av mikroorganismer. Med andra ord är det väldigt svårt att optimera tillväxtförhållandena för samtliga organismer samtidigt och därmed svårt att uppnå en optimal process.

Detta arbete har riktats till att studera och evaluera såväl processprestanda som mikrobiologin i biogasprocesser med höga ammoniakhalter. Anledningen är att proteinrika material anses vara ett attraktivt substrat (mat till biogasprocessen). Dels för att biogaspotentialen i dessa material är förhållandevis hög men också för att rötresten från ett sådant material blir extra rik på kväveföreningar, vilket gör den lämplig att använda som gödningsmedel. Dock finns en baksida med dessa material. När proteiner bryts ned frigörs ammoniak, en substans som inhiberar aktiviteten av de metanproducerande mikroorganismerna (metanogener) i processen. Dessa organismer är mycket känsliga, så det kan räcka med mycket små störningar för att skapa en kaskadeffekt som riskerar att hela processen kollapsar.

Målet med denna studie har därför varit att experimentellt alternera driftparametrarna hos biogasprocesser med högt kväveinnehåll för att hitta de optimala förutsättningarna som dels

ger en hög biogasproduktion men också medger en stabil och ohämmad process. Med hjälp av molekylära tekniker var syftet också att få en närmare förståelse för hur kopplingen mellan driftparametrar och kompositionen av det mikrobiella samhället hänger samman.

Resultaten från studien påvisar att en drifttemperatursänkning från termofil (52°C) till mesofil (37°C) är möjligt att göra i en redan uppstartad biogasprocess samt att detta dramatiskt sänker ammoniakhalten i reaktorn och minimerar därmed risken för hämning av de metanproducerande mikroorganismerna. Temperaturskiftet fick dock oönskade konsekvenser i form av en långsammare biogasproduktion, mindre effektiv nedbrytning av substratet samt en högre känslighet för ökad organisk belastning (matning).

Då man vet att syntrofa acetatoxiderande bakterier (SAOB) delvis eller helt kan ersätta de hämmade metanbildarnas funktion var en idé att justera processtemperaturen till den optimala tillväxttemperaturen för SAOB och på så sätt få en positiv effekt. Precis som man hoppats visade resultaten att SAOB ökade med cirka 20 gånger men utan synbar effekt på processprestandan.

Effekten av att tillsätta spårämnen i substratet var förvånadsvärt stora. Detta ökade metanproduktionen med så mycket som 30% jämfört med kontrollreaktorn som inte gavs spårämnen. En förklaring kan vara det ökade antalet metanbildare av släktet *Methanomicrobiales* eftersom detta bara sågs vid processen som gavs spårämnen.

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List of abbreviations

BMP – Biochemical methane potential

CSTR – Continuously stirred tank reactor

HRT – Hydraulic retention time, the time the substrate is staying inside the reactor

Nml – Normal millilitre, volume at standard temperature and pressure (0°C, 1 atm)

OLR – Organic loading rate, the amount of organic material given per litre and day

SAO – Syntrophic acetate oxidation

SAOB – Syntrophic acetate oxidising bacteria

TS – Total solids

VFA – Volatile fatty acids

VS – Volatile solids (i.e. organic fraction)

1 Introduction

1.1 The research question

The choice of substrate (or feed) used for biogas production is crucial for the amount and quality of the gas that will be produced. A favourable substrate is household waste and slaughterhouse waste because of their high protein and fat content (Baserga 1998). These substrates hold higher methane potentials than many other substrates and as protein is degraded nitrogen compounds are released. This contribute to a nutritious residue (also called digestate) which can be used as a bio-fertilizer in agriculture (Haraldsen *et al.* 2011). The major part of the nitrogen compounds released is ammonium and ammonia. Ammonium is relatively harmless to the microorganisms in the biogas process (Schnürer & Nordberg 2008, Sprott & Patel 1986) while ammonia has an inhibitory effect on important microbes involved in biogas formation, even at low concentrations i.e. above 30mg l^{-1} (Fricke *et al.* 2007). One way to control the ammonia level is to displace the equilibrium between ammonia and ammonium towards ammonium by lowering the temperature. However, lower temperature may instead slow down the microbial activity and thus reduce the biogas production rate.

Studying the microbial community is a necessity for understanding how this system works, especially which environmental conditions that are beneficial for the microorganisms to maximize the gas production efficiency. The microbial community structure of a biogas process is dependent on both the origin of the inoculum, as well as, the process parameters (e.g. substrate composition and retention time) and environmental parameters (e.g. temperature and pH). Changes of such parameters lead to changes in microbial community structure and as long as the parameters are altered gradually the community will have time to adapt and find a new steady state.

Concerning this project, concentration of ammonia has shown to be an important parameter that affects the structure of microbial communities. When the ammonia reaches above a certain level (i.e. 128-330mg l^{-1}, Schnürer & Jarvis 2010) an alternative pathway is opened, the syntrophic acetate oxidising (SAO) pathway, which was first proposed by Barker (1936). At high ammonia levels, aceticlastic methanogens are most easily disturbed and cannot continue to degrade acetate into methane. Instead, acetate is cleaved into hydrogen and carbon dioxide by the high ammonia tolerant syntrophic acetate oxidising bacteria (SAOB) to further be converted to methane by the hydrogenotrophic methanogens (Figure 1).

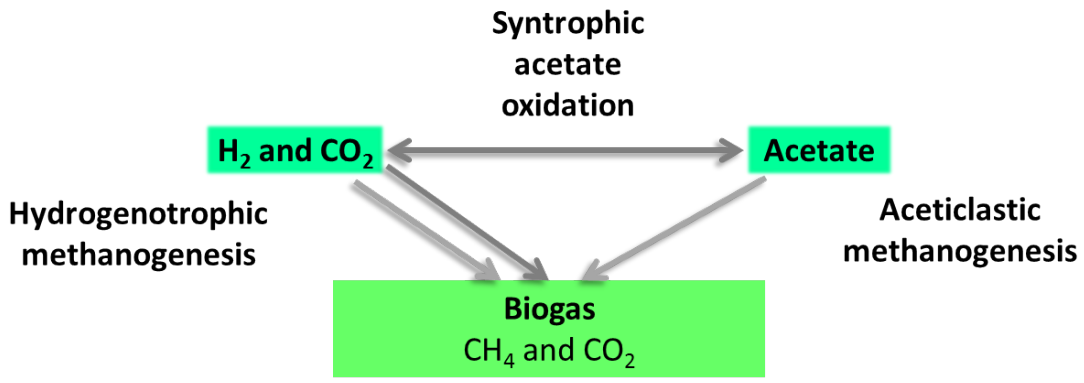

Figure 1.*The three different methane formation pathways, hydrogenotrophic, aceticlastic and the syntrophic acetate oxidising pathway.*

This SAO pathway is very important for methane formation at high ammonia levels since as much as 70% of the biogas produced originates from acetate (Kotsyurbenko *et al.* 2004). For the reaction to go in the right direction SAOB requires a close cooperation with hydrogen-consuming methanogens, as long as the hydrogen levels are kept low the SAOB will continue splitting acetate into hydrogen and carbon dioxide (Schink 1997, Schink 2002, Stams 1994). Today, a lot is known about the SAOB, but more research is required in order to control the biogas process at high ammonia levels.

1.2 Biogas as an energy source

The production of renewable energy has become an important issue because of our changing climate. Focus has long been on the generation of electricity through environmentally friendly processes like wind, water, and solar power plants. Along with electricity, the society is also highly dependent on energy carriers in terms of fuels. The extensive transport sector, for example, is one big climate problem which cannot be replaced with electricity in a near future.

One biofuel that has been developed and produced successfully at full scales is biogas, a gas mixture of methane and carbon dioxide with high energy content. Biogas is a completely renewable fuel with no net addition of carbon dioxide. When combusted, it also has the lowest emission of particles and greenhouse gases of all commercial fuels (Börjesson & Berglund 2003).

Biogas is produced through anaerobic digestion of almost any organic material (except woody materials and plastics). Preferably, biogas is produced from waste products such as sludge from waste water treatment plants and household wastes but also from many other sources of organic wastes. The advantage of using waste products as substrates is that it encourages a more controlled waste disposal and, furthermore, the rest products from the biogas process are rich in phosphorous as well as nitrogen that act as an excellent biofertilizer for agriculture (Arthurson 2009). In this way, we can replace the use of artificial fertilizers and thereby further reduce greenhouse gas emissions. Natural fertilizers, such as different kinds of manure, also acts as a climate change villain when it is stored since it releases large amount of me-

thane gas into the atmosphere. By taking advantage of the manure through anaerobic fermentation instead, valuable methane can be utilised and the fertilizing properties will be remained (IEA-Bioenergy 2010).

1.3 Biogas in Sweden and Europe

In the past few years, Sweden has made efforts to build and develop biogas plants and today Sweden is seen as a pioneer in the process of refining biogas to vehicle fuel quality. Other countries in Europe have also invested in the production of biogas. Germany alone has over 7200 large-scale biogas plants that produce more biogas-recovered energy than all the rest of the European countries put together. However, the usage of gas differs from Sweden. The major part of biogas produced is Germany, and many other European countries, is used for electricity and heat production while Sweden primarily uses the gas for vehicle fuel refinement (EurObserv'ER 2012).

The development potential is still large, not least in Sweden, where new projects are constantly being initiated. Expanding the national gas pipeline system along with the development of techniques for thermal gasification from forest industry by-products seem to be the most important steps in future development (SGC 2013). Biogas seems to have a bright future, much research and development are taking place throughout Europe and long-term goals are set to achieve a sustainable society (Biogasportalen 2013).

1.4 Biochemical process

The biochemical activity during biogas processing is very complex and far from being fully understood. The process involves hundreds of different microorganisms from all kinds of genera, each with unique metabolic systems and importance for the biogas process. These microbes are together responsible for the entire biochemical decomposition chain (Figure 2), from macromolecules (e.g. protein and carbohydrates) to the final products (i.e. methane and carbon dioxide).

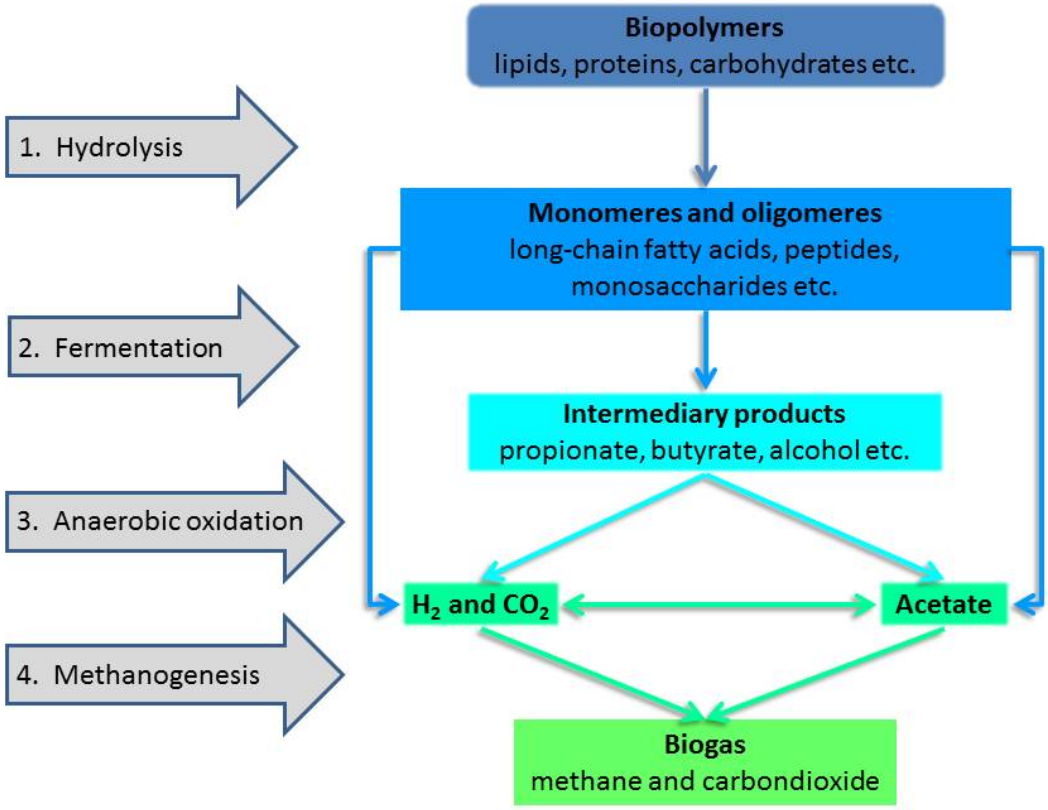

Figure 2. *Flow scheme of the degradation chain in the biogas process (modified from Weiland 2010).*

1.4.1 Hydrolysis

Hydrolysis refers to the depolymerisation of large insoluble organic compounds, such as carbohydrates, lipids and proteins, into more soluble oligomers or monomers e.g. monosaccharides, long chain fatty acids and amino acids respectively (Conrad 1999, Parawira *et al.* 2008). Hydrolysis is mediated by extracellular enzymes, either attached to the surface of bacteria or secreted into the external environment by a complex consortium of microorganisms (Angelidaki *et al.* 2011). A common feature of these organisms is that they generally are strictly anaerobic bacteria, such as *Clostridia* or *Bacteriocides* (Weiland 2010). Due to the complexity of the organic macromolecules, this step is considered the slowest and therefore the rate limiting step (Angelidaki *et al.* 2011). The degradation rate is highly dependent on substrate composition, but generally fats, cellulose and proteins are decomposed slowly while certain carbohydrates, such as starch, are more easily broken down. There are many ways to speed up this step, which has been examined in several studies. For example, pre-treatment of the substrate has proven to be a simple solution with clear improvements of speeding up the hydrolysis. Methods that use ultrasound or microwaves to increase the biodegradability look promising, but the best pre-treatment method so far is mechanical grinding of the substrate along with thermal processing at 70°C (Appels *et al.* 2013, Chang *et al.* 2011, Nges & Liu 2009).

1.4.2 Fermentation

Biochemically, fermentation is defined as the conversion of organic materials in absence of any inorganic electron acceptors e.g. sulphate, nitrate and oxygen (Angelidaki *et al.* 2011). In this stage, the by-products (monosaccharides, long-chain fatty acids and peptides) from hydrolysis are used as substrate by a broad spectrum of bacteria, many of them participating in both hydrolysis and fermentation (Weiland 2010).

The amino acids undergo the conversion into different organic acids, ammonia and hydrogen during fermentation. The saccharides are also converted into organic acids, from two carbon compounds to as long as six carbon compounds. The majority of the fermentation products consist of acetate, butyrate and ethanol but other acids could form as well depending on microbial community (Angelidaki *et al.* 2011). The long-chain fatty acids, on the other hand, are rarely fermented but can be further processed in the next stage of the process, i.e. anaerobic oxidation (Schnürer & Jarvis 2010).

1.4.3 Anaerobic oxidation

The next step in the biochemical food chain is the anaerobic oxidation. Byproducts from the previous step, i.e. volatile fatty acids and alcohols, are converted into acetate, hydrogen and CO_2. Microorganisms responsible for these reactions are most often obligate hydrogen-producing acetogenic bacteria, which are not well characterized in the literature (Weiland 2010). What we do know though, is that this group of bacteria is very sensitive to hydrogen it produces (i.e. the hydrogen inhibits their own bacterial metabolism due to thermodynamic reasons). To get around this problem, these acetogenic bacteria work in close contact with another microorganisms whose function is to consume hydrogen and produce methane (Schnürer & Jarvis 2010, Weiland 2010). These microbes are further described in the next and final stage of the process.

1.4.4 Methanogenesis

The main producers of methane are microorganisms of the phylogenetic domain Archaea. They are called methanogens and are found naturally in freshwater sediment, landfills and in intestinal tracts of many animals, including 1/3 of the human population (Miller & Wolin 1986). Similar to the previous steps, the product formed in the earlier step serves as a substrate; in this case methanogens assimilate hydrogen, carbon dioxide and acetate from the anaerobic oxidation stage to ultimately form methane and carbon dioxide. The conversion of hydrogen and carbon dioxide into methane follows a different pathway from the decomposition of acetate to methane. Both these pathways are thus carried out by different groups of methanogens: hydrogenotrophic and acetotrophic (Angelidaki *et al.* 2011, Schnürer & Jarvis 2010). Both pathways are important for methanogenesis, but studies have shown that the acetotrophic pathway often dominates and can represent as much as 70% of the biogas produced in a biogas reactor (Angelidaki *et al.* 2011, Schnürer & Jarvis 2010). Up until now, six phylogenetic orders of methanogens has been characterized. Among these, only two of the groups are known to utilize the acetotrophic pathway, namely *Methanosaeta* and *Methanosarcina*. Even though these acetotrophic methanogens use similar metabolic pathways, they do not compete with each other. Instead they have developed their own special niches; *Meth-*

anosaeta grow better and dominate when the acetate level is sufficiently low, i.e. under 20 mg/L (Zinder 1993), and *Methanosarcina* dominate when the value reaches above this limit. The disadvantage of being able to survive at low substrate levels is that the growth rate becomes slower (Schnürer & Jarvis 2010), but the advantage of *Methanosaeta* is that it utilize acetate more effectively and provide a more stable digestion (Conklin *et al.* 2006). In contrast to aerobic microorganisms, anaerobic microbes (especially methanogenic archaea) normally have a longer regeneration time because they can only convert a small fraction of the energy content of the substrate into biomass. In turn, all the remaining energy is stored in reduced products such as methane, ethanol, hydrogen and various volatile fatty acids (Angelidaki *et al.* 2011). It is not unusual with regeneration times of around 10 days for methanogens. This fact makes it impossible to have a hydraulic retention time shorter than 10-15 days (depending on operation temperature) without risking washing out these microbes. A further consequence of the long generation time of methanogens is that a reactor start-up becomes very slow. At least 3 months are required before a strong and stable microbial community structure can be reached (Deublein & Steinhauser 2008).

In general, the methanogens are also the most sensitive consortia in the biogas process. When changes in pH, temperature or when contaminants enter the process, methanogens is the first to be influenced by the change. This sensitivity is due to the large differences in cell structure among archaea compared to bacteria. For instance, archaea often lack an outer cell membrane. The slow growth of the methanogens also contributes to the sensitivity, which is why the methanogenisis step is regarded as the rate limiting step in the biogas process (Schnürer & Jarvis 2010).

1.5 Important process parameters

1.5.1 Substrate

All the chemical substances together that are needed to maintain vitality of a microbe is called a substrate. In other words, a substrate is the microbial food. To be a useful substrate for microbes it must contain both energy-rich substances for survival and building blocks for proliferation. Metals, minerals and vitamins are also important for carrying out chemical reactions and to stabilize certain enzymes for example. Because of the complex microbial flora involved in a biogas process, a very versatile substrate is required to fulfil all nutrient needs. By maintaining a high complexity of the substrate a greater biodiversity is allowed, this in turn makes the microbial community more resilient to changes of external parameters i.e. a more stable process is achieved. Also, if the complexity of the substrate is reduced the overall biodiversity tends to be reduced as well (Schnürer & Jarvis 2010). Besides being versatile and nutrient rich, a substrate should also be easily digested for effective biogas production. The overall methane potential a certain substrate carries (using a certain inoculum) can be revealed in a simple test described below.

1.5.2 Biochemical methane potential

A biochemical methane potential test, or BMP, is a standard method to determine the maximum amount of methane produced from a certain type of substrate-inoculum pair. The sub-

strate is only added once and allows the possibility to follow gas production from beginning until end when the substrate is not viable anymore. Furthermore, through the batch test the methane production rate can be determined, as well as, indicate a suitable hydraulic retention time (HRT) for the process. These tests are generally recommended before the start of a new continuous biogas process, or when substrate compositions are changed during biogas processing, in order to give the best possible operational conditions. Below is a list of common substrate components and their BMP, see Table 1.

Table 1. Methane potential of common substrate components (information adopted from Angelidaki & Sanders (2004)).

Material	**Methane potential** [Nml gVS^{-1}]	**CH_4 in biogas** [%]	**CO_2 in biogas** [%]
Carbohydrates	415	50	50
Proteins	496	50	50
Lipids	1 014	70	30

N – Normal temperature (0 °C) and pressure (1 atm).

1.5.3 Digester type

In the choice of digester type there are essentially two basic principles: continuous stirred tank reactor (CSTR) or batch reactor. The difference between these lies in how frequent the substrate is added and withdrawn. A batch process is the most straightforward type; substrate is added only once when the digester is started. Gas produced are let out to be collected, otherwise the reactor is isolated from the external environment. Eventually, the substrate will be consumed and the gas production will cease, then a new batch can be started. Understandably, this technique will make the biogas production irregular and staccato and thus not suitable for large-scale plants. For a smoother biogas production modifications of the batch reactor can be applied. This involves several digesters that are at different stages of the digestion process. Most commonly used in large-scale biogas plants though, is the CSTR. Here substrate is continuously (e.g. once to several times a day) fed to the reactor while material simultaneously is drained. Consequently, this means that the microbial growth does not reach a steady state, but keeps within the exponential growth phase and eliminates the risk of inhibition by overpopulation. The main cost for having a smoother gas production with a CSTR is that the substrate digestion is less complete due to the shorter time inside the reactor. Moreover, the CSTR also requires an increased frequency of monitoring since the substrate added can differ slightly from time to time and cause internal environmental changes e.g. pH-value, VFA accumulations and nutrient availability (Schnürer & Jarvis 2010).

1.5.4 Organic loading rate

Organic loading rate (OLR) is the amount of degradable material that is fed to the digester per unit of time and digester volume. Grams of volatile solids (i.e. organic matter) per litre digester and day (g VS l^{-1} day^{-1}) are often used as a standard unit.

The organic loading rate is selected depending on substrate composition, viscosity and reactor design, as well as, biogas production profile that is sought. To achieve optimum organic loading rates, a balance between feeding rate and microbial growth rate must be found. An OLR higher than what the microorganisms are capable of degrading can lead to disturbances of the process. Methanogens, which are slow in their work, could simply not decompose acetate and other volatile fatty acids at the rate which they are formed. This leads to an accumulation of VFA that lower the pH dramatically and ultimately inhibit the biogas process. Also, the OLR must not be too low, as the microbes do not get enough food to maintain their growth rate and are ultimately washed out. In that sense, the OLR is dependent of the process temperature as well (Mattocks 1984, Schnürer & Jarvis 2010). In order to meet these criteria, the knowledge of VS content of the substrate is of essential importance, this is discussed in a later paragraph.

1.5.5 Hydraulic retention time

The average time the substrate stays inside the digester refers to the hydraulic retention time (HRT). This parameter is closely coupled with OLR since a greater feeding rate results in a fast replacement of the reactor volume and thus a shorter HRT is obtained. Except for changing the OLR the hydraulic retention time can be altered through changes in TS of the substrate, simply by diluting or dewatering the substrate. The HRT can vary from 10 to 60 days (Mattocks 1984) depending on the substrate composition as well as reactor size and working temperature (Schnürer & Jarvis 2010). If the substrate has a high content of easily decomposable substances, like carbohydrates, the HRT is allowed to be very short and then the organic loading rate can be increased which results in a very high biogas production rate. Substrate containing a larger portion of fibres, such as cellulose, will be harder for the microorganisms to digest and thus requires a longer retention time. To avoid washing out microorganisms, the HRT should exceed at least 2 times the doubling time of the slowest growing microorganism. An alternative method is to add inert structures (i.e. biofilm carrier, see Figure 3) that allow immobilization of microorganisms which in turn enables a low HRT without the risk of washing species out (Angelidaki *et al.* 2011, Weiland 2010). The HRT can be calculated using the equation below.

Figure 3. *Carrier material for immobilization of microorganisms.*

$$\boldsymbol{HRT}[days] = \frac{\boldsymbol{V_{active}}\ [ml]}{\boldsymbol{V_{daily\ feed}}\ [ml\ day^{-1}]} \qquad \textbf{(eq. 1)}$$

1.5.6 Total solids and volatile solids

For convenience and simplicity of comparison, standard methods have been introduced by American public health association, APHA, for the measurements of total solids and volatile solids or TS and VS for short (APHA 2005). These measurements are commonly used for describing the content of solid materials in substrates but can also be applied to digestates. When comparing biogas performances, values are often normalised to a VS basis.

The TS is defined as the total proportion of solids in the substrate, both inorganic and organic, while VS only includes the organic part of the solids. Following equations describe the determination of these factors:

$$TS = \frac{m_{dry}}{m_{wet}} \quad \textbf{(eq. 2)}$$

where m_{wet} and m_{dry} is the mass of the raw substrate and the dried sample respectively.

$$VS = TS - \frac{m_{ash}}{m_{wet}} \quad \textbf{(eq. 3)}$$

where m_{ash} is the mass of all inorganic residues after annealing at 550 °C.

It is important to know these values when determining or adjusting the OLR, as well as, the HRT of a biogas reactor. A very wet substrate for example has a small VS and TS and to reach a certain loading rate, a greater volume has to be used than if the substrate would have had higher VS. Of course the substrate could either be diluted or dehydrated to achieve the desired VS and TS which would decrease or increase these values respectively.

1.5.7 Degree of degradation

The degree of degradation is a measure of the proportion of organic material that is converted to (and emitted) as gas from a certain substrate. It is a useful parameter to consider when comparing the efficiencies of different inoculums or if you only want to know how well the inoculum degrades a specific substrate. A common way to measure the degree of degradation is to take the difference of VS of the substrate and VS of the digestate, which represents the total loss of organic material during the process. Comparing with the VS data from the substrate will then give us the proportion of digested material. Mathematically it could be presented as the following equation:

$$Degree\ of\ degradation = 1 - \frac{VS_{digestate}}{VS_{substrate}} \quad \textbf{(eq. 4)}$$

where $VS_{substrate}$ is the mass of the organic part in the substrate and $VS_{digestate}$ is the mass of the organic part in the fermentation residue.

1.5.8 Nitrogen content

The substrate used in an anaerobic digester plays a decisive role for the gas composition and the intermediate products that will be formed in the process. Using protein-rich materials,

such as slaughterhouse wastes and household wastes, will release large amounts of nitrogen compounds (mainly ammonia and ammonium) during the degradation process. Ammonia has shown to have an inhibitory effect on microorganisms involved in methane production, even at very low concentrations (30 mg l^{-1} (Fricke *et al.* 2007)), whereas ammonium is harmless until it reaches much higher concentrations. These two compounds are in equilibrium, but can be displaced by changes in pH and temperature, as equation 5 below describes. As the temperature increases, the equilibrium will shift towards ammonia which also occurs when pH elevates (Fricke *et al.* 2007), see figure 4. The reason for the inhibition is not yet fully understood but Sprott & Patel (1986) refers to the explanation that the ammonia (NH_3), which is uncharged, can penetrate the cell wall freely and inside the cell, ammonia is converted to ammonium (NH_4^+) with the addition of a proton. The cell will then actively pump protons into the cell to maintain the natural pH and this active transport is done in exchange for potassium ions are pumped out. Ultimately, this leads to the lack of potassium inside the cell and the activity will drop. At what concentration the activity will fall depends on the organism since the amount of potassium in their natural state differs.

$$NH_3\ [g\ L^{-1}] = \frac{NH_4^+ - N\ [g\ L^{-1}]}{(1 + 10^{(pKa - pH)})} \qquad \textbf{(eq.5)}$$

$$pKa = 0.09018 + \frac{2729.92}{T\ [K]}$$

Where the pKa value is the dissociation constant for ammonium (NH_4^+).

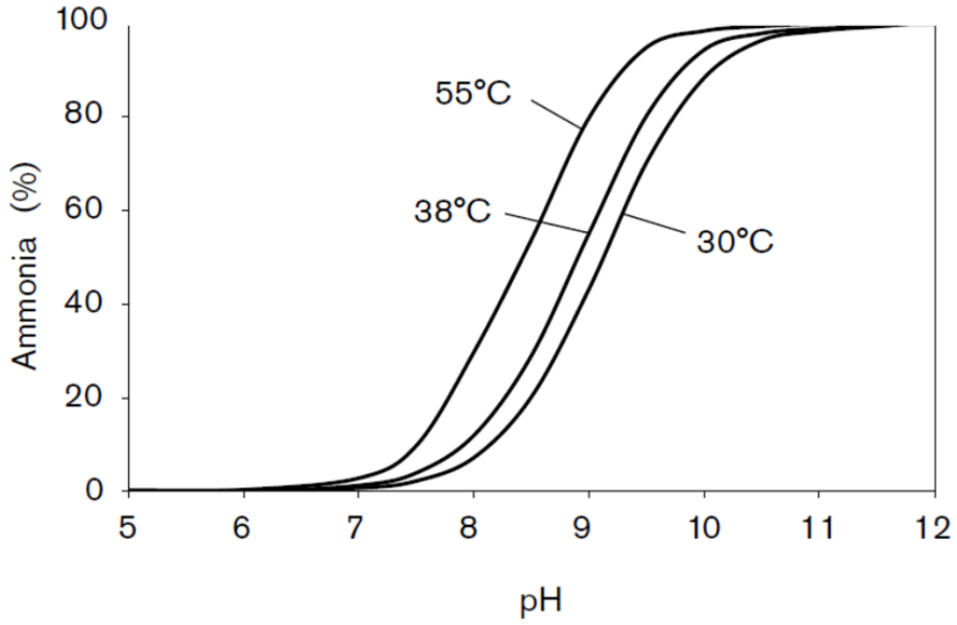

Figure 4. *Temperature and pH affecting the ammonia/ammonium ratio. Increasing pH results in a higher proportion of ammonia, a higher temperature also results in a larger proportion of ammonia. Figure modified from Fricke et al. (2007).*

1.5.9 pH and light

For methane to be formed, the pH must be carefully controlled. Within the pH range of 6.5 and 8.5 these reactions can be performed, but optimally the pH is held between 7 and 8. Typically, the substrate has a sufficient buffering capacity and could maintain a stable pH during temporary process disturbances, such as VFA accumulations and ammonia accumulations (Weiland 2010). Another parameter to consider when operating a biogas process is to keep the system in darkness. UV light is of course lethal for microorganisms but also other wavelength can inhibit the activity of methanogens (Deublein & Steinhauser 2008). Some cofactors, such as F420 and F430, are important for cell growth in methanogenic archaea and can become granulated and inhibited upon light stress (Olson *et al.* 1991).

1.5.10 Temperature

Most biogas processes operate at temperatures of around 37°C (mesophilic) or around 55°C (thermophilic). Microorganisms involved in the digestion have different temperatures for optimum growth depending on species, but can normally grow in a broad temperature range. When a microbe population structure is stabilized to a certain temperature, it is usually not recommended to have larger temperature oscillations than two or three degree Celsius. As mentioned before, methanogenic microbes are the first to be affected by changes of operating parameters and this is also true for the temperature. To keep methanogens active, it is important to maintain a constant, but also uniformly distributed temperature (Schnürer & Jarvis 2010).

1.5.11 Trace elements

In anaerobic digestion, as well as in all other living systems the process relies on the work of proteins. Many proteins, like enzymes, need help from coenzymes or cofactors to work properly or to contribute to their catalytic ability and in the methane formation process (van der Veen *et al.* 2007). For an example, trace elements, such as Co, Ni and Fe, are important cofactors of carbon dioxide dehydrogenase (CODH), which is found in all methanogens. Also, acetogenic bacteria need these cofactors to produce acetate, as well as the acetate-utilising methanogens for decomposition of acetate (Hattori *et al.* 2005). The very small quantities needed of these metals for proper use is why they are called trace elements.

Many studies have showed positive effects on the reactor performance (e.g. higher gas production as well as a higher degree of degradation) by the addition of trace elements (Feng *et al.* 2010, Jarvis *et al.* 1997, Kida *et al.* 2001, Zandvoort *et al.* 2006) and according to Karlsson *et al.* (2012) the trace elements improved both methane yield and lowered the accumulation of acetate and propionate. Ultimately, lower VFA enables a higher OLR to be used and even higher methane could be produced.

1.5.12 Sulphur compounds

As protein rich materials are fermented, a large amount of sulphide is released. This dianion of sulphur has a high capability of binding and precipitating heavy metals. In wastewater treatment plants, this is an important reaction and is used as a treatment to reduce the high

concentration of dissolved heavy metals. For other types of anaerobic digesters, metals are important micronutrients (trace elements) for microbes and required level should be met. It is important to inactivate sulphide and a proved method to do this is by adding metals, such as iron, in excess in order to be precipitated with the sulphide, which makes the other trace elements bioavailable (van der Veen *et al.* 2007).

Sulphide also forms a complex with hydrogen to make hydrogen sulphide, H_2S, which is the major contaminant in biogas production. Hydrogen sulphide has extreme corrosive effects on metal parts, an effect which is enhanced by elevated temperatures and when mixed with water (IEA-Bioenergy). When combusted hydrogen sulphur also turns into a toxic compound: sulphur dioxide, SO_2 (Cherosky & Li 2013). Therefore, before biogas could be used as fuel the gas had to be purified from H_2S to avoid corrosion damages. A number of methods have been published to remove hydrogen sulphide in large scale, including physical, chemical and biological methods (Horikawa *et al.* 2004, Nishimura & Yoda 1997, Yuan & Bandosz 2007). A common way is to utilise the water solubility properties and simply bubbling the biogas through water, which means that only methane remains because both H_2S and CO_2 have a higher water solubility than methane, this refining method is referred to as water scrubbing (IEA-Bioenergy).

2 Thesis objectives

The main goal of this study was to evaluate the importance and the effect of temperature and trace elements in the processes operating at high ammonia levels. In this attempt, two different set-ups was used which divides this thesis into two parts.

2.1 Implementations and limitations

The first part (**Approach 1**) of this work was to start a thermophilic (52°C) biogas process, with the large-scale biogas plant of Uppsala as a model reactor. Then, gradually decrease the temperature to mesophilic conditions. By this change, the proportion of ammonia in relation to ammonium would theoretically decrease and result in less toxic effects on the microorganisms inside the reactor.

The second part (**Approach 2**) focuses on a mesophilic biogas process (37°C) at high ammonia level. This process was subjected three trials. In the first, the process was given additions of trace elements which have been reported to be an effective method to avoid problems associated with high levels of ammonia. In the second, the same process was subjected of a temperature change to 42°C which was chosen because the SAOB has shown its optimum growth at that temperature. In the third, both of these changes were induced to see a potential synergy effect.

The microbial community structure involved in methane formation was determined and compared between these processes. By this mean, the existence of correlations between microbial structure and reactor performance will also be determined.

The work was performed at SLU (Swedish University of Agricultural Sciences) in Uppsala, at the department of Microbiology. Lab-scale biogas reactors were used to maintain continuous long-term experiments. During the experiments the reactor performance was monitored with various chemical analyses. To limit the work, only 8 groups of microorganisms were selected to be quantified and followed over time by real-time quantitative PCR, 5 groups of methane producers and 3 groups of SAOB, were chosen because they all play an important role in the methane formation step of the process. Since the time of this master thesis was limited (20 weeks), data and samples from earlier time points had to be collected to obtain a sufficiently large data set. For the same reason, the experiments were forced to be closed within this timeframe.

3 Materials and methods

3.1 Anaerobic digester

For running the anaerobic digesters or biogas reactors a continuously stirred tank reactor system (Belach Bioteknik AB, Sweden) was used. The digester chambers have a total volume of 8 litres which allows 5 litres of active volume to be used. The reactors were connected to a computer where temperature and stirring speed can be controlled and the gas production can be read. Since it is a continuously process that is being sought, the digester is equipped with an inlet valve where substrate goes in and an outlet valve where digestate can be taken out to maintain a constant internal volume. For proper stirring of the digester an impeller together with a baffle was used (Figure 5). The substrate and inoculum was collected from the commercial co-digestion biogas plant of Uppsala Vatten och Avfall AB. The substrate consists of a mixture of sorted household waste and slaughterhouse waste.

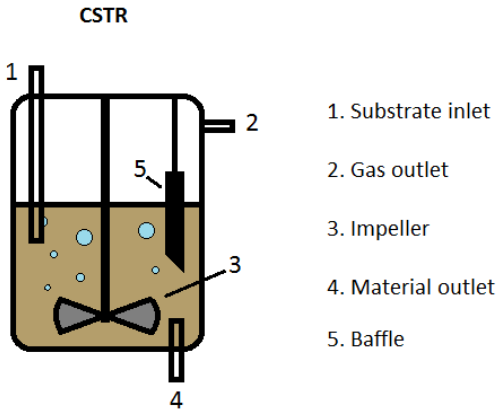

Figure 5. *Schematic illustration of the core components in a CSTR.*

3.1.1 Approach 1 – Equilibrium displacement

To start these CSTRs, 5L of the inoculum was put into the 8L lab-scale bioreactor, from here on called GP2. Next step was to adjust the process parameters to achieve the same performance as the large-scale plant to ensure a representative starting point.

With all process parameters adjusted, a period of observation and maintenance of the reactor performance was needed. The digesters were fed semi-continuously (once a day). At the same frequencies, digestate was discarded to keep a constant digester volume and carbon dioxide and gas production was measured. At a weekly basis, digester samples were analysed for volatile fatty acids and pH.

A temperature drop of two degrees per week was decided based on the temperature difference and experimental period. Such temperature change is not too fast, risking the microorganisms to go into shock, but still quickly enough to finish the experiment within the indicated timeframe of the project.

3.1.1.1 Parameter settings

TS of the substrate used for GP2 was determined by heating a pre-weighted sample of the substrate in a low temperature oven at 105°C over night. The dry sample was then weighted again to obtain the dry mass and used to calculate the TS ratio with equation 1. However, any volatile fatty acids are inevitably evaporated during this process, although these should be included in the TS content. This error was to be corrected when the VFA content analysis was determined. Triplicate sampling was made to give a more reliable mean value; this is summarized in table 1.

To obtain the VS-value of the substrate the dry sample from TS measuring was subjected to a high-temperature oven where all the organic compounds were vaporized at 550°C for 12 hours, leaving only the fixed solids behind such as sand, salt and other inorganic materials. The inorganic ash weights were then inserted to equation 2 to calculate the VS ratio.

The nitrogen level inside the reactor was analysed using the Foss Tecator AN 30D method.

The degree of degradation was calculated based on reactor digestate samples and substrate samples. Both samples were subjected to a TS-analysis followed by a VS-analysis (the procedure is the same as described in section “Determine TS and VS of the substrate”) from which the degree of degradation was calculated by equation 3.

3.1.1.2 Load increase study

As the GP2 reactor reached operational stability at mesophilic temperature a load increase study was performed to determine the load tolerance. The OLR was increased from 3 to 6 gVS l^{-1} day^{-1} gradually with 0.25 gVS every third day. For stability verification, the process was kept at constant OLR for 1 HRT after each unit of gVS increased (i.e. at an OLR of 4, 5 and 6 gVS).

3.1.2 Approach 2 – SAOB enrichment and trace element additives

A total of four digesters were operating simultaneously at two different temperatures, a reference temperature of 37°C and a SAOB-optimised temperature of 42°C. At both temperatures one reactor was given trace element supplementations while the other was not. Remaining parameters were kept constant between the reactors (Table 2). The trace element mixtures used were KEMIRA BDP-820 and KEMIRA BDP-Start-up 100. Those were both mixed with the substrate to a concentration of 1% w/w. Inoculum was taken from reactors of an earlier study (Schnürer & Nordberg 2008) and household wastes were given as substrate.

Table 2. Initial parameter values of the four biogas reactors. HW – Household waste.

Reactor	**Operating temperature** [°C]	**Trace elements**	**Substrate type**	**OLR** [gVS l^{-1} day^{-1}]	**HRT** [days]	**Ammonium -nitrogen** [g/l]
GR1	42	Added	HW	3	30	5
GR2	37	Added	HW	3	30	5
GQ1	42	Not added	HW	3	30	5
GQ2	37	Not added	HW	3	30	5

The initial start-up period lasted for 60 days (2 HRT). During this time the OLR was slowly increased to 3 g VS l^{-1} day^{-1} and was then kept constant. After this period, trace elements were added to two of the four reactors, GR1 and GR2. After another 150 days of operation, additional iron were given to GR1 and GR2 to precipitate potential sulphides (see section "Sulphur compounds") over an additional 30 days. Throughout this period, 8 HRT in total, the microbial communities had the chance to stabilize and acclimate to the new environments.

3.2 VFA analysis

Volatile fatty acids were analysed by high-performance liquid chromatography (HPLC), Agilent 1100 Series HPLC with a refractive index detector and an ion exclusion column (Rezex ROA – Organic Acid H+, 300 x 7.80 mm). The mobile phase used was 0.005M H_2SO_4 and a flow rate of 0.6 ml min^{-1} was used. For VFA preparation, 2ml raw digester sample was centrifuged at 14 000 rcf for 15 minutes, then the supernatant was transferred to a new 2ml tube and then frozen for at least 4 hours to help precipitation. The sample was thawed and spun down again at 14 000 rcf for 10 minutes this time, 450µl of the supernatant was collected and transferred to a new 2ml tube. For further precipitation, 50µl of 5M H_2SO_4 was added and the sample was centrifuged again at 14 000 rcf for 7 minutes. Supernatant was collected and transferred to a glass vial to be analysed in HPLC.

3.3 Gas analysis

The CO_2, CH_4 and H_2S content of the gas produced by the digesters and batches were analysed. Carbon dioxide was measured by letting a gas sample of 5ml bubbling trough sodium hydroxide (NaOH, 7M), the carbon dioxide dissolved in the liquid while the remaining gas volume was measured. The difference between input volume and remaining volume is the volume of carbon dioxide. A gas chromatograph was used for determining the methane content, 2ml gas sample was analysed in a PerkinElmerARNEL Clarus 500 gas chromatograph with flame ionization detector. The mobile phase used was N_2 that transfers the sample through an ELITE-PLOT Q column (length 30m and diameter 0.53mm). For hydrogen sulphur analysis the gas analyser GA 2000 (Geotechnical Instrument) was used.

3.4 Molecular methods

DNA extraction of bioreactor samples were carried out using FastDNA® SPIN Kit for Soil (MP Biomedicals) according to the manufacturer´s instructions. This method involves cell lysis through mechanical force, purification through multiple steps of centrifugations to get rid of cell debris and filter centrifugations to bind the DNA. Finally the purified DNA was eluted with DNase free water.

Polymerase chain reaction, PCR, was used extensively during the project. For example, to amplify DNA fragment and to find optimal annealing temperatures for new designed primers. BIO-RAD C1000™ Thermal Cycler was the machine of choice which could handle a 5°C temperature gradient. DreamTaq green master mix from Thermo Scientific and the Ready to go beads from GE were both used for PCR. 1pmol of each primer, about 20ng template DNA and dH_2O were added to the total volume of 25µl PCR reaction mixture.

Agarose gel electrophoresis was used to detect DNA fragments from PCR reactions and confirm its length but also to check for impurities like unspecific PCR product and primer dimers. An agarose concentration of 1 - 2% mixed with 0.5x TBE buffer formed the gel. At all times GeneRuler™ 1kb DNA ladder flanked the row of samples.

In order to obtain sufficient purity for sequencing; PCR product was purified with QIAquick® PCR Purification Kit (QIAGEN) according to the manufacturer´s instructions.

3.5 Microbe quantification

For the molecular investigation, samples from the four bioreactors were continuously taken during the experimental period to allow detection of changes of various kinds through molecular assays. From these samples, DNA was extracted and was subsequently used for qPCR analysis. Triplicate extractions were done from each sampling point in order to neglect the importance of random deviations. Each replicate was also diluted to different levels (10, 100, 500 and 1000 times) to ensure detection in the measuring range, but also to detect any poten-

tial PCR inhibition. If gene abundance results to be lower in lower diluted samples, it suggests inhibitory factors were present and a higher dilution would be chosen.

For the qPCR machine to do a quantitative estimation of the samples; a well-made standard curve is required as a reference. This consists of previously purified PCR product ligated into pGEM vectors with known copy number (10^8 copies per µl). Ten times dilutions of this stock solution made up the standard curve series (10^8, 10^7, 10^6 and so forth down to 10^0 copies per µl).

Predesigned primers, targeting the 16S ribosomal RNA gene of each species or methanogenic group, were used for the qPCR reaction (summarized in Table 3). The qPCR protocol is described in (Westerholm 2012) and consists of an initial temperature phase at 95°C for 7 minutes, followed by 55 cycles of the denaturation phase at 95°C for 40 seconds, annealing phase (primer specific temperatures, see table 3) for 60 seconds and the elongation phase at 72°C for 40 seconds. The run finishes with an increasing temperature gradient to create a melting curve to ensure the quality of the PCR (55-95°C, $\Delta T=0.1°C\ s^{-1}$).

To perform the reaction a BIO-RAD CFX96™ Real-Time System was used. It handles a 96-well plate to which reaction solution, template DNA and primers are added as before. For result compilation, Microsoft Excel was used to plot graphs and calculate statistical numbers like standard deviations and student t-tests.

Table 3. List of primers used to quantify SAOB and methanogenic groups of archaea.

Microbial Group	Target species	Primer	Sequence (5´→3´)	T_m [°C]
SAOB	*T. acetatoxydans*	Tp-f[1] Tp-r[1]	AGG TAG TAG AGA GCG GAA AC TGT CGC CCA GAC CAT AAA	63
	S. schinkii	THAC-f[1] THAC-r[1]	ATC AAC CCC ATC TGT GCC CAG AAT TCG CAG GAT GTC	61
	C. ultunense	Cult-f[1] Cult-r[1]	CCT TCG GGT GGA ATG ATA AA TCA TGC GAT TGC TAA GTT TCA	57
	T. phaeum	TH795-f[2] TH985-r[2]	GGG TGG TGT GAA GCC ATC AGG TCC GCA GAG ATG TCA AG	68
Methanogen	Methanomicrobiales	MMB-f[1] MMB-r[1]	ATC GRT ACG GGT TGT GGG CAC CTA ACG CRC ATH GTT TAC	66
	Methanoculleus	MAB-f[3] MAB-r[3]	GGA ATG CCC TGT AAT CCA AA CAC CTG AAC AGC CTG CAT T	64
	Methanobacteriales	MBT-f[3] MBT-r[3]	CGW AGG GAA GCT GTT AAG T TAC CGT CGT CCA CTC CTT	58
	Methanosaetaceae	Mst-f[1] Mst-r[1]	TAA TCC TYG ARG GAC CAC CA CCT ACG GCA CCR ACM AC	61
	Methanosarcinaceae	Msc-f[1] Msc-r[1]	GAA ACC GYG ATA AGG GGA TAG CGA RCA TCG TTT ACG	60

[1]Source: Westerholm *et al.*, 2011.
[2]Source: Sun *et al.*, 2012.
[3]Source: Westerholm *et al.*, 2012.

3.6 Determine the dominant pathway

To certify the dominant degradation pathway that is used to convert acetate into methane, substrates were radioactive labelled, which can be detected and followed through the molecular conversions. In this case, radiocarbon-labelled acetate molecules ($^{14}CH_3OO^-$) were subjected to the digester and the dominant pathway was determined by analysis of the emitted gases. If the SAO pathway was dominant the radiocarbon would be found in both the resulting carbon dioxide as well as in the methane according to reaction 1 and 2 below. If instead the aceticlastic pathway dominated, all radiocarbon would end up in the methane, following reaction 3 below.

$$^{14}CH_3COO^- + 4H_2O \rightarrow H^{14}CO_3^- + HCO_3^- + 4H_2 + H^+ \qquad \text{Reaction 1}$$

$$4H_2 + H^{14}CO_3^- + H^+ \rightarrow {}^{14}CH_4 + 3H_2O \qquad \text{Reaction 2}$$

$$^{14}CH_3COO^- + 4H_2O \rightarrow HCO_3^- + {}^{14}CH_4 + 4H_2 + H^+ \qquad \text{Reaction 3}$$

This method cannot give a quantitative ratio between these pathways, but instead a discrete answer was received. Either the $\frac{^{14}CO_2}{^{14}CH_4}$ ratio is higher than 1 and the SAO pathway is dominating, otherwise the $\frac{^{14}CO_2}{^{14}CH_4}$ ratio is lower than 1 and the aceticlastic pathway is identified as the dominant.

3.7 Biochemical Methane Potential test (BMP-test)

BMP-test, or a batch test, was carried out in small bottles (250ml) where both inoculum and substrate were added. For all batch test performed in this work, these components were added in a 2 to 1 ratio in the terms of gram VS per litre. More precisely, a substrate concentration of 3g VS l^{-1} and an inoculum concentration of 6g VS l^{-1} were used. Along with triplicate of these sample batches, a set of control batches (also triplicate) were prepared in parallel. These controls did only contain the inoculum and were used to eliminate the background noise from the sample batches. The inoculum still contained some undegraded substrate from previously feedings and thereby still produces some gas. Therefore, to actually know how much gas a certain addition of substrate can generate the background gas production, obtained by these controls, has to be measured and subtracted from the samples.

3.7.1 BMP-test of the substrate (approach 1)

In order to establish the influence by the decrease in temperature on the methane production rate, a batch test was prepared both just before the temperature decrease and another when the final temperature was reached and the reactor had stabilized at this temperature for 2 HRT.

For each test, six bottles were prepared with 6gVS l^{-1} inoculum from the GP2 reactor, 3 of them were given substrate to a concentration of 3gVS l^{-1} while the other 3 were control samples to represent the background gas production (not given any substrate). All batches were diluted with water to the final volume of 193ml, which was used to slow down the process to manageable conditions. This corresponded to 63.28g of inoculum and 5.85g of substrate in each of the three sample bottles.

The batches were then incubated at 52°C, for the pre-temperature change, and at 37°C, for the post-temperature change. Each day the gas pressure inside the bottles was measured with a digital pressure meter (Greisinger GMH 3111), a 2ml gas sample was collected and the pressure was neutralized to atmospheric pressure. The gas samples were analysed in a gas chromatograph (GC) where the methane content was determined. With the measured pressure, the amount of produced gas can be defined through the ideal gas law and this data along with the methane content gives the specific methane production.

After an operating temperature change (from thermophilic to mesophilic) of the continuous reactor GP2; a new batch experiment was set up in accordance with the thermophilic batch test. The only difference was that the bottles were incubated at 37°C instead of 52°C. All the preparation, measurements and analysis steps were performed as described in the previous paragraph.

3.7.2 Volatile fatty acid degradation test (approach 2)

Also a third batch test was set up to follow VFA degradation capabilities of different inoculums (reactor GR1, GR2, GQ1 and GQ2). This experiment was assembled in accordance to the previously mentioned batch tests. Inoculum was taken from the four experimental reactors and along with different kinds of fatty acids these were enclosed in 250 ml bottles, see preparation scheme (Table 4). In agreement with the four CSTRs, the batches from GR1 and GQ1 were kept at 42°C while the batches from GR2 and GQ2 were kept at 37°C.

Table 4. Preparation scheme over batch test for VFA degradation.

Batch	**Inoculum** [ml]	**Initial acetate** [g l^{-1}]	**Initial propionate** [g l^{-1}]	**Sodium acetate added (3M)** [ml]	**Sodium propionate added (3M)** [ml]	**Final acetate** [g l^{-1}]	**Final propionate** [g l^{-1}]
GR1	40	0.15	4.25	0.46	0.24	3	6
GR2	40	0	0	0.49	0.83	3	6
GQ1	40	1.5	6	0.24	-	3	6
GQ2	40	3	6	-	-	3	6
GR2* *Acetate only*	40	0	0	0.49	-	3	0
GR2** *Propionate only*	40	0	0	-	0.83	0	6

Liquid samples were taken from each of the bottles at regular intervals. Samples were then analysed for VFA content on a HPLC (described in previous section). The experimental set-up was made to represent the situation in reactor GQ2 that had the greatest accumulation of both acetate and propionate. For the other batches, acetate and propionate were added to match the levels in GQ2.

Note that there were three different batches with inoculum prepared from the GR2 reactor. One batch only included acetate (GR2*), another where only propionate were added (GR2**) and a third where both fatty acids were added (GR2). In this manner, potential VFA degradation dependent relationship would be detected. For example, if propionate was only degraded when acetate was present, the propionate in batch GR2 would be stable over time.

4 Results and discussion

4.1 Approach 1 – Equilibrium displacement

4.1.1 Evaluation of start-up

Before any experiments were performed, a series of tests were carried out to ensure that process parameters and reactor performance was representative for the large scale biogas plant. These tests are described in the next sections. After adjustments due to these evaluation tests, the reactor was allowed to show stability for 30 days before further experiments were carried out.

While the inoculum was acclimated to its new environmental conditions prevailing in the reactor, a TS and VS analysis was conducted of the substrate. The test measured 11% dry materials (TS) and 10% organic materials (VS), as these values refer to the wet weight of the substrate.

We knew that the large scale biogas plant operated with an OLR of 3gVS l^{-1} day^{-1}. The same OLR should thus be achieved in the lab scale reactors. The amount of wet substrate to feed the 5 litre inoculum on a daily basis to reach this OLR was calculated as follows:

$$m_{substrate} = \frac{OLR_{wanted} * V_{inoculum}}{VS_{substrate}} = \frac{3\ [gVS\ l^{-1}\ day^{-1}] * 5\ [l]}{0{,}100} = 150g\ day^{-1}$$

However, for technical reasons, the reactor was operated only six days a week which meant that the seventh day load was spread over the other six days, resulting in:

$$m_{substrate}(6\ days\ a\ week) = \frac{7[days] * 150[g\ day^{-1}]}{6[days]} = 175g\ day^{-1}$$

This, in turn, lead to a hydraulic retention time of approximately 28.6 days, using equation 4. This is in accordance with the commercial facility where the hydraulic retention time is about 30 days.

$$HRT = \frac{V_{inoculum}}{V_{substrate}} = \frac{5000[ml]}{175[ml\ day^{-1}]} = 28.6\ days$$

For this calculation it was assumed that the substrate had an equivalent density of water due to the low TS of the substrate.

The result from a digester sample taken during start-up and sent for nitrogen analysis showed a total amount of nitrogen in the digester sludge of 4206mg l^{-1} and the amount of ammonium bound nitrogen was 2418mg l^{-1}. Both these values are generally considered high, but are within the normal range of what is measured in the large scale plant of Uppsala Vatten och Avfall AB.

The degree of degradation was measured twice, each time with three replicates. Unambiguously, the results showed a degree of degradation of 81%. This means that 81% of the organic material inside the substrate was converted to biogas (on average) by this continuous digester. Calculations were performed using equation 3.

Analysis of VFA during the up-start phase showed very low levels below detection limit (<0.1g l^{-1}) which indicates a non-disturbed and well performing process.

After 30 days (1 HRT) of operation the reactor performance was stable and agreed well with the large-scale biogas plant. The performance data is summarized in Table 5 below.

Table 5. Thermophilic lab-scale reactor performance summarized together with data from the large-scale biogas plant of Uppsala. Figures reported from large scale plant are approximate since the variations are fairly high.

	Specific methane production [Nml day^{-1} gVS^{-1}]	**CH_4 in biogas** [%]	**CO_2 in biogas** [%]	**pH**	**VFA** [g l^{-1}]
Lab-scale reactor	562	68	30	8.13-8.29	<0.1
Large-scale reactor	570	60	38	8.00-8.10	<0.1

4.1.2 Temperature shift

On day 35, the change in operating temperature was started but what could be seen as an immediate reduction in methane production (Figure 6) was just an effect of the larger amount of inoculum that was taken out for a parallel batch experiment. However, the remaining inoculum slowly recovered to full volume again unaffected by the temperature decrease. The first sign of influence by the gradual temperature decrease came when the reactor had reached

42°C. At this point (day 63) a steady downward trend was seen in methane production while the carbon dioxide content slowly increased. Without taking any precautions, the experiment proceeded as predetermined and resulted in a large gas production decline at day 80 (38°C). The carbon dioxide content increased up to 48% at one point and VFA accumulations were also found. Acetate levels reached 2.1 g l^{-1} while propionate exceeded 2.9 g l^{-1}. Actions to reverse this trend had to be taken in order to avoid a collapse of the whole microbial system. The organic load was thus halved from day 80 to day 84 to give the microorganisms time to decompose the inhibitory VFA. The OLR were slowly increased to the original level (day 95) and the reactor slowly recovered and showed stability again at day 138.

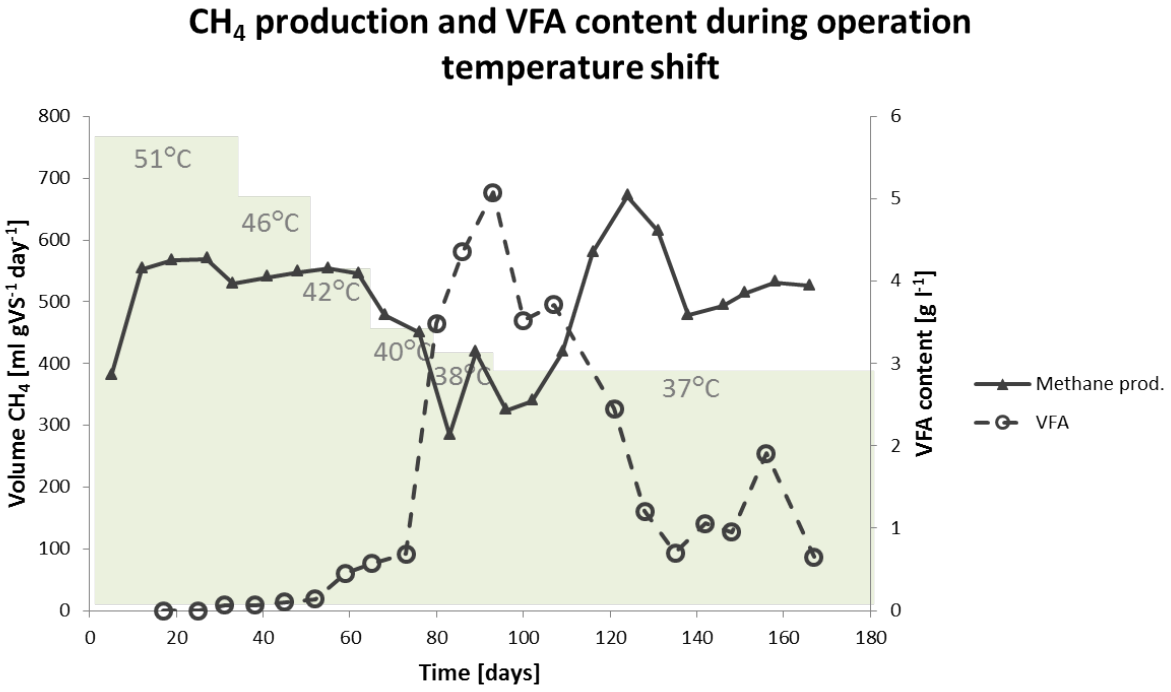

Figure 6. *Average daily specific methane production (triangles) is shown together with VFA content (open circles) over time during gradual temperature decrease (background). An inversely correlation between methane production and VFA accumulations was clearly reviled. At 38°C (day 80) methane production was rapidly slowed down and at the same moment fatty acids was accumulated. When microbial population adapted to the lower temperature the VFA degradation speed up again which explains a methane production peak (at day 124) higher than the maximum potential. The time-distance between two points is 7 days, each point represents the mean value of the previous 7 days.*

The slowdown in methane production at 38°C (day 80) was likely an effect of a shift in microbial population structure. Thermophilic microbes weakens and mesophilic microbes becomes more viable at that point. This would cause a lag phase where fewer microbes can take care of the substrate completely until the mesophilic population had grown strong enough, a situation that probably was reached at day 100.

When the final temperature was reached, the reactor produced over 700ml CH_4 gVS^{-1} day^{-1} for a while, until it flattens out to steadily produce around 528ml CH_4 gVS^{-1} day^{-1}. That peak in methane production was even higher than the one at the initial temperature. Moreover, this value even exceeded the maximum methane potential that was measured initially to be 601ml

gVS^{-1} day^{-1}. A reasonable explanation to this result is that the accumulated VFA was decomposed into methane as the new microbial community arose. This explanation corresponds nicely to what was seen in figure 6 where the VFA content follow methane production inversely.

For comparison, a new TS and VS were measured after operation at mesophilic temperature (37°C) for 65 days. The degree of degradation was also calculated again. TS and VS for the substrate remained the same which was predicted since the same substrate was used over the whole experimental period. The digestate, on the other hand, showed both a higher TS content as well as a greater VS content. This means that this lower operation temperature process has difficulties to degrade the organic part in the same extent as at the thermophilic process. However, this difference was not very big, the degradation efficiency at thermophilic and mesophilic operation were only separated by 7% but may still be considered a factor when trying to improve the process stability by temperature reduction. This analysis should be repeated a few more times to evaluate the significance of this change and to obtain a statistical support.

A degree of degradation of 81% at mesophilic temperature and 74% at mesophilic temperature seems to be reasonable figures if comparing to similar processes throughout the literature. A study at Linköping biogas plant in Sweden, for example, used a similar substrate (food waste and slaughter waste) to a large scale reactor operating at 38°C. That study presents an 80% degree of degradation which they claim to be very efficient (Ek *et al.* 2011).

Nitrogen content from the continuous reactor was also measured again after the temperature shift. As Table 6 shows, the total amount of nitrogen in the sludge remained almost the same throughout the shift. A greater difference was seen in the ammonium bounded nitrogen. This reduction of ammonium nitrogen from thermophilic to mesophilic operation was most probably the effect of decreased degradation of nitrogen-containing compounds.

Table 6. Nitrogen content in thermophilic and mesophilic operation.

Operation	**Total nitrogen** [mg l^{-1}]	**Ammonium nitrogen** [mg l^{-1}]	**Free ammonia** [g l^{-1}]	**pH**
Thermophilic	4206	2418	0.77	8.2
Mesophilic	4010	1311	0.07	7.7

As described in the theoretical part of this work, the ammonium-to-ammonia equilibrium is dependent on the temperature and pH-value. This theory suggests that a decrease in temperature will displace the equilibrium towards ammonium (Fricke *et al.* 2007). A decrease in pH would in the same way contribute to a higher ammonium proportion. These experimental data shows that the temperature and pH are strongly correlated (Pearson´s r = 94.6%). As the temperature steadily declines, a clear decrease in pH was also seen (Figure 7, left). During the whole experiment the temperature was dropped 15°C resulting in a pH shift of 0.8 units. As

both of these factors affect to lower the free ammonia level, a change from about 0.8 g L^{-1} (at the highest operating temperature) down to 0.07 g L^{-1} (at the lowest operating temperature) was seen (Figure 7, right). The ammonia content was calculated using the ammonium-nitrogen value through equation 5.

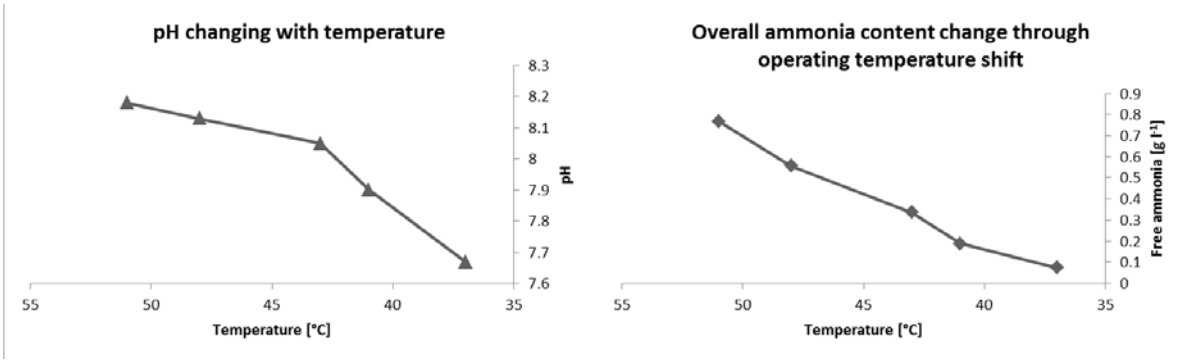

Figure 7. *The leftmost figure shows how the pH was consequently dropped throughout the operating temperature reduction. In the rightmost figure, a clear decreasing trend of the ammonia content in the reactor (GP2) is described. Over the whole temperature reduction (ΔT=15°C) the ammonia level was reduced from 0.8g l^{-1} to 0.07g l^{-1}.*

4.1.2.1 BMP tests

Comparing the BMP test made before and after the temperature decrease it is obvious that the capacity of the inoculum has changed considerably. Looking at the grey line (Figure 8) reveals that after 10 days, over 90% of the maximal methane production (556 ml out of 601 ml) was reached at thermophilic temperatures. Maximum yield was reached after 25 days, which is shorter than the hydraulic retention time of the experimental reactor (28 days). This means that the substrate stays in the reactor long enough to be broken down completely. This result also indicates that the methane production could be improved by increasing the loading as long as the HRT does not decrease below 25 days.

After 30 days of incubation, an unexpected declining trend was observed. It is most likely an effect of microbial starvation. Since the substrate is a mixture of different compounds some that are easily degradable and some that are less easily decomposed. When the easily degraded substrate is running out, metabolic changes within the microorganisms have to be done in order to proceed with the less nutrient-rich materials. This transition phase is probably what puts the gas production to a lag phase inside the experimental batches whereas in the control batches the microbes are already adapted to the more complex substrate. That is why the controls produce more gas than the samples and the graph shows a reduction in gas production.

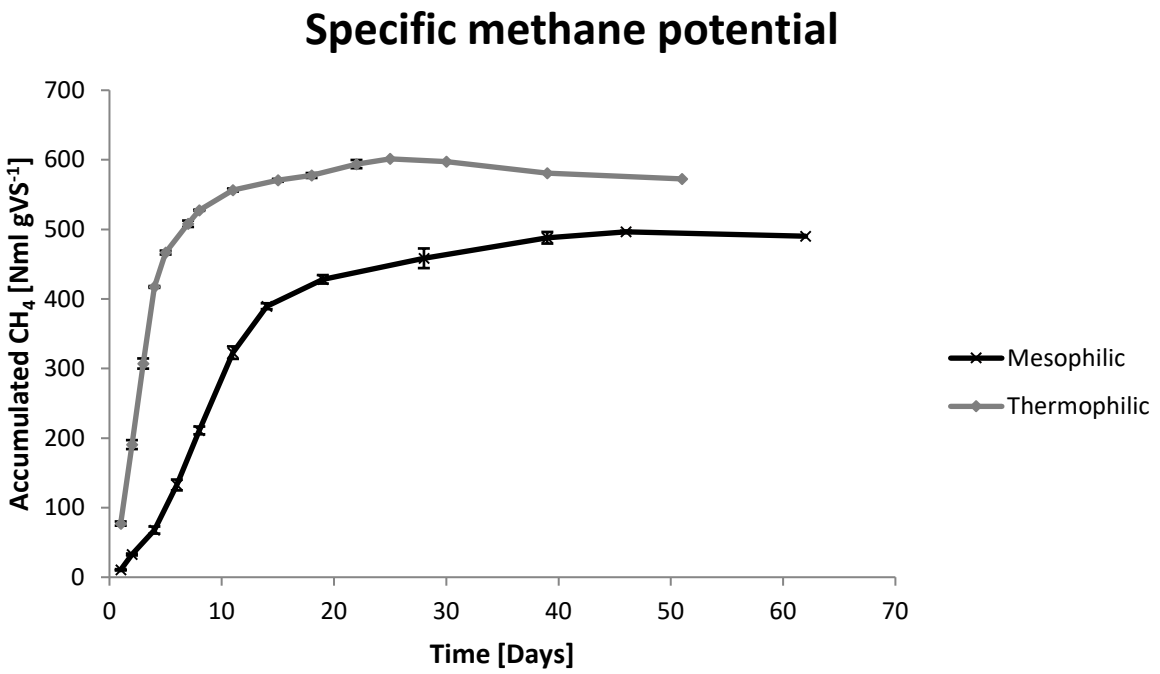

Figure 8. *Accumulated specific methane production from batch test. Gray line (diamond marker) represents thermophilic process (52°C) and black line (cross marker) describing mesophilic process (37°C).*

After reaching the final temperature of the continuous reactor and thereafter had a stable run for 2 HRT, a new batch experiment was started (day 155 of operation). At mesophilic operating temperature (37°C), a decrease in gas production was observed. This is likely the result of slower microbial proliferation and activity. Initially, a much slower methane production rate was seen (figure 8, black line), 38Nml CH_4 per day. A 66% drop in production speed compared to the thermophilic process. As the experiment proceeded, 90% of the maximum methane production was reached at day 25 and the maximum methane yield at 496 ml was not reached until day 46.

As mentioned before the HRT of the reactor was about 28 days and for this mesophilic process only 92% of the methane potential was reached within that period. Consequently, this means that potential methane is lost through substrate washout which also explains why the degree of degradation is lower at mesophilic process temperature.

4.1.3 Load increase study

As the load gradually was raised, an immediately response was seen in the methane production (Figure 9). The decreased methane production was fully regained (520 Nml gVS^{-1} day^{-1}) during the first stabilization period (at 4 gVS l^{-1} day^{-1}), but when the second stabilization period was reached (5 gVS l^{-1} day^{-1}) the methane yield was lagged behind, reaching only 315 Nml gVS^{-1} day^{-1}. As the organic loading rate was further increased to 6 gVS l^{-1} day^{-1} a rapid collapse occurred. The specific methane production dropped down to 10 Nml gVS^{-1} day^{-1}, carbon dioxide content exceeded 65% and the pH was just below 5.4 at this point. A previous study showed that a thermophilic process (52°C), using the same inoculum and substrate as for this study, managed a load increase from 3 to 6 gVS l^{-1} day^{-1} without showing any perfor-

mance disturbances at all (Frid 2012). The main reason for the reduced load tolerance in mesophilic systems may be the limited microbe activity rate.

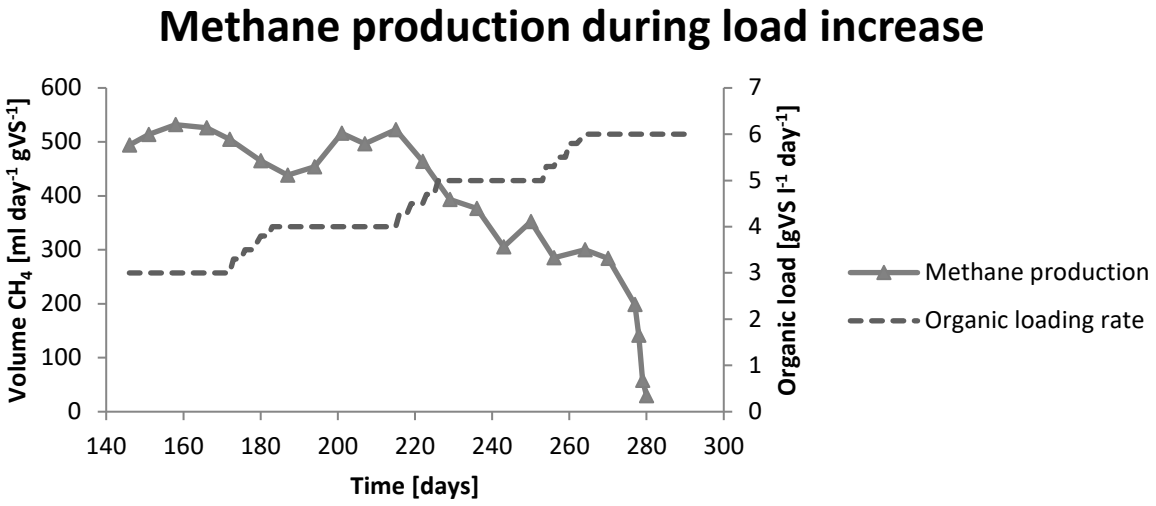

Figure 9. *The specific methane production (triangles) was followed throughout a load increment (dotted line) of the mesophilic operated reactor, leading to a major process collapse when reaching 6 gVS l^{-1} day^{-1}.*

4.2 Approach 2 – SAOB enrichment and trace element additives

A second experiment was performed in parallel to the first one. As in the first experiment the influence of different operating temperature on reactor performance was investigated. Moreover, this experiment also included the examination of the influence of trace element additives. To understand the difference in digester performance due to external parameter changes, i.e. temperature and trace elements, a closer look at the underlying microbiology was done. Using quantitative PCR together with species-specific or order-specific primers, the microbial composition was determined and quantified. To limit the work, this test was restricted to only include species involved in methane formation, namely methanogens and SAOB.

4.2.1 Reactor performance

The performance of four laboratory reactors was stable throughout the experimental period, but fluctuation occurred within the system parameters. Because of that, average values (between day 232 and day 440 of operation) were used for presenting the results in this section.

Identification of how much influence each of the investigated factors, i.e. temperature and trace element additives, had on the process performance was made by comparisons between the four test reactors (data from each of the four reactors are summarized in Figure 10). The temperature factor (37°C and 42°C) did not lead to any significant performance difference without trace element additives (figure 10, top, GQ2 vs. GQ1). However, when trace elements were added, an 11% increase in methane production was found at the lower temperature (figure 10, top, GR1 vs. GR2). Another observation was the difference in accumulation of VFA between the operation temperatures. Notable levels of VFA were found in the GR1 reactor, while the levels in GR2 were negligible. That kind of temperature dependent VFA

level was repeated in the GQ-reactors (figure 10, bottom). This indicates an inhibited degradation of fatty acids at 42°C. The impact of trace element showed even higher importance than the temperature factor for the reactor performance. 14% higher methane production was obtained through trace element additives at 42°C (figure 10, top, GR1 vs. GQ1) compared to 30% higher methane yield at 37°C (figure 10, top, GR2 vs. GQ2). Reactors with trace elements also showed a decrease in VFA, independent of temperature (figure 10, bottom). My speculations is that the VFA degrading organisms, SAOB and aceticlastic methanogens for example, must be enhanced by the additives and thus degrade the VFA in a more efficient way.

Since higher temperature generally is associated with higher microbial activity and higher growth rate (Madigan & Michael 2005) it was surprising to find a higher methane production at the lower temperature where trace element was added (GR2). A correlation between higher methane production and lower VFA content seems to exist.

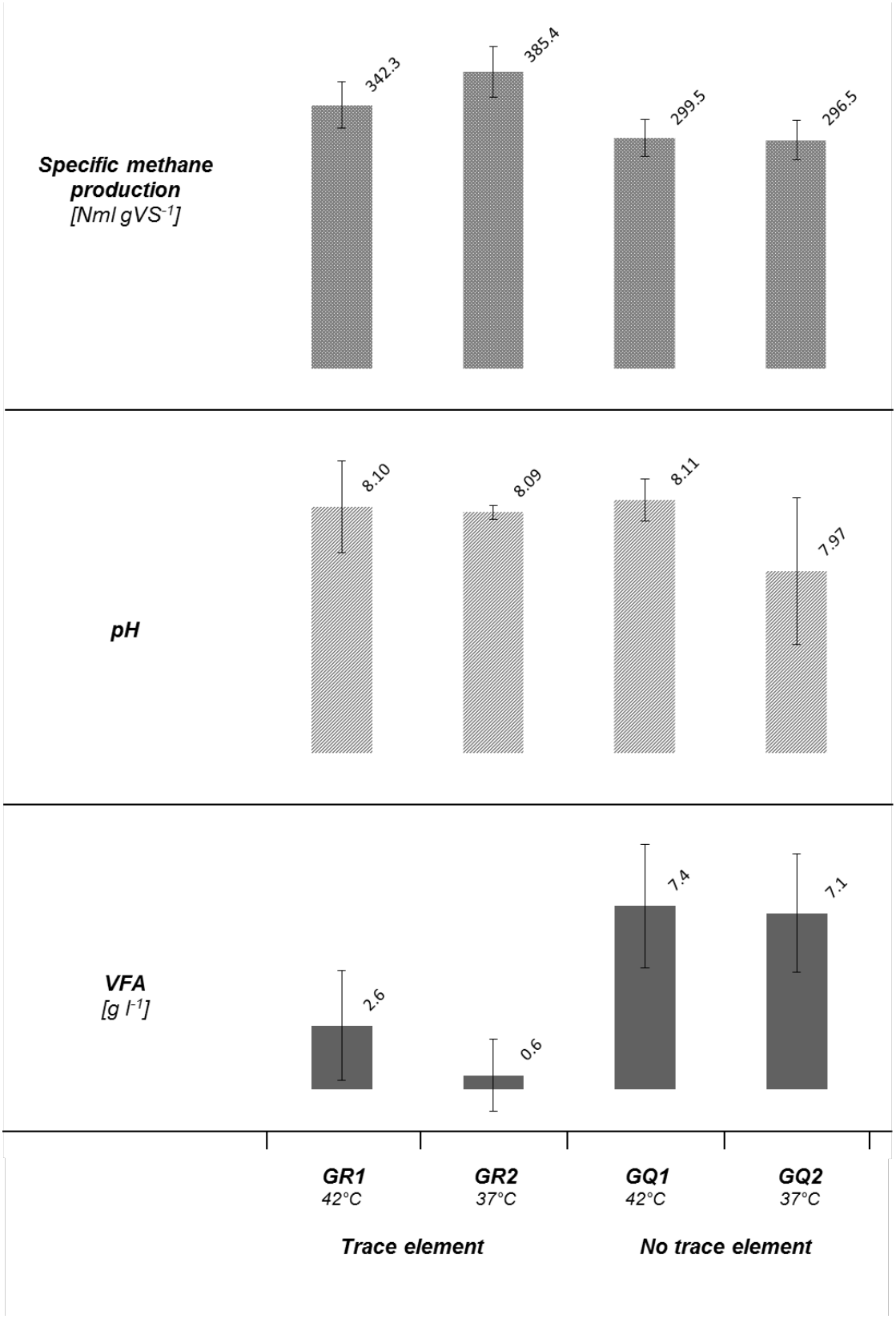

Figure 10. *Summary of the performance parameters of the four experimental reactors.*

4.2.2 Pathway identification by radioactive tracers

For all of the four test reactors this method showed a radioactive labelled carbon dioxide to methane ratio above 1, which undoubtedly suggests the syntrophic acetate oxidising pathway to be the dominant in all cases.

4.2.3 Microbe community investigation

Reactor samples taken from six different time points were analysed to quantify SAOB and methanogenic archaea. All standard curves used in qPCR had a linear correlation factor (r^2) in the range of 0.981 to 0.999 and the calculated efficiency of the reaction varied between 80.0% – 100.2%.

4.2.3.1 Syntrophic acetate oxidising bacteria

The three syntrophic acetate oxidizing bacteria to be investigated in qPCR were *Tepidanaerobacter acetatoxydans, Clostridium ultunense and Syntrophaceticus schinkii*. A fourth member of SAOB is *Thermacetogenium phaeum,* a thermophilic species that is not often present in mesophilic biogas reactors since its growth optimum is 58°C (Hattori *et al.* 2000).The presence of these species was tested using regular PCR with primers TH795-f and TH985-r (Sun *et al.* 2012). PCR followed by gel electrophoresis showed no presence of *T. phaeum* in any of the reactors at any given time. 16S ribosomal RNA gene amplified from genomic DNA of *T. phaeum* was included as positive control.

4.2.3.2 Quantification of SAOB

The quantification of 16S gene abundance associated with SAOB was done for each of the four reactors. Common to all of the reactors was that the SAOB community structure remained stable over the experimental period but also that a pronounced species evenness was seen. Each of the SAOB species abundance in GR1 ranged between $5.0*10^7$ ml^{-1} and $3.2*10^8$ ml^{-1}, in GR2 these bacteria ranged from $2.5*10^6$ to $2.0*10^7$ ml^{-1}. Looking at the GQ1 and GQ2 (Figure 11) a significant higher abundance was found for *S. schinkii* compared to the other two, *T. acetatoxydans* and *C. ultunense*. In GQ1 *S. schinkii* varied between $1.3*10^9$ ml^{-1} and $2.0*10^9$ ml^{-1} while the other two species varied between $6.3*10^7$ ml^{-1} and $7.9*10^8$ ml^{-1}. Reactor GQ2 showed a level of *S. schinkii* between $1.0*10^8$ – $7.9*10^8$ ml^{-1} whereas *T. acetatoxydans* and *C. ultunense* had a lower abundance, $6.3*10^6$ – $7.9*10^7$ ml^{-1}.

Figure 11 indicates that all the reactors are using the syntrophic acetate oxidizing pathway due to the overall high abundance of SAOB. This idea is further strengthened by a radioactive tracer experiment described in the previous section.

When comparing the different operating temperatures, a noteworthy influence of SAOB was found. At the higher temperature (42°C) all tested SAOB had increased abundance. This was in fact the case in both reactors with trace elements (GR1 and 2) as well as for those without the additive (GQ1 and 2). A t-test confirmed the significance of these, the P-value ranged between 0.0008 and 0.02. With trace elements around at 42°C the *T. acetatoxydans* were induced to an almost 5 times higher abundance compared to 37°C. In the absence of trace elements *T. acetatoxydans* was more than 45 times more abundant in the higher temperature. For *S. schinkii* the same pattern was seen, at the 42°C reactor the abundance were around 10

times higher than in the 37°C, both with and without trace elements. *C. ultunense* also showed a 10-fold increased abundance both with and without trace elements at the higher temperature.

To investigate how the addition of trace elements affect the populations of SAOB, qPCR results between reactors GR1 with GQ1 were compared. Both processes are running at 42°C, but GR1 was given trace element additives, whereas GQ1 operated in the absence of these. The reactors GR2 and GQ2 were compared in the same way, though with both running at 37 degrees. At 37 degrees, a significant reduction of *S. schinkii* was seen (28 times reduction, P-value = 0.002) while both *T. acetatoxydans* and *C. ultunense* showed no significant change due to trace additives. At 42 degrees Celsius, only *C. ultunense* was unaffected, *T. acetatoxydans* and *S. schinkii* showed a significant decrease in occurrence where trace elements were added, 6 and 17 times reduction respectively.

It is important to note that only 16S gene abundance was analysed and not the actual number of organisms. This does not affect comparisons within the same species. To consider the actual number of organisms instead of gene abundance, the 16S gene copy number must be known for each organism. For the SAOB this is known, *T. acetatoxydans* has two 16S genes per cell (Manzoor *et al.* 2013a), *S. schinkii* contains only one copy per cell (unpublished data), *C. ultunense* also contains one copy (Manzoor *et al.* 2013b). For the methanogens, it is harder to convert gene abundance to actual number of cells. That is because the primers used are not species-specific and instead are order-specific. Since the ratio between different species inside the order is unknown, such conversion is impossible to do with precision.

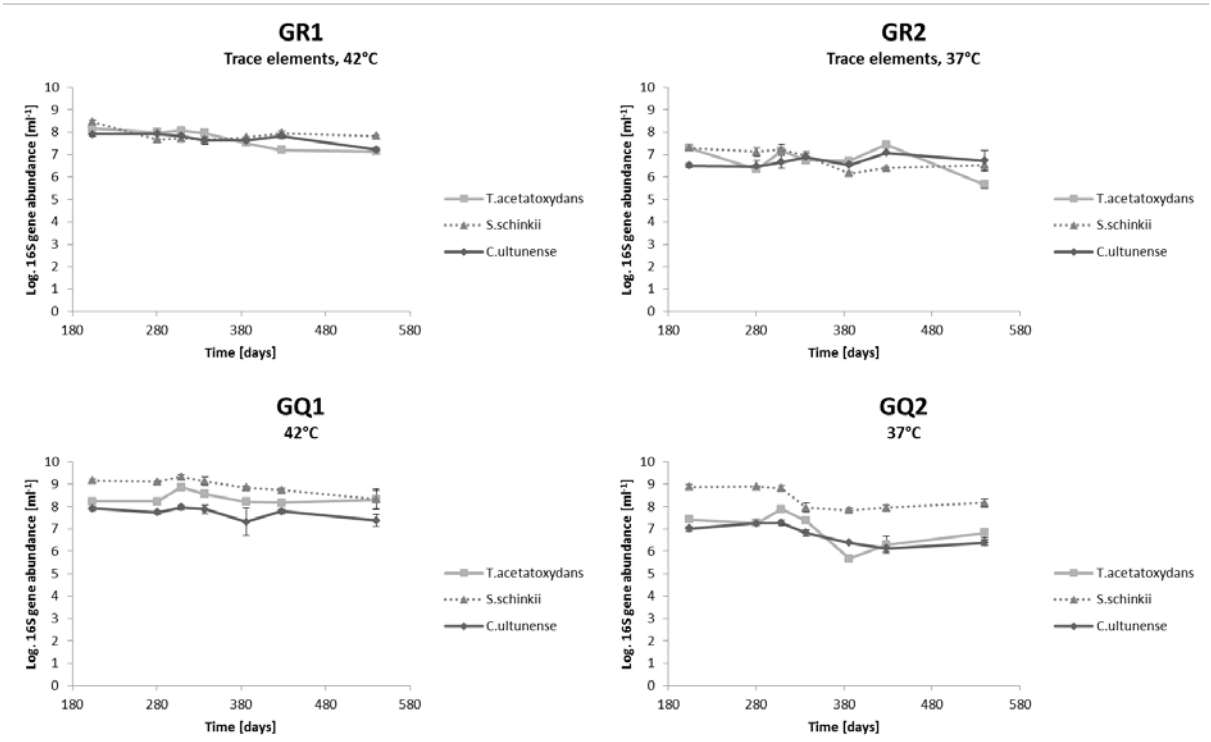

Figure 11. *Result of qPCR displaying SAOB 16S gene abundance in different reactor operating conditions.*

4.2.3.3 Methanogenic archaea

A total number of five families of archaea were investigated with qPCR from each of the four reactors. All these microorganisms have proved to be important in the methane producing step. Some are aceticlastic methanogens, such as *Methanosarcinaceae* and *Methanosaetaceae* spp., and some are hydrogenotrophic methanogens, such as *Methanomicrobiales, Methanoculleus* and *Methanobacteriales*. Primers to target these mentioned orders were used in the qPCR analysis to quantify them, see table 3.

4.2.3.4 Quantification of methanogens

The quantitative PCR results are visualized in Figure 11 and tabulated in Table 7. Unlike the SAOB quantification, the community structure of these methanogenic groups were varying over time, during the whole experimental period of 540 days. This was a surprising insight since previous experiences have pointed out that a stable process and so a stable community usually would establish during the first 3 HRT of operation.

Table 7. Tabulated average 16S gene abundance of the five methanogenic orders in each experimental reactor. *NO* – not observed, TE – Trace elements added.

	Average gene abundance *[copies ml^{-1} reactor sludge]*			
Methanogenic order	**GR1** 42°C, TE	**GR2** 37°C, TE	**GQ1** 42°C	**GQ2** 37°C
Methanosarcinaceae	$3.87 * 10^6$ (21.0)	$1.27 * 10^5$ (19.8)	$8.46 * 10^5$ (9.75)	$3.86 * 10^3$ (59.3)
Methanosaetaceae	*NO*	*NO*	*NO*	*NO*
Methanomicrobiales	$1.27 * 10^{10}$ (3.71)	$9.22 * 10^8$ (5.61)	$1.19 * 10^{10}$(4.50)	$6.64 * 10^7$(51.7)
Methanoculleus	$3.00 * 10^6$ (0.76)	$2.18 * 10^8$ (0.85)	$3.15 * 10^6$ (0.80)	$3.32 * 10^7$ (1.06)
Methanobacteriales	$1.23 * 10^6$ (0.50)	$1.67 * 10^6$ (0.45)	$1.30 * 10^6$ (0.32)	$1.80 * 10^6$ (0.40)

Methanosaetaceae was detected in the qPCR using Mst-primers, but were after further investigation declared to be incorrect in all cases, due to wrong amplicon size. The explanation for the lack of Methanosaetaceae was partly due to its sensitivity to high nitrogen levels, which decreases their viability, and partly due to the outcompeting organisms (i.e. Methanosarcinaceae) that are more resistant to high nitrogen levels and utilizes the same route of degradation (Karakashev *et al.* 2005).

The quantification analysis indicated that a lower temperature causes a lower species evenness i.e. larger difference in abundance between species. The acetoclastic methanogens tend to be less abundant while the hydrogenotrophic methanogens getting more abundant at 37°C compared to 42°C, regardless if trace element was added or not.

Trace element addition does not seem to affect the methanogens significantly. No significant difference was seen at 42°C with trace elements as compared to the identical reactor without trace elements. The same goes for methanogens at 37°C with the exception of the *Methanomicrobiales* (MMB) which actually shows a significant decrease in abundance where no trace elements are present. As this reduction was not explained by the subgroup MAB it must be explained by another subfamily.

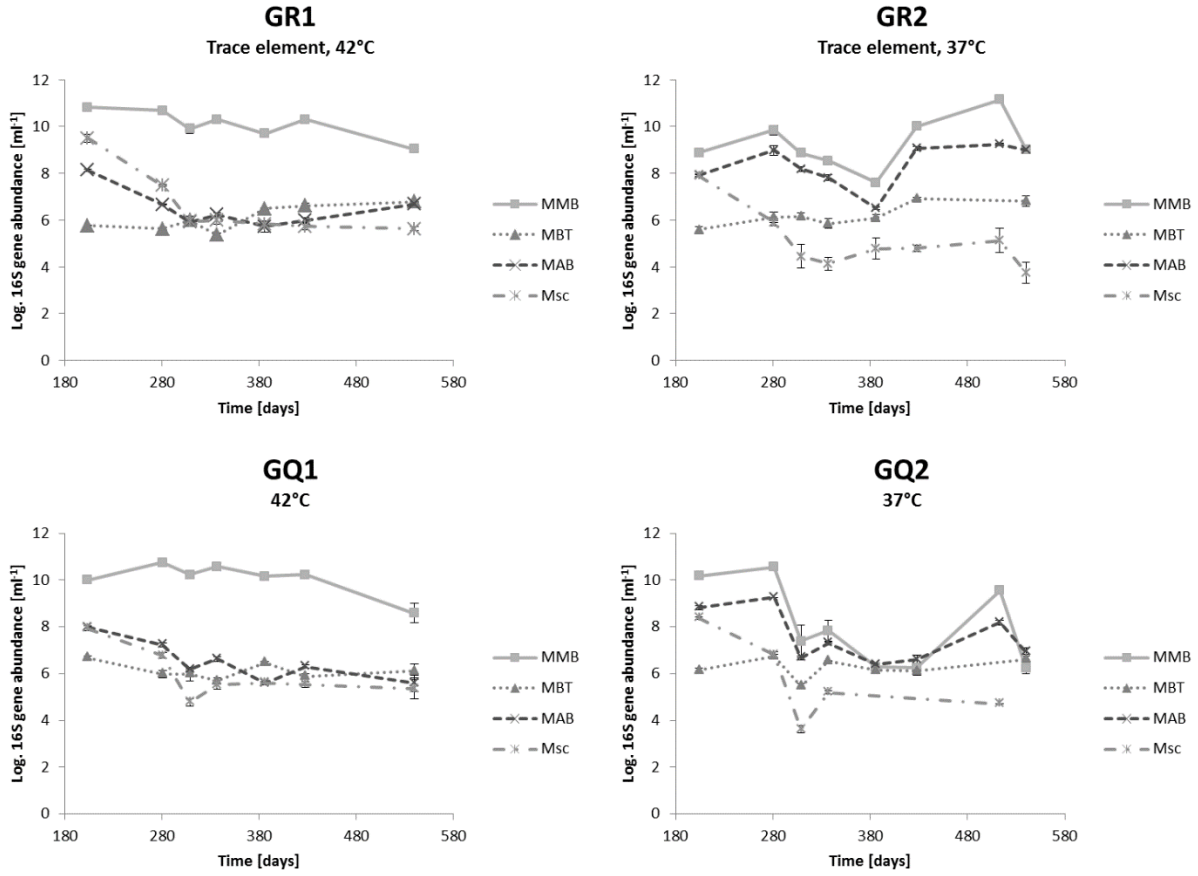

Figure 12. *Illustrated qPCR results for the different methanogenic orders in the 4 different reactors over time. Community structure was mostly affected by temperature and not to trace element.*

4.2.4 Fatty acid degradation test

To investigate whether the different VFA accumulations in the reactors was due to a high VFA production or difficulties during degradation of these, a new experiment was performed.

The VFA degradation from each of the batches is illustrated in figure 12 – 14. The batches from trace element treated reactors (GR1 and GR2) both degraded acetate and propionate

completely and relatively fast. Note that the time axis is presented in logarithmic scale so the GR2 actually breaks down all the VFA at half the time compared to GR1, 9 and 19 days respectively. Batches from GQ1 and GQ2, however, did not show a tendency of breaking down propionate. Besides, a third fatty acid (I-valerat) was also found in these batches which can be degraded in GQ2 but not in GQ1.

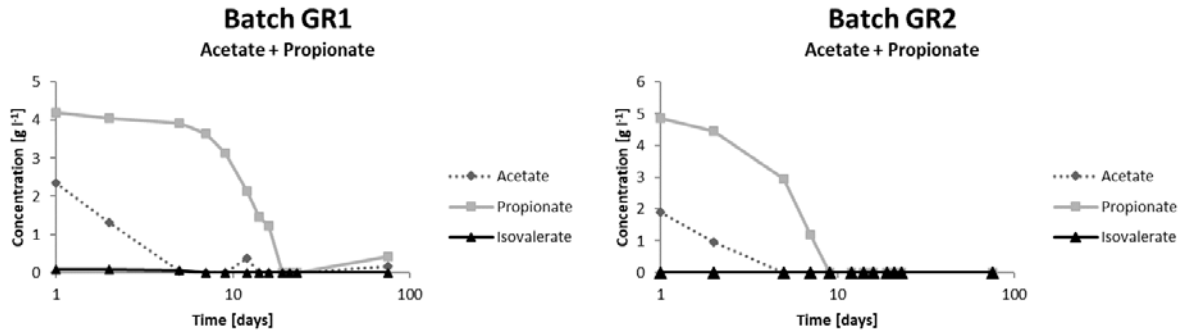

Figure 13. *VFA degradation over time in batch systems treated with trace elements. Both acetate, propionate and isovalerate was decomposed completely even though the lower temperature (batch GR2) showed a more effective decomposition.*

Batch GR1 degraded acetate with a rate of 0.59 g l^{-1} day^{-1}. The propionate in the same batch had a degradation rate of approximately 0.23g l^{-1} day^{-1}. GR2, on the other hand, showed a degradation rate of 0.48g l^{-1} day^{-1} for acetate and 0.61 g l^{-1} day^{-1} for propionate (Figure 13). GQ1 and GQ2 did not manage to break down propionate but instead a small accumulation can be seen (Figure 14). Acetate is degraded almost completely in both of these even though it is a slow process in the GQ2 batch, 0.19 g l^{-1} day^{-1}. GQ1 had the acetate degradation rate of 0.34 g l^{-1} day^{-1}.

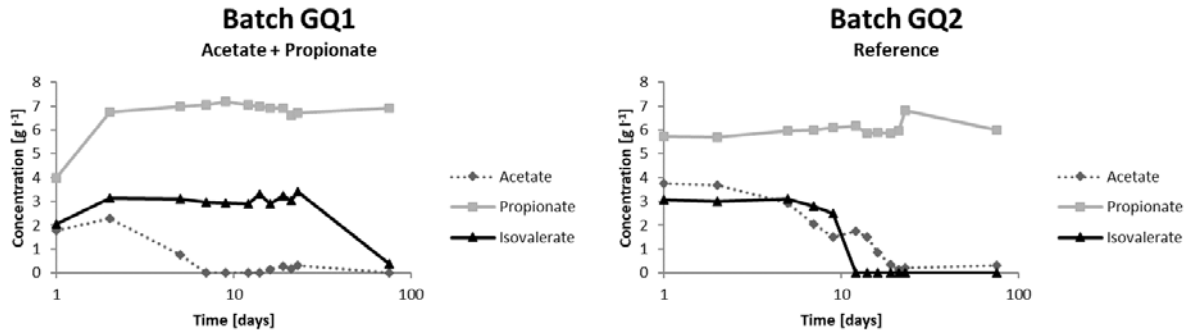

Figure 14. *Degradation of fatty acids in batch systems without trace element additives.*

The two replicates of GR2 with either acetate or propionate added showed both rapid degradation rate 0.36 g l^{-1} day^{-1} for acetate and 0.55 g l^{-1} day^{-1} for propionate (Figure 15). This suggests that a dependency of the degradation between acetate and propionate can be ruled out. This is because both acetate and propionate exhibit a rapid breakdown without the other fatty acid present. The only dependency visible is the emergence of acetate when propionate is degraded (see figure 14, Batch GQ2). This is nothing more than the natural degradation pathway where propionate partly turns into acetate. A consistent trend is that acetate decom-

poses more quickly at higher operating temperature, while the opposite seems to be true according to propionate decomposition. Another interesting observation is that valeric acid, indicated as I-valerate, is only present in the digesters where trace elements are absent (GQ1 and GQ2).

Since this experiment contained very few data sets the uncertainties could not be determined and for this reason no comparative figure is presented. These data only indicates how the volatile fatty acids were processed in these systems.

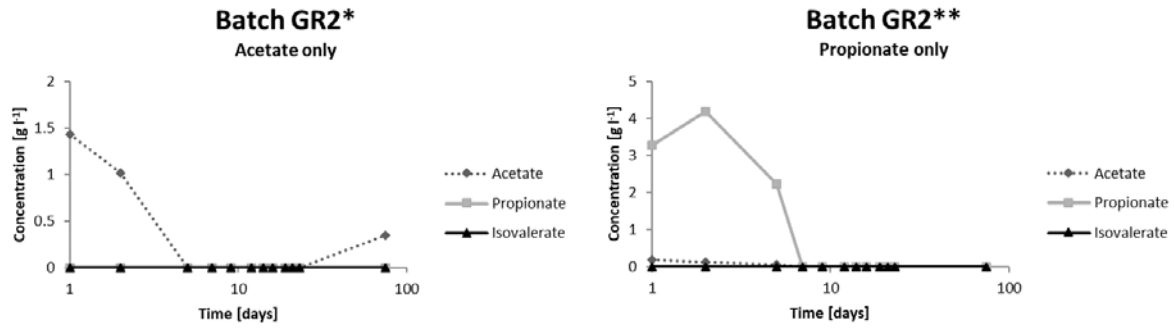

Figure 15. *Replicate of GR2 batch with either only acetate added (left) or only propionate added (right). Both systems showed rapid decomposition of either VFA, indicating independent degradation routes.*

5 Conclusion

A shift from thermophilic to mesophilic operating temperature during an ongoing biogas process was in this study demonstrated to be possible through a gradual temperature decrease. To reconnect to the initial research question whether this temperature shift would lower the ammonia content in the system and thus increase the process stability, the answer would be yes and no respectively. The overall result from the temperature shift was a 90% decrease in ammonia level, from thermophilic (0.80 g l^{-1}) to mesophilic (0.07 g l^{-1}) operation. This change allows decomposition of nitrogen-rich substrates without the risk of ammonia-induced inhibition. However, the system became much slower at the mesophilic temperature. For example, the maximum specific methane potential was negatively affected by 17% and the time to reach this potential was almost doubled at the mesophilic temperature. Through the load increase study of the mesophilic process it was clear that the methane production could not match the thermophilic process through increased loading rate. Instead, the process collapsed due to the slow degradation of volatile fatty acids which caused a large pH drop.

The addition of trace elements resulted in an increased biogas production, possibly due to the change in the syntrophic acetate oxidising bacteria population structure that was found. The trace elements seems to have a greater impact at 37°C operating temperature, where a 30% higher methane production was obtained compared to the control reactor given no additives. The accumulation of volatile fatty acids was also found to be affected by the addition of trace

elements. Regardless of operating temperature (37°C or 42°C), the VFA levels were kept lower in reactors treated with trace element.

To optimise the biogas production at high nitrogen levels more tests are needed. However, this study hopefully provides knowledge in the right direction of finding the optimal process conditions. Moreover, I think a deeper understanding of the microbial structures, functions and interactions is an important topic, not only to optimize the existing process, but to further develop the technique of methanogenesis.

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