Sveriges lantbruksuniversitet
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Abstract

This study investigated the problem of ammonia inhibition in biogas reactors, commonly occurring when protein rich substrates are used in anaerobic digestion processes. Ammonia inhibition is one of the foremost causes of failure in anaerobic digestion, leading to process instability, with symptoms commonly expressed as; reduction in biogas produced, decreased methane content in the gas and accumulation of volatile fatty acids. Increasing ammonia concentrations lead to a shift in the dominant pathways for methane production, from acetoclastic methanogens to syntrophic acetate-oxidizing bacteria, working with hydrogenotrophic methanogens. This shift occurs as the acetoclastic methanogens are sensitive to increasing ammonia concentrations. Both pathways utilize acetate, which apart from ammonia, is one of the factors that also is believed to regulate the level of the different pathways.

The aim of this study was to evaluate the impact of subjecting anaerobic digestion processes in biogas reactors to increasing levels of ammonia and to study the performance of the reactors as well as the potential microbial changes that occur when reactors are supplemented with acetate simultaneous with the ammonia increases.

The total ammonium-nitrogen was successfully increased in the reactors to concentrations known to cause ammonia inhibition towards the end of one hydraulic retention time. The increase in total ammonium-nitrogen correlated with increased H_2S concentrations in the reactors and an accumulation of volatile fatty acids. Acetate addition resulted in higher acetate concentration in the reactors, but without accumulation. The acetate addition possibly caused an increase in the gene abundance of hydrogenotrophic methanogenic order Methanomicrobiales and the genus *Methanoculleus*. No significant change in gene abundance of acetoclastic methanogens or syntrophic acetate-oxidising bacteria occurred in response to the increase in ammonia and acetate in this study.

The lack of microbial compositional changes could have been due time of the study, being too short for changes in the microbial composition to happen. Moreover, the acetate-enrichment was possibly too low to stimulate growth of syntrophic acetate-oxidising bacteria when compared to concentrations in other studies.

Keywords: Anaerobic digestion, ammonia inhibition, acetate enrichment.

Populärvetenskaplig sammanfattning

Biogas består till största delen av metan, men också av koldioxid, och mindre nivåer av svavelväte och andra gaser. Biogas kan framställas från en mängd olika ämnen såsom hushållsavfall, slaktavfall och rester från t.ex. livsmedelsindustrin. Biogasprocessen är attraktiv då den medger möjligheten att framställa förnybar energi från många olika avfallsmaterial och är användbar som ett alternativ till fossila bränslen. Biogas kan användas för elproduktion, uppvärmning eller matlagning i städer eller uppgraderas för att ta bort andra gaser än metan och därefter användas som fordonsbränsle av bilar och bussar. Från resterna av biogasprocessen bildas det även en näringsrik nedbrytningsrest (rötrest) som kan användas inom jordbruket som gödningsmedel.

Bildning av biogas från olika material sker i en rötningsprocess som är anaerob (fri från syre till största grad). Det kan då uppstå vissa problem om man använder proteinrikt material i sin process, som i övrigt är eftertraktade på grund av sin höga metanpotential. Under nedbrytning av protein frigörs ammoniak, som kan hämma processen. Problemen kan vara så allvarliga att processen inte längre fungerar som den ska.

Syftet med den här studien var att se om det går att förbättra biogasprocessen vid användning av proteinrikt material. Vissa av de mikroorganismer som är delaktiga i processen blir lättare inhiberade av höga ammoniak-nivåer än andra mikroorganismer, vilket leder till ett skifte i vilka grupper som dominerar och hur metanbildningen sker.

Exjobbet genomfördes vid Sveriges Lantbruksuniversitet under hösten 2019. I studien höjdes ammoniaknivåerna medvetet i fem reaktorer i labskala (nedskalade reaktorer för biogasproduktion). Samtidigt som ammoniaknivån höjdes tillsattes också ättiksyra. De höjda nivåerna av ättiksyra syftade till att främja tillväxten av en av mikroorganismgrupperna som är tåliga vid höjda ammoniakkoncentrationer. På så sätt var målet att se om vissa av de problem som vanligtvis uppstår kan motverkas. Data från reaktorerna samlades in för att se om prestandan förändrades under studien, och för att se om några förändringar i den mikrobiella sammansättningen skedde.

Resultatet från studien visar att den medvetna höjningen av ammoniak-nivåerna lyckades, men att tillsatsen av ättiksyra inte var tillräckligt hög för att den tåligare gruppen skulle tillväxa signifikant. I studien uppvisade reaktorerna vissa av de dokumenterade problem som uppstår i samband med höjda ammoniaknivåer.

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Abbreviations

AM – acetoclastic methanogenesis

DAD – Dry anaerobic digestion

DD - Degree of degradation

HM – Hydrogenotrophic methanogenesis

HRT – Hydraulic retention time

OLR – Organic loading rate

SAO – Syntrophic acetate oxidation

SAOB – Syntrophic acetate oxidising bacteria

TS – Total solids

VS – Volatile solids

VFA – Volatile fatty acids

WAD – Wet anaerobic digestion

1 Introduction

As we move into a new year, 2020, we are only ten years from 2030 and the goal of decreasing greenhouse gas emissions by 63%, compared to the 1990 levels. We are also 25 years from the 2045 long term goal of having zero net emissions. (Naturvårdsverket, 2018)

One approach to reach these goals is to develop alternatives which can replace fossil fuels. As of today, there is a high global demand in such alternative fuels. (Lönnqvist et al., 2019) The energy sector, including transportation, accounts for 71% of total greenhouse gas emissions in Sweden. Transportation accounts for about a third of total greenhouse gas emissions (Morel, 2018). Meanwhile, the share of transportation running on renewable fuels only makes up 22% of the total in 2017. (Fagerström et al., 2019)

Since the year 2000, there has been an increase in the number of busses fuelled by biogas and natural gas. In 2017, 17% of all busses in Sweden were powered by biogas or natural gas compared to 0% in 2000 (Bussföretag, 2018). The trend continuous to increase as many municipalities that have their own production of biogas choose to invest in biogas busses, often in connection with local treatment plants.

Biogas is one of the fuels that can play an important role in the coming transition to renewable fuels used in transportation, as well as for generating electricity and heat (Malmkvist, 2018). In addition, the liquid residue from biogas production is rich in ammonia and suitable to use in agriculture as fertilizer, replenishing the soil with nutrients (Sogn et al., 2018).

1.1 Background: Fuelling biogas reactors

Biogas reactors in Sweden are fuelled using waste products and residues from municipal treatment plants, manure, food waste and various residues from industrial food production and slaughterhouses (Klackenberg, 2019). Household waste and

slaughterhouse waste typically have high protein content. Protein rich materials have been shown to yield large quantities of biogas in anaerobic digestors, due to its high specific methane potential (Westerholm et al., 2016). Sorted household waste can yield 461-650 m^3 methane/ton VS and is composed of approximately 12 g/kg fat and 32 g/kg protein. Slaughterhouse waste can yield from 434 m^3 methane/ ton VS substrate depending on the parts used from the slaughter process (Carlsson and Uldal, 2009). Moreover, using substrates with high level of proteins additionally improve the fertilizing quality of the digestate (Risberg, 2015).

1.2 Ammonia inhibition

Even though protein rich organic material has a high methane potential, there are problems related to such substrates. A side effect of high protein substrate is that it can result in the accumulations of ammonia from the fermentation of amino acids. Ammonia is one of the foremost causes of process failure in anaerobic digestors (Rajagopal et al., 2013). The accumulation of ammonia has inhibiting effects on the microbial community found in anaerobic digestion processes, and is especially toxic to methanogens, who have a low tolerance to ammonia. The accumulation of ammonia often lead to process instability, with symptoms commonly expressed as; a reduction in biogas produced, decreased methane content in the gas, alkalinity and accumulation of volatile fatty acids (Rajagopal et al., 2013). High protein substrates are also rich in sulphur (Yan et al., 2018) which is released as hydrogen sulphide during the degradation. Excessive amounts of hydrogen sulphide can be inhibitory to microorganisms involved in anaerobic digestion (Chen et al., 2008). In addition, high sulphide content can cause corrosion to metal parts used in the biogas process and engines, and therefore needs to be removed (Erdirencelebi and Kucukhemek, 2018). The upgrading of the biogas to remove sulphide adds to production costs.

Towards the end of the anaerobic digestion, methane is formed mainly either by acetate or hydrogen-utilizing archaea. Traditionally, methane production via acetate have been reckoned as the main metabolic pathway in biogas production (Sun et al., 2014, Zinder and Koch, 1984). At high ammonia concentration however, an alternative metabolization occurs where acetate is first oxidized into hydrogen and carbon dioxide by syntrophic acetogenic oxidizing bacteria (SAOB), followed by a reduction of hydrogen by hydrogenotrophic methanogens into methane (Westerholm et al., 2016). SAOB have a higher relative tolerance against high ammonia concentrations compared to acetate-utilizing methanogens, which induces a metabolic shift in the biogas process where the dominant methane producing pathway changes at high ammonia concentrations (Schnürer and Nordberg, 2008).

1.3 Acetate enrichment

Acetoclastic methanogens and SAOB both utilize acetate. However, there seems to be conditions that affect the competitivity between the two groups when it comes to acetate concentration. One study (Hao et al., 2013) showed that concentrations of acetate (>50mM) inhibited acetoclastic methanogenic activity. However, SAOB and the hydrogenic methanogens Methanomicrobiales and Methanobacteriales showed tolerance towards high acetate concentrations and high pH. At higher concentrations of acetate (150-200mM) and after resumption of the pH to normal levels (pH<8,0), the growth of SAOB and hydrogenic methanogens was stimulated, while the acetoclastic methanogens remained impaired (Hao et al., 2013). This led to a shift in the dominant pathway from acetoclastic methanogens to the SAO pathway.

Another study reported that a shift to the syntrophic pathway was complete when the acetate concentrations was 250 mmol/L and the ammonium levels of 6-7 g-N/L (Lü et al., 2013). At ammonium levels of 1-4 g-N/L and acetate concentrations from 150 to 250 mmol/L, acetate was degraded by both acetoclasic methanogens and SAO together with hydrogenotrophic methanogens while the latter dominated at 250mmol/L. Moreover, in addition to high ammonia concentrations, high acetate concentrations was suggested as one of the factors contributing to the shift from acetoclastic methanogenesis to SAO and hydrogenotrophic methanogenesis (Westerholm et al., 2012)

1.4 Thesis objective

The purpose of this study was to evaluate the impact of subjecting anaerobic digestion processes to increasing levels of ammonia and to study the performance of the reactors as well as the potential microbial changes that occur when the reactors are pulsed with acetic acid to enrich the reactors with acetate, along with the ammonia increase. The pulsing of acetic acid to the reactors aimed at increasing the biomass of SAOB, which have a higher tolerance towards ammonia. The increased biomass of SAOB were hypothesized to generate a positive effect in the reactors when the ammonia concentration increases and the metabolic shift from acetoclastic methanogenesis to SAOB and hydrogenotrophic methanogenesis occurred.

Furthermore, the objective of this study was to study the factors contributing to reactor instability and changes in reactor conditions correlating with changes in the microbial population in anaerobic digestors.

1.5 Project implementation

At the Swedish University of Agricultural Sciences in Uppsala, five lab-scale biogas reactors for anaerobic digestion were available for this study. The reactors had previously been operated at similar conditions as used in present study, such as similar substrate, temperature, stirring speed, original inoculum et cetera. Initially, an analysis of VFA production and methane formation from the substrate was done to investigate the similarities and/or differences in the reactors and to get information of the acetate production/consumption kinetics.

After the VFA production and methane formation in the reactors had been analyzed, the project focused on increasing the ammonia levels in four of the five reactors during one retention time, while the last fifth reactor operated as a non-changed control. Two of the four reactors that were subject to an increase in ammonia were pulsed with acetic acid to promote growth in biomass of SAOB by acetate enrichment.

Data and samples were gathered in order to evaluate reactor performance and analyze the microbial changes before and during the retention time.

1.6 Expected impact of this study

The results of this study will hopefully contribute to the understanding of anaerobic digestion processes and how one of the main current problems affecting biogas processes today might be prevented.

2 Biogas production

Biogas is the product of anaerobic digestion and is composed of methane, carbon dioxide and smaller amounts of other gases such as nitrogen gas and hydrogen sulphide. From the point when substrate is added to the process, multiple reactions are performed by several different groups of microorganisms, which finally results in biogas. The microorganisms involved in the biogas process work together in a synchronized manner and are sensitive to disturbances. The products of one organism is the food for another, and what has an effect on one microorganism can have a rippling effect in the intertwined network that makes up the microbial population in anaerobic digestion reactors (Amani et al., 2010).

2.1 Organic degradation in anaerobic digestors

There are many processes that transform substrates into biogas. This section describes the degradation of complex organic materials such as protein and polysaccharides to the formation of biogas. The degradation is done by several microorganisms that each take part in different stages of the degradation. Organic compounds, including carbohydrates, proteins and fats are broken down in a series of processes that include hydrolysis, fermentation, anaerobic oxidation and methanogenesis, illustrated in figure 1.

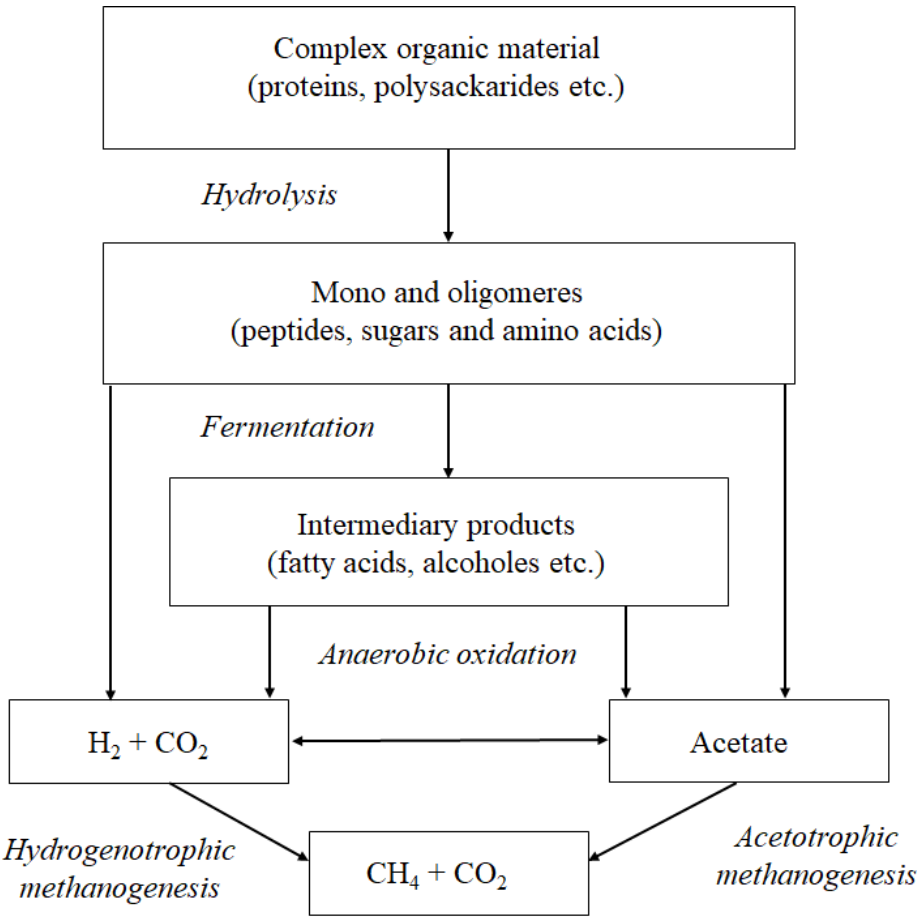

Figure 1. A flow scheme depicting the metabolic processes that lead to the formation of biogas (based on figure by (Schnürer and Jarvis, 2017).

2.1.1 Hydrolysis

The first step of the organic degradation is hydrolysis, which is a term which describes processes where organic compounds are broken down from polymers into monomers (Angelidaki et al., 2011). This process is needed as the polymers is too large to be taken up and consumed as energy and nutrients by the organisms. The organisms excrete extracellular enzymes that either is discharged in the space around the cell or attaches to the cell wall of the organism. The enzymes break down the organic compounds into smaller parts, with proteins, fats and carbohydrates being converted into amino acids, fatty acids and sugars, such as glucose, respectively.

Hydrolysis is often regarded as a limiting factor in the overall degradation. However the rate of degradation varies between compounds, cellulose being more limiting than for example soluble starch (Noike et al., 1985).

2.1.2 Fermentation

During fermentation, the products from the hydrolysis are being used for a new set of reactions by the organisms. Sugars and amino acids are transformed into various organic acids such as acetic acid, propionic acid, butyric acid, as well as alcohols such as ethanol, and gases, such as carbon dioxide, hydrogen. In addition ammonia and hydrogen sulphide is released from the degradation of amino acids. (Sikora et al., 2017). The fatty acids that are produced from the fermentation are not processed further by any of the microorganisms performing the reactions but are instead used in the proceeding anaerobic oxidation (Schnürer and Jarvis, 2017).

The state of acids that are formed during fermentation from sugars, amino acids and alcohols are highly dependent on the prevailing pH-values in correlation with its pKa-value (Schnürer and Jarvis, 2017). The acids will be anions when the pH is higher than the acids respective pKa-value. Anions are negatively charged and therefore more likely to react with ions such as natrium and form salts.

2.1.3 Anaerobic oxidations

Anaerobic oxidation is the next step following fermentation where predominantly the acids, but also alcohols, aromatic compounds and some amino acids are transformed into hydrogen, acetate or carbon dioxide (Schnürer and Jarvis, 2017). The anaerobic oxidation is depending on the relationship between different bacteria and the current hydrogen concentration. The anaerobic oxidation is highly dependent on hydrogen being consumed by methanogens, since the formation of new hydrogen is not possible unless the current hydrogen concentration is kept low, due to thermodynamic laws.

2.1.4 Methanogenesis

During methanogenesis methane is formed from mainly hydrogen, acetate and carbon dioxide by methanogenic archaea (Schnürer and Jarvis, 2017). Methanogens can be divided into two general groups depending on whether they utilize hydrogen (hydrogenotrophic methanogens) or compounds with methyl groups (methylotrophic methanogens). Methylotrophic methanogens can then be further be specified as acetotrophic methanogens if they use acetate as substrate. Methanogens in general have low growth rates and are sensitive to disturbances in the reactor, for example contaminations of heavy metals and fluctuations in temperature and pH. Ammonia is another common disturbance factor causing inhibition of methanogens. Methanogens are more sensitive to ammonia compared than other microorganisms, such as different bacteria. Disturbances of the methanogens often create ripples in

the entire process. Mainly as the methanogens consume hydrogen and keep the hydrogen concentration low, crucial for the anaerobic oxidations.

The metabolism of methanogens differs from other organisms and is related to unique enzymes and coenzymes. Acetate was long believed to be the most important substrate for methanogens and suggested to accounts for 70% of the methane produced, while hydrogen, carbon dioxide and format accounts for the other 30% of produced methane. (Angelidaki et al., 2011). However, today it is clear that hydrogen is the main substrate for methanogenesis under certain conditions.

Known species of hydrogenotrophic methanogens are found within the order Methanobacteriales and Methanomicrobiales. One specific genus of Methanomicrobiales commonly detected in biogas processes are *Methanoculleus*. Known acetoclastic genera *Methanosaeta* and *Methanosarcina* (Angelidaki et al., 2011).

2.1.5 Syntrophic acetate oxidation

Acetate is the one major product metabolized into methane and carbon dioxide in anaerobic digestion (Sikora et al., 2017). The metabolization is done by acetoclastic methanogens. However, an alternative pathway exists where acetate is converted by syntrophic oxidizing bacteria into hydrogen and carbon dioxide. Syntrophic acetate oxidation (SAO) is performed by two groups of organisms; hydrogenotrophic methanogens and acetate oxidizing bacteria that work in a syntrophic relationship.

The first reaction in SAO is the oxidation of acetate to hydrogen, carbon dioxide and format by syntrophic acetate oxidizing bacteria (SAOB) (Westerholm et al., 2016). The hydrogen and carbon dioxide can then be used as substrate by hydrogenotrophic methanogens to form methane.

SAO is often referred to as an alternative pathway, yet it has been shown to be the major pathway for methane production in anaerobic digestion systems with high ammonia (Müller et al., 2016, Westerholm et al., 2016). SAOB have also been shown to be significantly more abundant in high ammonia processes where SAO is the dominant pathway (Westerholm et al., 2011). Among the microorganisms active in the biogas process, the acetate utilizing methanogens are said to have the comparably highest sensitivity to ammonia (Westerholm et al., 2016).

Species of bacteria that have been shown to be syntrophic acetate oxidizers include; *Thermacetogenium phaeum*, *Pseudothermotoga lettingae*, *Tepidanaerobacter acetoxydans*, *Clostridium ultunense*, and *Syntrophaceticus schinkii* (Westerholm et al., 2016). SAOB are more tolerant to ammonia compared to acetate utilizing methanogens, yet they have in comparison a slow growth rate (Westerholm et al., 2016).

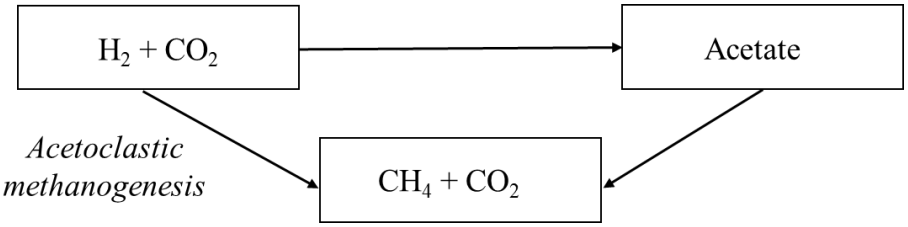

Figure 2. The pathway of acetoclastic methanogenesis (Schnürer and Jarvis, 2017).

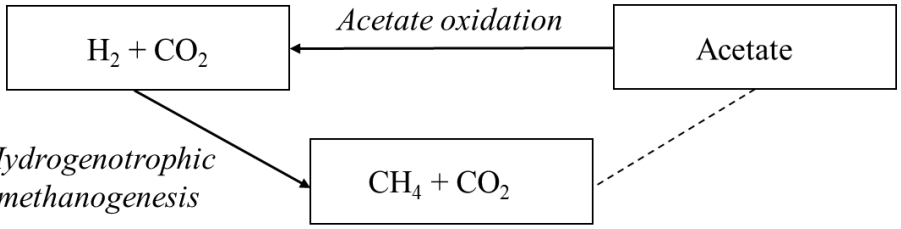

Figure 3. The pathway of syntrophic acetate oxidation (Schnürer and Jarvis, 2017).

2.2 Operating biogas reactors

The following chapter will cover some of the most important parameters relevant in the daily operations and surveillance of biogas reactors for anaerobic digestion.

2.2.1 Anaerobic digestors

Anaerobic digesters are designed to create an ideal environment for the organisms to thrive in. This means that various parameters need to be regulated according the organisms needs. After the start of an anaerobic digester it might take a while for the process to stabilize. The inoculum is suggestively collected from another already functional biogas process with a microbial population suitable for the new biogas process. This increases the chances of the new biogas process to have a good functionality (Schnürer and Jarvis, 2017).

The digestors are designed to simulate an environment which naturally is free from oxygen and can be designed in many ways. There are continuous processes where the reactor has a regular inflow and outflow. In batch processes, substrate is added to the reactors only in the start-up and the reactors are not emptied until the end of the process.

A further distinction can be made between wet anaerobic digestion (WAD) and dry anaerobic digestion (DAD). WAD requires comparably more water (see below

2.2.5), which can be scarce in some countries, which is why DAD has increased in popularity (Kothari et al., 2014).

2.2.2 Temperature

The correct temperature for anaerobic digestion varies according to the conditions preferred by the organisms. The temperatures that microorganism prosper in can be divided roughly into three regions: psychrophilic (0-15°C), mesophilic (20-45°C) and thermophilic (45-70°C) conditions. The conditions that bacteria thrive in are often similar from where it is naturally occurring, for example in the gut of cows (39°C). The temperature highly affects the growth rate of the bacteria, however, warmer is not always better as the organisms each have their specific optimal range (Schnürer and Jarvis, 2017).

2.2.3 Substrate

Substrates commonly used in biogas processes include various residues from industrial food production, slaughterhouse waste, sludge from municipal waste water treatment plants and manure (Klackenberg, 2019).

Choosing which source of organic material to use as substrate in biogas processes comes down to several considerations; access to the substrate, its potential to cause side effects in the process and its gas potential. Another important consideration when choosing substrate is how it affects the quality and composition of the formed digestate. The gas potential can be calculated using formulas considering the amounts of carbon, nitrogen and oxygen molecules in the substrate (Schnürer and Jarvis, 2017). However, theoretical gas potential often differs from actual produced gas, for several reasons. The time the substrate remains in the reactor (retention time) affects the gas potential as the reactor contents are constantly interchanged in continuously designed processes. This means that since some of the reactor content is exchanged for new substrate daily, the drained reactor material could contain substrate that has not been completely utilized. This lowers the gas potential of the substrate. This is not the case in batch processes where the substrate has the theoretical potential of being fully utilized, since it stays in the reactor during the whole process and as the reactor content is not exchanged until the end (Schnürer and Jarvis, 2017). Most organic materials are mixtures of fats, proteins and carbohydrates. Only using one substrate (mono-digestion) has in some cases been shown to have certain disadvantages compared to co-digestion (using multiple sources of substrates). For example, animal waste with low carbon to nitrogen ratio (C/N ratio) can be difficult to use as a mono substrate (Velásquez Piñas et al., 2018).

Co-digestion has been shown to produce more gas compared to single substrates (Feyisetan Adekunle and Awele Okolie, 2015). Using co-digestion of complementary substrates has certain advantages compared to the use of single substrates as this increases the possibility of meeting the nutritional needs of the microorganisms. Co-digestion could add more vitamins and trace elements depending on the substrates used, and has been shown to improve process stability and biogas production. For example, mixing substrates that are rich in carbon (for example agricultural waste) with substrates rich in nitrogen (like animal manure and household waste) can balance out the C/N ratio and allow a more optimal process. The optimum C/N ratio is in the range of 20-30. High C/N ratios has been linked to poor buffer capacity and formation of large quantities of VFAs while substrates with low C/N ratios often have higher buffer capacities but also higher ammonia concentrations. (Hagos et al., 2017)

In some cases, pretreatment is needed before adding substrate to digesters to sanitize the substrate and reduce risks for contaminations. Pretreatment might also be needed to modify the composition of the substrate. For example; removing or adding liquids to alter the solid to liquid composition, removing chunks that might inhibit the digester stirring, and grinding of the substrate to increase the bioavailability. (Schnürer and Jarvis, 2017)

2.2.4 Organic loading rate

Organic loading rate (OLR) is the total load of organic material that is used to feed the digesters. It relates to volatile solids (VS), the amount of organic material in the daily feed (equation 1 (Nagao et al., 2012). If the OLR is too low, the organisms will not have enough nutrients and energy to grow. If the OLR is lowered from a regular rate in a running process, the organisms will not be able to produce as much biogas as previously and if kept even lower they might not be able to sustain themselves and start to die off. On the contrary, if the OLR is too high this might lead to accumulation of volatile fatty acids (VFAs), which has an inhibiting effect on the process. Produced biogas in relation to the OLR in the process can be used as a valuable performance evaluator to see if the process is efficient.

$$\mathrm{OLR} = \frac{g * VS}{L * day} \qquad (1.)$$

2.2.5 Total- and volatile solids

Total solids (TS) and volatile solids (VS) are standards used when feeding reactors. Biogas production is often related to added VS per volume unit and day (OLR) as it clarifies the amount of organic matter that generate a certain amount of gas. TS is defined as the proportion of the substrate, which is made up of solid material, both organic and inorganic.

The TS of the substrates is often used to determine the type of digestion technology most suitable to use, with DAD being applied to substrate with TS at 20% or above. Semi-dry processes utilizes substrates with a TS of 10-20% and conventional wet processes are applied to substrates with a TS less than or equal to 10% (Yi et al., 2014).

VS is defined as the proportion of substrate that is made up solely of organic material, excluding inorganic materials. If either TS or VS vary in the ingoing substrate, the processes might need more or less of the substrate in order to maintain the same OLR. This could also affect the hydraulic retention time in the process, which has to be taken into consideration.

TS are determined by heating the substrate to 105°C and comparing the dry sample to its wet weight. VS is determined after burning the dry sample to 550°C and measuring the weight of the ashes (Schnürer and Jarvis, 2017). VS is then calculated by taking the difference between the dry mass after 105°C minus the weight of the ashes after 550°C. (equations 2 and 3).

$$TS = \frac{Dry\ mass\ (105°)}{Wet\ mass} \tag{2.}$$

$$\mathrm{VS} = \frac{Dry\ mass\ (105°) - Ash\ (550°)}{Wet\ mass} \tag{3.}$$

2.2.6 Hydraulic retention time

In continuous processes, the digester content is constantly being replenish by the regular inflow of substrate and outflow of digestate. The time that the average substrate compound remains in the process is named the hydraulic retention time (HRT) and it is an important process parameter. If the HRT is too short (low), this might lead to a decrease in microbial concentration as the organisms are flushed out faster than they can grow. On the other hand, a longer HRT means that less substrate can be added each day to the reactors as the active reactor volume is to be kept constant. The HRT can affect the conversion of VS into biogas, and achieving a

shorter HRT while maintaining the biogas production is a way of increasing process efficiency (Shi et al., 2017). The appropriate HRT for a process depends on the degradability of the material used as substrate, with the goal of achieving a good circulation of substrate and biogas production without using a too large reactor, which is expensive.

$$\text{HRT} = \frac{Digester\ volume\ (approximate\ working\ volume)}{Daily\ feeding\ volume} \quad (4.)$$

2.2.7 Degree of degradation

Degree of degradation (DD) is a common evaluator of performance in biogas reactors (equation 5). A process which is efficient and utilizes most of the substrate, has a high degree of degradation (DD). A high degree of degradation means that much of the organic material in the substrate has been converted into gas. A low DD on the other hand, suggests that material remains to be converted to biogas in the process. Some substrates take longer time to break down than others, for example lignocellulose, and the DD varies from 30-90% depending on the substrate. Also, continuous processes tend to have lower DDs than batch processes. Yet when running both types of processes, it is worth taking into consideration whether it is worth waiting for the substrate to be further degraded as it usually takes a long time to fully convert substrates (Schnürer and Jarvis, 2017).

$$\text{Degree of degradation } (\%) = 100 * (1 - \frac{VS_{digestate}}{VS_{substrate}}) \quad (5.)$$

2.2.8 Reactor circulation

Circulation of reactor liquid is a way to facilitate the interaction between microorganisms and the digester sludge with substrate (Schnürer and Jarvis, 2017). It eases the interactions in the reactor, for example the ingestion of macronutrients for the organisms. It also makes reactions happen more rapidly than would otherwise be the case. One common way to circulate the reactor content is to stir the reactor using a set of paddles that are rotated on an axle. This is common in continuously stirred tank reactors. To enhance the stirring, baffles can be added to the inside of the reactor.

There are other types of reactors and mixing strategies and choosing which type to use often depends on the TS of the used substrate.

2.2.9 Ammonia and ammonium

When substrate rich in protein is fed to digesters it is broken down in the hydrolysis to amino acids. From the amino acids, ammonia (NH_3) is released in the fermentation. Ammonia (NH_3) is in equilibrium with ammonium (NH_4^+). The relative equilibrium between ammonia and ammonium depend on the temperature and pH levels, which drive the equilibrium to either end of the spectrum. An increase in temperature and pH pushes the equilibrium towards ammonia (figure 4) (Schnürer and Jarvis, 2017).

The estimated concentrations of ammonia can be calculated using equation (6 and 7) (Schnürer and Jarvis, 2017).

$$\mathrm{NH_3}(g/L) = \frac{NH_4^+ - N\,(g/L)}{(1 + 10^{(pKa - pH)})} \qquad (6.)$$

$$pKa = 0{,}09018 + \frac{2729{,}92}{T + 273{,}15} \qquad (7.)$$

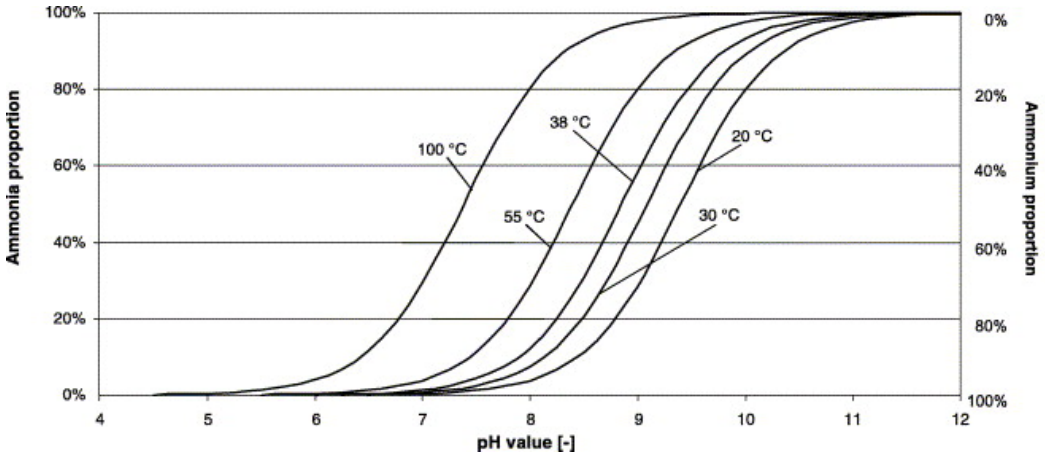

Figure 4. Balance between ammonia and ammonium depending on pH and temperature (Fricke et al., 2007).

2.2.10 Inhibitors in anaerobic digestion

There are some elements that could have negative effects on the anaerobic digestion. Some of the elements that are known to have inhibiting effects include salts, heavy metals, long chain fatty acids, sulphides and ammonia. These elements could either be in the reactor as they were introduced together with the substrate, or could be compounds formed as a result of internal reactions in the various stages of the anaerobic digestion. The effects are potentially irreversible, leading to a permanently compromised process. However, organisms can also to some extent acclimatize to the new conditions, after sometimes an initial phase with low growth and compromised gas production (Schnürer and Jarvis, 2017). The adaption can be caused of organisms growing as they can thrive in the new conditions, eventually making up a larger part of the microbial population than prior to the new conditions.

High concentrations of salts could lead to death of cells as osmosis occurs when the salt concentrations become too high and the cell pumps out its water. Some organisms can counter this by forming osmolytes, which stabilize the cell during in those conditions (Schnürer and Jarvis, 2017).

Another common inhibitor in anaerobic digestion is ammonia. The inhibition of ammonia have been proposed to related to the fact that ammonia is a non-charged molecule that can enter the cell (Sprott and Patel, 1986). In the cell it binds to hydrogen ions and forms ammonium. A compensation for the loss of hydrogen ions takes place as the cells pump in new hydrogen ions from the environment to regulate its pH-levels (Rajagopal et al., 2013). To pump hydrogen ions into the cells, the cells pump out potassium ions, which results in a loss of potassium ions. It was also suggested that the acetoclastic methanogens have lower potassium concentrations than hydrogenic methanogens which put them in a worse situation when the

ammonia levels increase. This different sensitivity to ammonia among methanogens is what is thought to induce the metabolic pathway shift for acetate conversion, as the acetoclastic methanogens are inhibited and the hydrogenotrophic methanogens together with SAOB are given room to grow (Westerholm et al., 2016).

Levels of total ammonium-nitrogen that have been documented to cause inhibition are values above 3 g/L (Schnürer and Nordberg, 2008), (Westerholm et al., 2016) and 3 g/L (Rajagopal et al., 2013). It has also been shown that the SAOB pathway is the dominant pathway in digesters operating around this level (2.8-4.6 g/L) (Fotidis et al., 2014).

2.2.11 Volatile fatty acids

Volatile fatty acids (VFAs), such as acetate, propionate and butyric acid, formed during the fermentation, are a reliable performance evaluators in anaerobic digestion systems (Franke-Whittle et al., 2014). Disturbances (e.g. an increase in organic loading rate) can affect the methanogens and cause a buildup of VFA. The buildup can then lead to an increase in the growth of hydrolyzing and fermenting bacteria, which is faster than the increase in the growth of methanogens (Schnürer and Jarvis, 2017). An accumulation in VFAs, can be a sign of inhibited methanogens. Increases in propionic acid and butyric acid have been shown to correlate with ammonia inhibition in anaerobic digestions (Bonk et al., 2018), which makes them worth measuring for monitoring purpose. Moreover, high VFAs can cause a decrease in pH in the reactor, which in turn can cause toxicity and process imbalance (Yuan et al., 2019).

2.2.12 Trace elements

Trace elements such as iron (Fe), nickel (Ni) and cobalt (Co) are some of the most studied trace elements in anaerobic digestion (Choong et al., 2016). Appropriate amounts of trace elements have been shown to contribute to a higher DD, higher biogas production and lower VFA concentrations. Trace elements mainly act as micronutrients in supporting the metabolism of the organisms where they function as co-factors in different enzymes, which keeps the anaerobic digestion process functional. Trace elements play a significant role in the function of methanogens. Supplementation of iron to anaerobic digestion has been especially studied as it is important as supplementation to reduce the levels of H_2S. H_2S forms metal sulphides, making trace metals unavailable for microorganisms, which can result in metal deficiencies (Thanh et al., 2016). By supplementing anaerobic processes with iron, the iron bind to the H_2S which frees other metals and makes them available for microorganisms.

3 Methodology and materials

The materials and methods used in this project are presented in this chapter.

3.1 Materials

The reactors that were used in this project where five continuously stirred biogas reactors made by Belach Bioteknik with a total volume of 10 liters and a working volume of 5 liters. The reactors were connected to a computer where the reactor could be controlled and monitored using a systems interface. The stirrer speed and temperature could be set and monitored, together with the biogas production, which could also be monitored.

Figure 5. The continuously stirred reactors that were used in this study.

The five reactors used in this study had been in use for some time at the and run at similar prerequisites. Some variations among the reactors existed but the handling

of the reactors had been identical for some time at the time of the start of this study. Prior to the test with increased ammonia, the reactors had been running for some time with stable conditions (day 0-139). The reactors were fed with the same substrate and OLR and operated with the same temperature and retention time. The original source of inoculum was the same for all reactors. At the start of this study the reactors had VFA levels of 0.89-2.05 g/L acetate and 0.03-0.09 g/L propionate (table 1). The reactors were fed substrate with an OLR of 2.5 g VS/L/day. The substrate consisted of a mixture of household and slaughterhouse waste and albumin (14 g/kg wet weight) and supplemented with BDP-865, which is a trace element additive (2.5 ml/L substrate). This mixture was used for the daily feed during days 0-140. The substrate had an average TS of 13% (SD: 0.0055) and VS of 12% (SD: 0.0056) two months prior to the start of the study. The substrate consisted of the same source of household and slaughterhouse mix throughout this study, but the added albumin and BDP-865 changed.

The stirrer speed was set to 90 rpm and the temperature was 37°C for all the reactors, making the conditions mesophilic.

Table 1. *The operating parameters of reactors C2, B2, A1, R1 and R2 prior to the start of this study and their respective acetate and propionate concentrations. The samples for the acetate and propionate samples were taken three days before day 0.*

Reactor	OLR [g VS/L/day]	HRT [days]	Temp [°C]	Stirring speed [rpm]	Acetate [g/L]	Propionate [g/L]
C2	2.5	30	37	90	1.59	0.08
B2	2.5	30	37	90	1.50	0.08
A1	2.5	30	37	90	0.89	0.03
R1	2.5	30	37	90	1.78	0.09
R2	2.5	30	37	90	2.05	0.09

3.2 Experimental setup

This study aimed at investigating the effect on the process when increasing the ammonia levels in four of the five reactors during one retention time (30 days), while using the fifth reactor as control. The goal was to reach an ammonium nitrogen concentration of 4 g/L, at least passing 3 g/L where the metabolic shift to SAO driven methanogenesis typically occur. Two of the four reactors subjected to an increase in total ammonium-nitrogen were furthermore pulsed with acetic acid to enrich the reactors with acetate, with the aim to promote growth in biomass of SAOB.

Prior to the increase in total ammonium-nitrogen and pulsing with acetic acid, a kinetics test where performed during which the acetate production and consumption as well as methane production was measured. A biomethane potential test and two tests where reactors C2 and B2 were pulsed with acetic acid were done as well. For details on the different experiments see below.

3.2.1 Increased total ammonium-nitrogen test

The increased ammonium-nitrogen test, and the pulsing of reactors C2 and B2, took place from day 140 to day 174. The reactors were fed with substrate once a day, six days a week. The substrate used was a mix of household waste and slaughterhouse waste from the co-digestion facility Kungsängens gård, Uppsala Vatten. The composition of the substrate was altered to reach the desired increase in ammonia-nitrogen in the reactors. To maintain approximately the same load but at the same time increase the nitrogen level some of the household waste was replaced with albumin in the daily feed. Still the change resulted in a slight increase of OLR in the daily feed from 2.5 g VS/L/day to 3.3 g/VS/L/day.

From day 140, Reactors C2, B2, A1 and R1 were fed this substrate, including 70 g albumin/L substrate, which was intended to raise the total ammonium-nitrogen in the reactor to 4.1 g/L. To estimate the amount of albumin needed to reach the desired ammonium level the degree of protein mineralization was calculated as described below. For reactor R2, which was the control reactor, the substrate was not changed as compared to the period before day 140. However, the OLR was adjusted to reach a similar level as the experimental reactors and reached 3.2 g VS/L/day. This change in OLR caused an increase in the ammonium nitrogen in the reactor to 2.4 g/L from 2 g/L, see table 2. An overview of the experimental setup is found in table 4.

The TS and VS of both the household and slaughterhouse substrate mix and the albumin was analyzed before calculating the amounts needed to achieve an increase in the supplied organic nitrogen (table 3). The TS and VS of the household waste and slaughterhouse waste was on average 13.0 ± 0.3% and 12.0 ± 0.4% of the wet weight. The VS of the albumin was 92 ± 0.01%.

The degree of nitrogen mineralization was calculated by considering the organic and inorganic content of nitrogen in the substrate (household and slaughterhouse mix and albumin) (table 2) and by using equation 8, which takes into account the ammonium nitrogen of the process digestate and substrate as well as the organic nitrogen of the substrate.

$$ML(\%) = \frac{(NH_4^+ - N)_{digestate}\,(g/L) - (NH_4^+ - N)_{substrate}\,(g/L)}{(Org - N)_{substrate}\,(g/L)} * 100 \qquad (8.)$$

At day 25, the nitrogen mineralization was calculated to be 48 ± 0.2%. This nitrogen mineralization was used in the calculations of the albumin supplementation to the substrate mix, in order to achieve increased amounts of supplied organic nitrogen.

Table 2. The analysis of the substrate (household waste and albumin) by Agrilab shows their organic nitrogen and ammonium nitrogen content.

	Organic nitrogen [g/kg]	**Ammonium nitrogen [g/kg]**
Household waste	3.9	0.2
Albumin	134.4	0.3

A trace element mixture was added to the substrate in order to assure reactor performance. In order to maintain the HRT at 30 days, water was added to the daily feed to reach the same total volume throughout the experiment.

Table 3. *The substrate composition used when feeding the reactors from day 0-140 and 140-174. The substrate composition differed for reactors C2, B2, A1 and R1 compared to the substrate fed to reactor R2 as the aim was to increase the total ammonium-nitrogen concentration in the former reactors.*

Reactor	**Period [days]**	**Substrate mix (household waste + albumin) [g/day]**	**Water [g/day]**	**Substrate albumin content [g/kg]**	**BDP [ml/L]**	**OLR [g VS/L/day]**
C2, B2, A1, R1, R2	0-140	115	80	14	8	2.5
C2, B2, A1, R1	140-174	107	88	70	8	3.3
R2	140-174	138	57	14	8	3.2

Reactors C2 and B2 were pulsed with acetic acid two times a day corresponding to a to a final concentration of 20 mmol/L reactor liquid (10 mmol/L each pulse). Reactors C2 and B2 were pulsed five days of the week at approximately 9:00 and 16:00 o´clock from day 140-174. Prior to day 140, the reactors had been run without pulsing except for the acetic acid pulsing tests on C2 and B2 (see 3.2.3).

Table 4. *The approach of the test where the total ammonium-nitrogen was increased and reactors C2 and B2 pulsed with acetic acid. Reactor R2 was not subjected to neither higher ammonia substrate nor pulsing with acetic acid, and thus acted as control.*

Reactor	**Ammonium-N increase**	**Acetic acid pulsing**	**Comment**
C2	Yes	Yes	
B2	Yes	Yes	
A1	Yes	No	
R1	Yes	No	
R2	No	No	Control reactor

Liquid and gas samples were taken in order to evaluate reactor performance during the increased ammonia. The samples were used to analyze VFA, biogas volume, CH_4, CO_2, and H_2S content in the gas pH, ammonium-nitrogen and total nitrogen.

3.2.2 Kinetics test

An initial kinetics test was done where samples for VFA and gas methane content were collected from the reactors during a period of 24 hours, starting in the morning. The test was done to analyze the VFA production/consumption and biogas production and its methane content in response to a feeding. The data from the kinetics test was used to design a suitable schedule for pulsing the reactors with acetic acid in order to achieve a regular supply of acetate in the reactors for the SAOB and hydrogenotrophic methanogens.

3.2.3 Acetic acid pulsing

A test where 5.7 ml acetic acid (≥ 99.8%, Sigma-Aldrich) was pulsed to the reactors was done to see the impact on the acetate degradation. The reactors were pulsed with an amount of acetic acid to achieve a molarity of 20 mM in each of the reactors. VFA samples and pH levels were taken continuously over the course of twenty-four hours. The test was done in order to see how the levels of acetate change and whether the acetate is degraded or accumulated in the reactors. The pulsing was only done to the reactors that later were to be pulsed with acetic acid during the increased total ammonium-nitrogen test, e g reactors C2 and B2.

3.2.4 Biomethane potential test

As part of discovering the kinetics of the system, a biomethane potential test (BMP) was done to study the ability of the process to degrade certain organic substrates and

at which rate. The substrates that were used in the BMP test was albumin, sodium acetate (Sigma-Aldrich), sodium propionate (Alfa Aesar), cellulose (two different batches where one was older than the other but no other differences, Sigma Life science) and rape seed oil (Eldorado).

The biomethane potential test was done using an AMPTS II (automatic methane potential test system) from Bioprocess Control Sweden AB. The BMP system is made up of three process units. The first is a sample incubation unit which contains the inoculum and substrate. The units suspended in a water bath at desired temperature, in this case 37°C and mixed by an agitator motor. The biogas which is produced is led through a tube to the second unit. The second unit is a CO_2 fixing unit. The unit contains saturated NaOH which fixates and retains all gases except methane which passes on to the third unit in the setup, which measures the produced gas as a lever is displaced by the buoyancy of the gas. The setup of the BMP-test is presented in table 5. The preparation of the BMP was done using pooled digestate from all the reactors taken at days 107-114 and done in triplicates. The digestate for the BMP was collected from the reactors before any pulsing with acetic acid had been done to the reactors and before the total ammonium-nitrogen increase test. The digestate from the reactors were therefore taken when the reactors were operated similarly. The results from the BMP-test is presented as Nml/gVS where the gas production from the digestate itself has been subtracted. The digestate for the BMP was collected from the reactors before any pulsing with acetic acid had been done to the reactors and before the total ammonium-nitrogen increase test. The digestate from the reactors were therefore taken when the reactors were operated similarly.

Table 5. The BMP test was prepared using the following substrates. The VS and added amount of inoculum, substrate and water for each flask is stated in the table. The inoculum had a VS of 1.7%.

Substrate	Substrate amount [g]	Substrate VS [%]	Inoculum [g]	Water [g]	Total weight [g]
Albumin	1.30	92.3	207	192	40.0
Sodium Acetate	1.67	73.2	207	191	400
Sodium Propionate	1.58	76.1	207	192	400
Cellulose (old)	1.26	95.5	207	192	400
Cellulose (new)	1.25	96.2	207	192	400
Rape seed oil	1.20	100	207	192	400

3.2.5 Microbial analysis

The microbial analysis was done in order to see if the increased ammonia and pulsing with acetic acids would impact the microbial population. The analysis

focused on three microbial groups; hydrogenotrophic methanogens, acetoclastic methanogens and SAOB, and how the concentrations of the groups changed over time from day 137 to 172. DNA was extracted from samples and quantified using qPCR. The quantification measured the number of copies of the 16s gene coding for the respective species/group per mL reactor sample.

3.3 Analyzation methods

3.3.1 VFA analysis

To determine the levels of VFAs, reactor samples were analyzed using high performance liquid chromatography (HPLC). The HPLC system was an Agilent Technologies 1100 series with refraction index detector. The column used was a Rezex ROA-Organic acid H+ (8%) interlinked. The mobile phase was 5mM H_2SO_4 and had a flow rate of 0,6 ml/min.

The samples that were analyzed using HPLC where prepared accordingly. The samples (2 mL reactor liquid) were centrifuged at11000 rpm for 15 minutes after which the supernatant was transferred to a new Eppendorf tube where it was mixed. 700 µL of the mixed supernatant was pipetted into a new Eppendorf tube and 70 µL of 5mM H_2SO_4 was added. The tube and its content were then frozen to allow precipitations to form. The frozen sample was allowed to thaw while being centrifuged for 10 minutes at 11000 rpm. As much supernatant as possible was extracted using a 1ml syringe, and then filtered through an Agilent technologies Captiva Econo 13mm, 0.2µm filter into a glass vial for HPLC analysis.

3.3.2 Gas chromatography

The methane analysis was done using a PerkinElmer Clarus 500 gas chromatography system with a flame ionization detector and mobile phase being helium (Westerholm et al., 2015).

1 ml samples for gas chromatography was taken using a syringe with a needle. The samples were taken through a membrane in the top of the reactor and then injected into a glass vial, before being run together with standards in the gas chromatograph.

3.3.3 Gas composition analysis

During the increasing ammonia test, the gas composition was measured using a Geotech Biogas 5000 instrument from Scantec Nordic. The instrument is made for surveillance of anaerobic digestion processes and measure CO_2 (0-100%), CH_4 (0-100%) and H_2S (0-5000 ppm).

CO_2 was also measured using an Einhorn saccharometer, filled with 7 M NaOH. A known volume of gas (5 ml) was injected into the tube, after which the CO_2 is dissolved in the NaOH and only the rest of the gas containing methane is left in the saccharometer. The concentration of CO_2 can then be estimated by comparing the injected volume to the amount of gas left.

3.3.4 Ammonium-nitrogen analysis

An ammonium-nitrogen analysis was done twice a week during the increasing total ammonium-nitrogen test to see if the concentrations increased towards 4 g/L as predicted. The test was done using a Merck Millipore Ammonium test and a Spectroquant Nova 60 spectrophotometer.

The samples were first diluted 100 times before being centrifuged (11 000 rpm for 10 minutes). The supernatant was filtered through a 2µm filter. 100 µL of the prepared sample was mixed with two reagents according to the manufacturer protocol in the Merck Millipore Sectroquant® Ammonium Test. In the test ammonium nitrogen is transferred to the form of ammonia by adjusting the pH to alkaline conditions (pH 11.5-11.8). The ammonia then reacts with hypochlorite ions and form monochloramine, which subsequently reacts with a phenol. The product was then photometrically analyzed in the Nova 60 spectrophotometer.

A further analysis of total nitrogen, organic nitrogen and ammonium-nitrogen was done externally by Agrilab.

3.3.5 Microbial population analysis

DNA extraction

DNA was extracted from 200 ml reactor sludge sample. The extraction was done in for one sample per time point using MP Biomedical FastDNA spin kit for soil samples according to the preparation protocol. The preparation included homogenization where the cells in the sample were lysed in the presence of reagents that protect and solubilize the nucleic acids. The DNA was then purified with a silica based GeneClean procedure where spin filters were used. The DNA was lastly eluted (with 70µL of DNase/Pyrogen-Free Water) into a clean Eppendorf tube and ready for further analyses.

Apart from the protocol, an extra step was done where 500 µL Guanadine-Thyocymine (5.5 M) was used to wash the DNA. The extra step was done before step 12 in the protocol, where the sample is washed with SEWS-M.

Qubit analysis

After DNA extraction, a DNA quantification was done in order to see that the extraction had been completed satisfactorily and to determine the amount of DNA extracted.

The quantification was done using a Thermo Fisher Invotrogen Qubit 3.0 Fluorometer. Samples were prepared according to the protocol (Thermo Fisher Invitrogen Life Technologies Qubit™ ds DNA BR Assay kit), where reagent and buffer was added with sample to a final qubit tube. The tube with sample, buffer and reagent was then measured according to their fluorescence in the Qubit Fluorometer and compared to standards for double standard DNA broad range.

qPCR analysis

The DNA was quantified and estimated using a quantitative polymerase chain reaction (qPCR) system from Thermo fisher, applied biosystems Quantstudio 5.

3 µL of extracted and diluted (500 & 750 times) DNA sample was added to a qPCR plate together with 10 µL HighQu ORA SEE qPCR GREEN ROX L mastermix, 1 µL Forward primer, 1 µL Reverse primer and 5 µL nuclease free water to a total volume of 20 µL in each plate well.

The qPCR running protocol was designed as followed; initially 95°C for 7 minutes, followed by 40 cycles of 95°C for 40 seconds, the specific annealing temperature for 1 minute and 72°C for 40 seconds. The last step was a melt curve stage where the plate was heated to 95°C for 15 seconds, followed by a 0.1°C increment per second from 55-95.

The primers used targeted the 16s rRNA gene. The annealing temperature, as well as the primer sequence used for each organism is further specified in table 6.

An initial dilution test was done to see whether inhibiting substances existed in the extracted DNA. The test showed that a dilution of DNA with 500-750 times was sufficient to avoid inhibition in the PCR reaction as opposed to dilutions of 50-200 times.

The standards for organisms/groups except total methanogens were prepared as described by (Westerholm et al., 2011). The preparation of the total methanogen standard is described in (Neubeck et al., 2019).

Table 6. *The archaea and bacteria which were studied in the microbial analysis using qPCR. The table includes the species of hydrogenotrophic methanogens, acetoclastic methanogens and syntrophic acetate oxidizing bacteria with their respective group affiliation, primer name, forward and revers primer sequence and annealing temperature.*

Organism/group	**Group**	**Annealing temp. [°C]**	**Primer**	**Primer Sequence**
Tot. Methanogens		60	Met 630-f	GGATTAGATACCCSGGTAGT
			Met 803-r	GTTGARTCCAATTAAACCGCA
Methanobacteriales	Hydrogenotrophic	58	Mbt 857-f	CGWAGGGAAGCTGTTAAG
			Mbt 1196-r	TACCGTCGTCCACTCCTT
Methanoculleus	Hydrogenotrophic	64	Mab-f	GGAATGCCCTGTAATCCAAA
			Mab-r	CACCTGAACAGCCTGCATT
Methanomicrobiales	Hydrogenotrophic	60	Mmb282-f	ATCGRTACGGGTTGTGGG
			Mmb832-r	CACCTAACGCRCATHGTTTAC
Methanosaeta	Acetoclastic	61	Mst-f	TAATCCTYGARGGACCACCA
			Mst862-r	CCTACGGCACCRACMAC
Methanosarcina	Acetoclastic	60	Msc 380-f	GAAACCGYGATAAGGGGA
			Msc-r	TAGCGARCATCGTTTACG
C. ultunense	SAOB	57	Cult56-f	CCTTCGGGTGGAATGATAAA
			Cult-r	TCATGCGATTGCTAAGTTTCA
S. schinkii	SAOB	61	Thac-f	ATCAACCCCATCTGTGCC
			Thac-r	CAGAATTCGCAGGATGTC
T. acetoxydans	SAOB	69	Th-f	GGGTGGTGTGAAGCCATC
			Th-r	CCCAGTGTTTCCACCAGGTC
T. phaeum	SAOB	63	Tp-f	AGGTAGTAGAGAGCGGAAAC
			Tp-r	TGTCGCCCAGACCATAAA

4 Results

In this chapter, the results from the tests in this study will be covered and presented. Starting with the results from the kinetics and acetic acid pulsing-test, followed by the results from the increasing ammonia test and the reactor performance, e g change in of biogas produced, pH, and VFAs. Last, the results from the microbial analysis will be presented.

4.1 Reactor kinetics

4.1.1 Kinetics test

The concentrations of acetate increase quickly after feeding and reached the maximum concentration of 1.6 g/L within 30 minutes to 2.5 hours (figure 6). The acetate was then slowly consumed during the course of 24 hours before reaching a concentration similar to that before the feeding (0.7 g/L). The acetate concentration in reactor B2 was slightly lower than the other reactors (figure 6).

The concentrations of propionate in the reactors reached its maximum concentrations of 0.19-0.33 g/L after about 2.5 hours after which the levels slowly declined (figure 7).

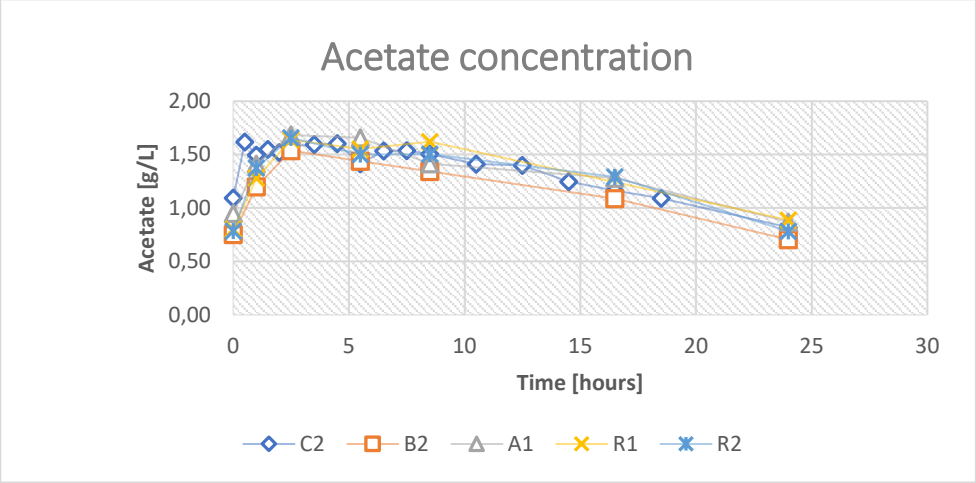

Figure 6. Acetate concentration after feeding in reactors C2, B2, A1, R1 and R2. The feeding took place right before hours 0.

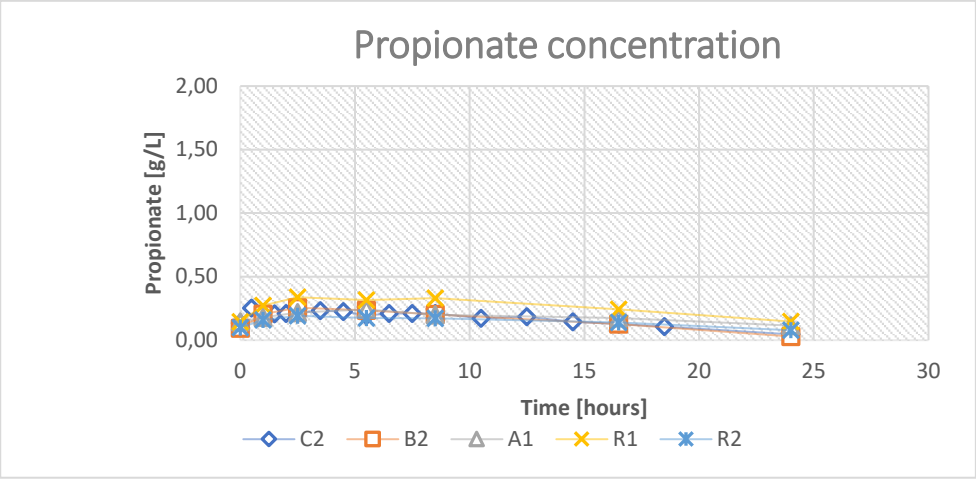

Figure 7. Propionate concentration after feeding in reactors C2, B2, A1, R1 and R2. The feeding took place right before hour 0.

The gas samples were taken shortly after the feeding of the reactor started. The reactors exhibit similar behaviour. In all reactors, the methane content of the biogas rose rapidly after feeding to levels between 81 and 88%, where after it subsided and reached a 24-hour minimum of 50% after 4.5 hours, before slowly rising to reach 59% after 24 hours (figure 8).

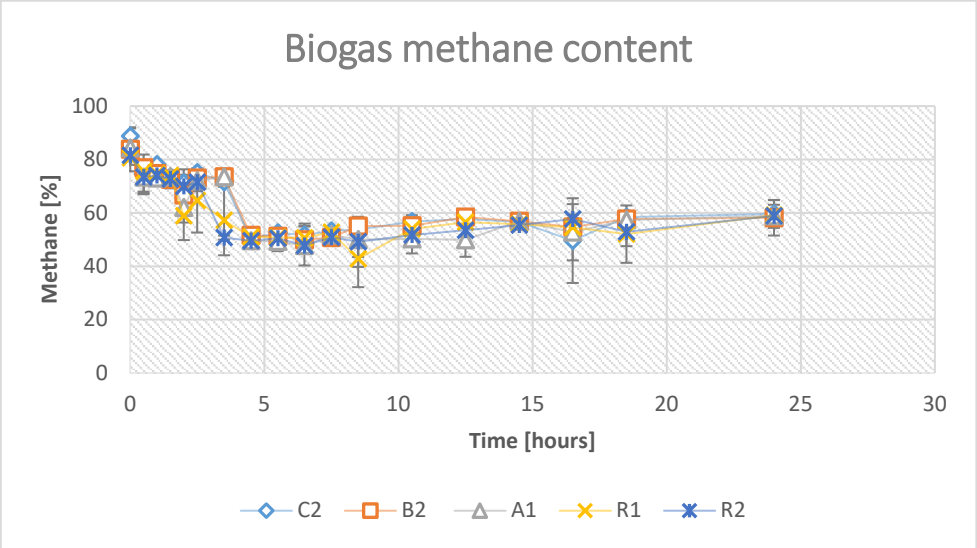

Figure 8. The methane content of the produced biogas in reactor C2, B2, A1, R1 and R2.

4.1.2 Bio-methane potential test

The maximum potential methane production was highest from rape seed oil, with a maximum average potential of 774 Nml/gVS (figure 9). The lowest BMP value was reached for albumin and sodium acetate with only 271 Nml/gVS and 268 Nml/gVS respectively.

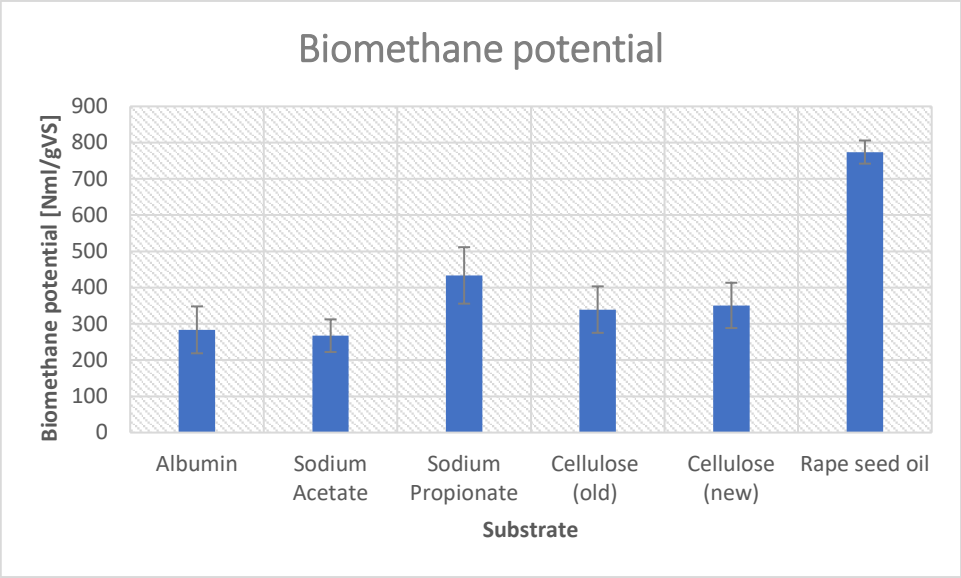

Figure 9. The biomethane potential from the substrates; albumin, sodium acetate, sodium propionate, two batches of cellulose (one older than the other but no other differences) and rape seed oil.

4.1.3 Acetic acid pulsing

After the tests where the feeding kinetics were investigated, two tests were done where two of the reactors were pulsed with acetic acid. The aim of these tests was to see how fast the acetic acid was produced and consumed and if any additional organic acids accumulated as a consequence of the pulsing.

Acetic acid pulsing test 1 (APT1)

During the first pulsing test (performed on day 129), the reactors were pulsed with 5.7 ml acetic acid (equation 10), equivalent to a concentration of 20 mmol/L (1.2 g/L) in the reactors. This meant that 0.1 mol of concentrated acetic acid was added to the reactors (equation 8). The acetic acid used has a density of 1.05 kg/L and a molecular weight of 60.05 g/mol, resulting in a concentration of 17.5 mol/L (equation 9). The reactors were pulsed with the acetic acid shortly after the first samples were taken (0 hours) and again fed with the substrate after hour 2. The test took place after a day where no feeding took place.

$$\frac{20 mmol/L}{5L} = 0.1\ mol \quad (9.)$$

$$\frac{1.04\ kg/L}{60.05 g/mol} = 17.5\ mol/L \quad (10.)$$

$$\frac{0.1\ mol}{17.47\ mol/L} = 5.7 mL \quad (11.)$$

The concentration of acetate increased rapidly after the pulsing to 1.0 g/L in C2 and 1.3 g/L in B2 (figure 10). The concentrations then slowly decline before increasing again as a result of the feeding.

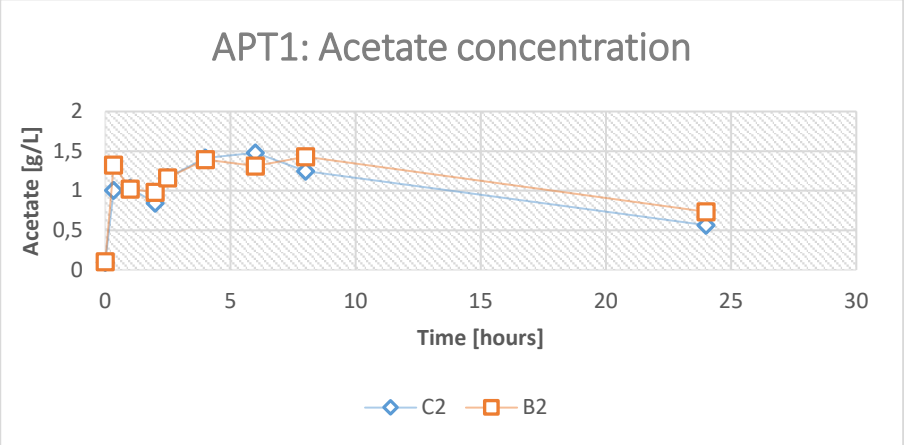

Figure 10. The concentrations of acetate in reactors C2 and B2 after being pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate after 2 hours during APT1 (acetic acid pulsing test 1).

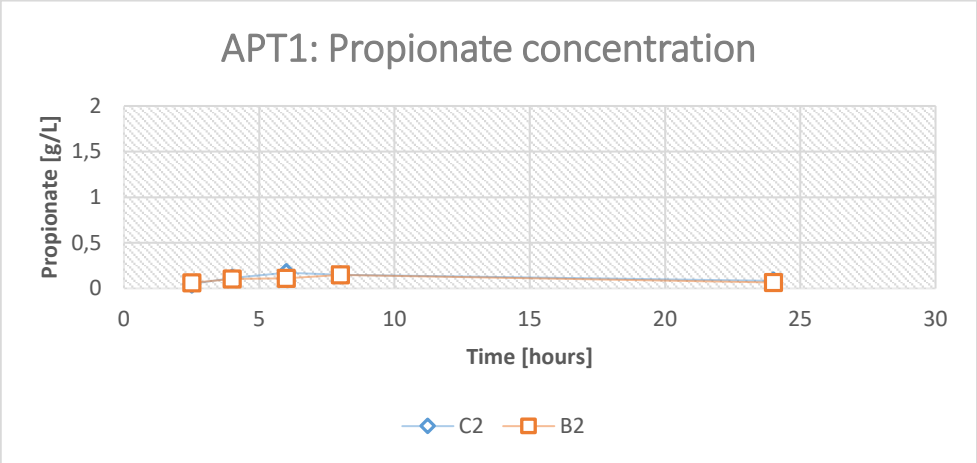

Figure 11. The concentration of propionate in reactors C2 and B2 after being pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate after 2 hours during APT1 (acetic acid pulsing test 1).

The concentration of propionate started to increase in both reactors after 2.5 hours in the test but reach only low levels (figure 11). The reactors showed similar behavior and the propionate slowly decreased in concentration, and remain low after 24 hours.

The pH for both the reactors was lowered shortly after the pulsing with acetic acid from 7.8 to 7.3 for C2, and from 7.9 to 7.2 for B2 (figure 12). The pH then increases somewhat again before feeding with the substrate, after which pH again decreased. The pH then slowly increases to about 7.5 for both reactors after 24 hours.

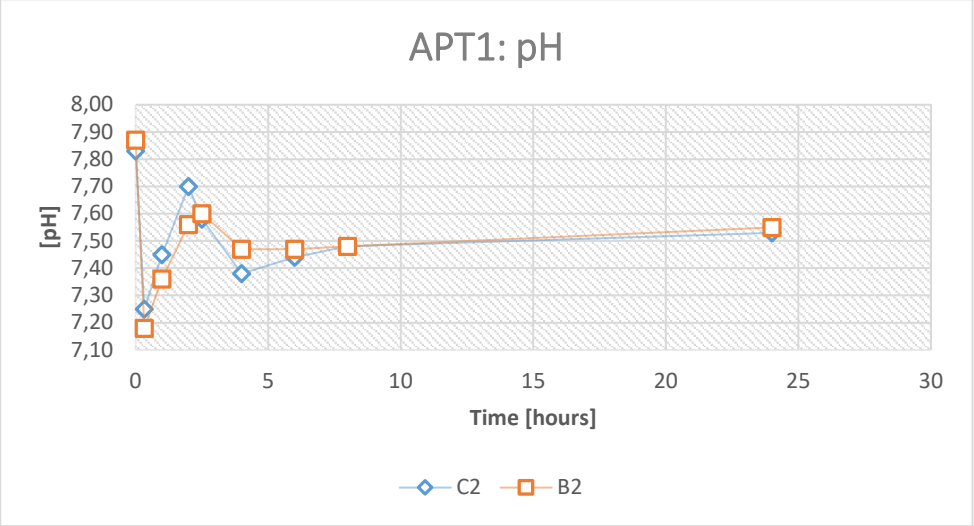

Figure 12. Variation of pH-level during the 24-hour test period of APT1 (acetic acid pulsing test 1), where reactors C2 and B2 were pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate after 2 hours.

Acetic acid pulsing test 2 (APT2)

The test where the reactors C2 and B2 were pulsed with acetic acid was reproduced again at day 134. The test took place after a day when no feeding occurred, like in APT1. The reactors were pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate between hours 1.25 and 2.25. Samples for VFA was taken as well as pH after 24 hours.

The reactors showed somewhat similar kinetics for both acetate and propionate as well as pH levels as in APT1. However, the acetate concentrations increased more in APT2 (figure 13) to a maximum of 1.9 g/L compared to 1.4 g/L in APT1, before decreasing again. The concentration of propionate (figure 14) was also higher in the second test compared to the first test for both reactors.

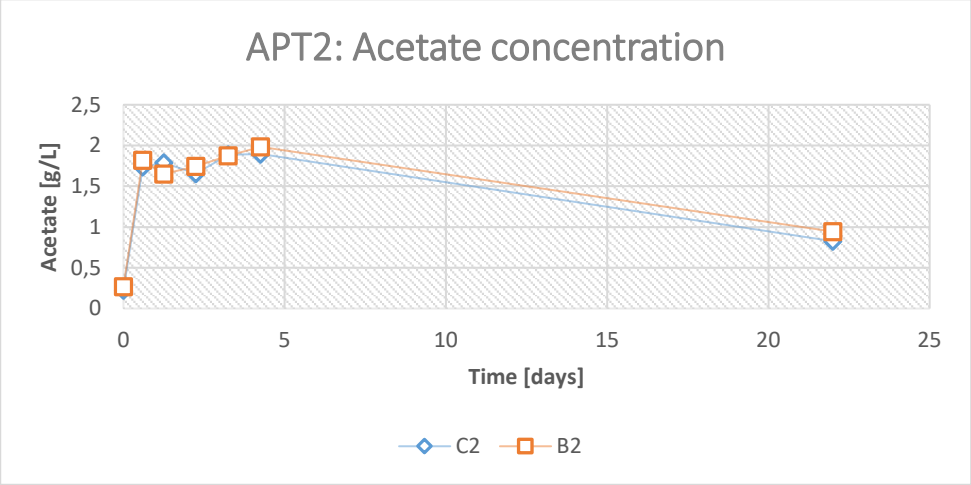

Figure 13. The concentration of acetate in reactors C2 and B2 after being pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate after 1.25 hours.

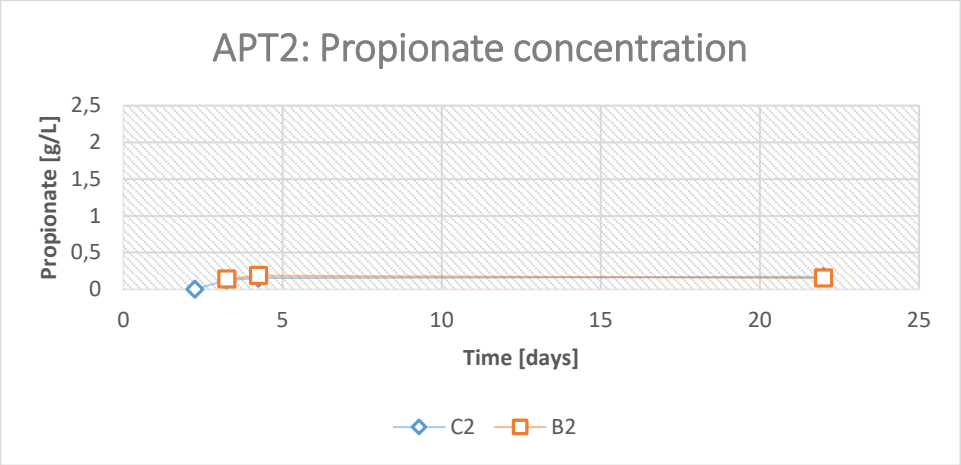

Figure 14. The concentration of propionate in reactors C2 and B2 after being pulsed with 5.7 ml acetic acid after 0 hours and fed with substrate after 1.25 hours.

The pH in the reactors were slightly lower at the beginning of the second test (APT2) at 7.52 (C2) and 7.56 (B2), compared to APT1. After pulsing the pH decreased to about 7.2 in both reactors but increased slightly again before the reactors were fed with substrate after 2.25 hours. After feeding, a slight decrease in pH was seen again after which the pH slowly increases during the rest of the 24-hour period (figure 15).

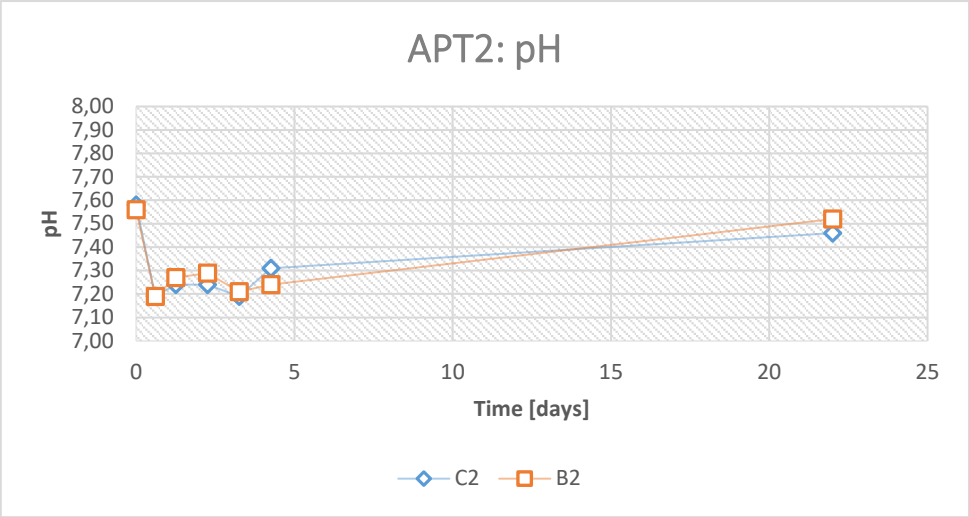

Figure 15. The pH levels for reactor C2 and B2 during the second acetic acid pulsing test.

4.2 Increasing ammonia test

In this section, the results from the increased ammonia-test are covered. The results include the concentrations of total ammonium-nitrogen in the reactors, the pH, the weekly average biogas production and the concentration of VFAs in all the reactors.

During this test, substrate supplemented with extra albumin was used to feed reactors C2, B2, A1 and R1, e g all reactors except R2, which acted as a control reactor. Reactors C2 and B2 were pulsed with acetic acid two times a day to a concentration of ca 10 mmol/L per pulse. Prior to the change in substrate, no pulsing was performed except for the pulsing tests on C2 and B2. The presentation of data below shows the behavior of the reactors where day 0 represent 139 days before the test with increasing ammonia starts. The increasing ammonia test, and the pulsing of reactors C2 and B2, took place from day 140 to day 174.

4.2.1 Total ammonium-nitrogen (TAN)

The total ammonium-nitrogen was measured from day 140 until day 169. An increase in TAN was be seen in all experimental reactors (about 1.9 g/L TAN to 4.1 g/L) except for reactor R2. The concentration of TAN in reactor R2 was 2.1 g/L at day 169 which is a slight increase as compared to day 141. All the reactors show a slight decline in the total ammonium-nitrogen concentration at days 151 and 155. The theoretical total ammonium-nitrogen concentration was 4.1 g/L (assuming the nitrogen mineralization increased at 50-60%) in reactors C2, B2, A1 and R1 and 2.4 g/L in reactor R2.

The effect of changing the substrate composition resulted in total-ammonium-nitrogen concentrations of 3.9 g/L (C2), 4.3 g/L (B2), 3.9 g/L (A1) and 4.1 g/L (R1) at day 169 according to the Merck Millipore Ammonium test Spectroquant Nova 60 spectrophotometer.

The analysis of samples by Agrilab shows the total nitrogen, organic nitrogen and ammonium nitrogen of the process digestate, table 7. The data from the analysis showed that the nitrogen mineralization increased from 48% at day 25 to 66% (A1+R1) and 64% (C2+B2) at day 193, table 8. The ammonium and organic nitrogen of the substrate mix was calculated using data from the Agrilab analysis for the individual substrates and amounts used in the mix (table 2, 3).

Table 7. Analysis of the process digestate after different days of operational for total nitrogen, organic nitrogen and ammonium nitrogen.

Reactor(s)	**Day**	**Total nitrogen [g/kg]**	**Organic nitrogen [g/kg]**	**Ammonium nitrogen [g/kg]**
R1	25	3.4	1.7	1.7
A1	25	3.6	1.8	1.7
R1	67	3.6	1.8	1.8
A1	67	3.6	1.8	1.8
A1+R1	149	4.6	2.0	2.6
C2+B2	149	4.8	2.0	2.8
A1+R1	168	6.3	2.3	4.0
C2+B2	168	6.3	2.2	4.1
R2	168	4.1	2.1	2.0
A1+R1	193	7.0	2.4	4.6
C2+B2	193	7.1	2.6	4.5
R2	193	4.2	2.0	2.2

Table 8. Analysis of the ammonium nitrogen from digestate samples and the ammonium and organic nitrogen from the substrate used the corresponding day. This data was used to calculate the nitrogen mineralization.

Reactor(s)	Day	Digestate ammonium nitrogen [g/kg]	Substrate ammonium nitrogen [g/kg]	Substrate organic nitrogen [g/kg]	Nitrogen mineralization [%]
R1	25	1.7	0.1	3.4	48
A1	25	1.7	0.1	3.4	48
R1	67	1.8	0.1	3.4	49
A1	67	1.8	0.1	3.4	50
A1+R1	149	2.6	0.1	6.8	36
C2+B2	149	2.8	0.1	6.8	39
A1+R1	168	4.0	0.1	6.8	57
C2+B2	168	4.1	0.1	6.8	59
R2	168	2.0	0.1	4.0	47
A1+R1	193	4.6	0.1	6.8	66
C2+B2	193	4.5	0.1	6.8	64
R2	193	2.2	0.1	4.0	53

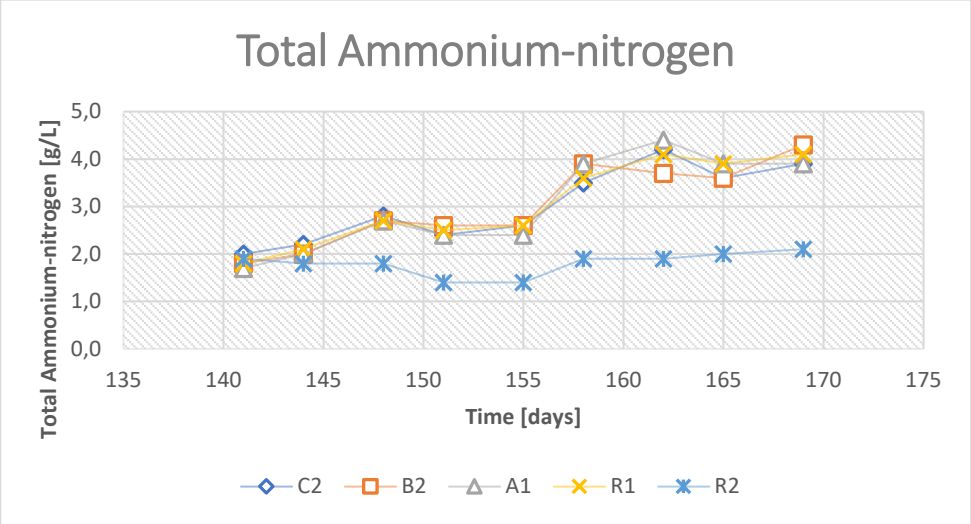

Figure 16. The total ammonium-nitrogen concentrations for reactors C2, B2, A1, R1 and R2 during the increased ammonia test (days 140-169).

The pH levels in the reactors were relatively stable in all reactors at around 7.3-7.6 before the increase in total ammonium nitrogen (figure 17). From day 140 the pH increased slightly in all reactors before decreasing again at day 169.

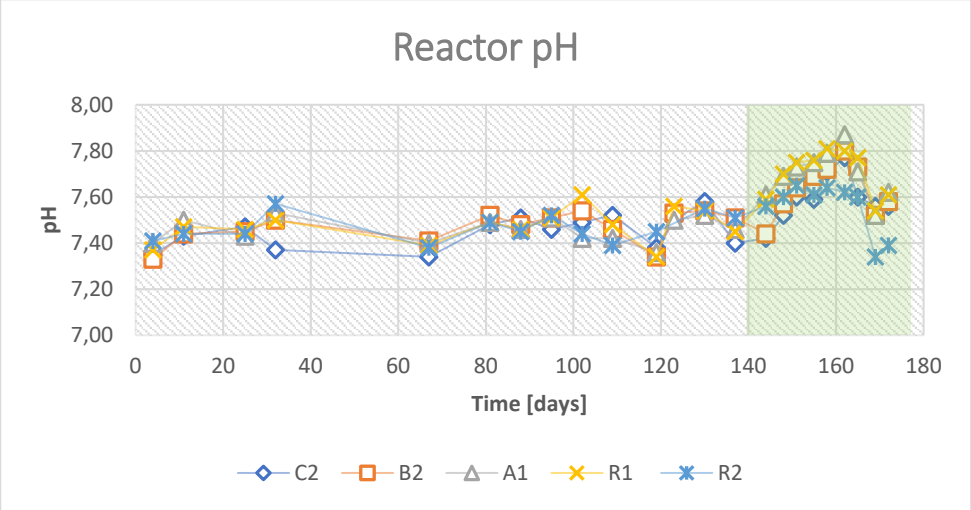

Figure 17. The pH levels in the investigated reactors. The green field illustrates the time when the total ammonium-nitrogen was increased.

4.2.2 Biogas production

The biogas production for the reactors were measured as an average weekly production. The average weekly biogas production was divided with the current gVS for the reactors (14.5 gVS for all reactors day 0-139 and 19.1 gVS (C2, B2, A1, R1) 19.0 gVS (R2) day 140-172). All the reactors C2, B2, A1, R1 and R2 behaved somewhat similar during the whole period, even from day 140 when the ammonia was increased and reactors C2 and B2 were pulsed with acetic acid.

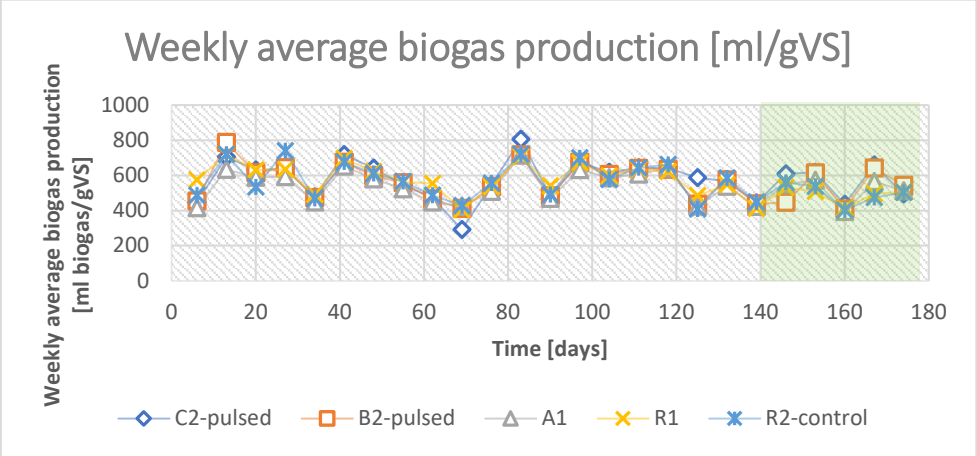

Figure 18. The weekly average biogas production for reactors C2, B2, A1, R1 and R2. The green field illustrates the time when the total ammonium-nitrogen was increased.

subjected to the new substrate with higher albumin content and before reactors C2 and B2 were pulsed with acetic acid. After days 141 the methane content in reactors C2 and B2 dropped to around 55% at the time of measurement where it remained stable until day 179 (figure 19). The added supplementation of albumin to the substrate did not seem to affect the methane content of the subjected reactors as the methane concentration remains at similar levels as before the albumin supplementation.

The CO_2 content in the gas was similar between the reactors at around 35% until day 141 when reactors C2, B2, A1 and R1 were subject to the new substrate with higher albumin and reactors C2 and B2 were pulsed with acetic acid (figure 20). The CO_2 content in reactors C2 and B2 then increase from 35% to 40% until day 179 when it decreases to 27% in C2 and 34% in B2.

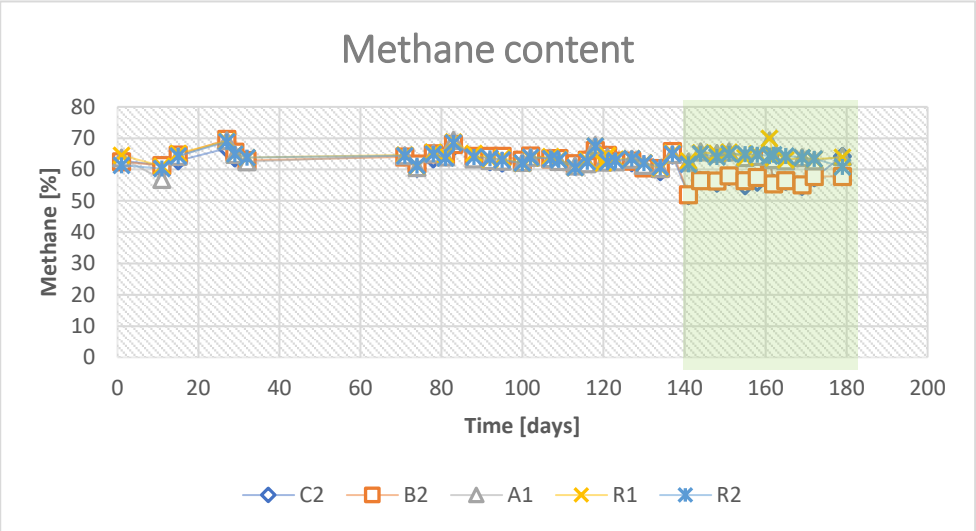

Figure 18. The methane content in the produced biogas from reactors C2, B2, A1, R1 and R2. The green field illustrates the time when the total ammonium-nitrogen was increased.

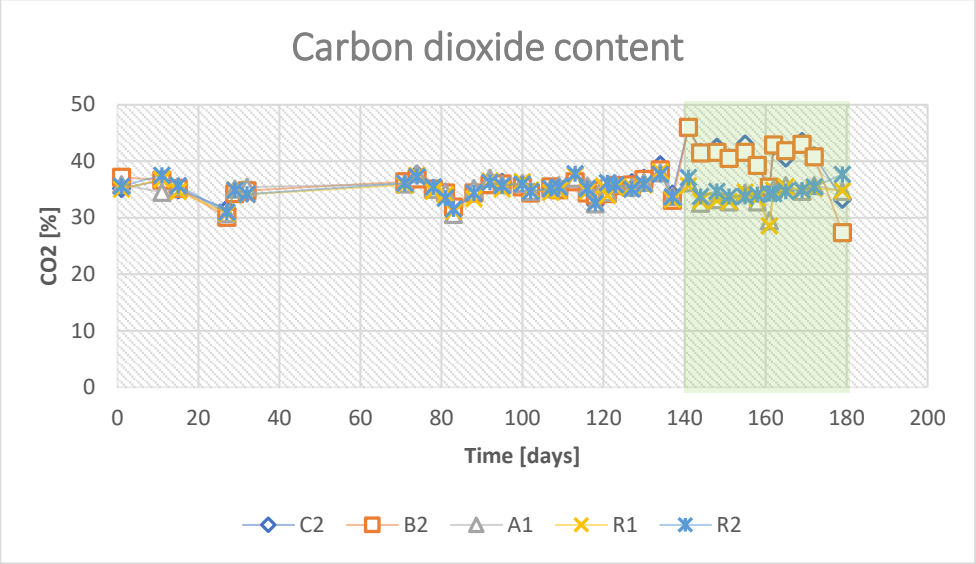

Figure 19. The carbon dioxide content in the produced biogas from reactors C2, B2, A1, R1 and R2. The green field illustrates the time when the total ammonium-nitrogen was increased.

The H_2S content in the reactors was below 350 ppm for all reactors from day 0 until day 141 when it increased to around 1600 ppm in reactors C2, B2, A1 and R1(figure 21). At this time point BDP supplementation was increased to counter the rise in H_2S. The concentrations of BDP was increased from 2.5 ml/L to 8 ml/L (2.5 ml/L to 6 ml/L to finally 8.0 ml/L). The H_2S then decreased from day 155 and at day 179 the level was 30 ppm in B2, 610 ppm in C2 and around 1000 ppm in A1 and R1.

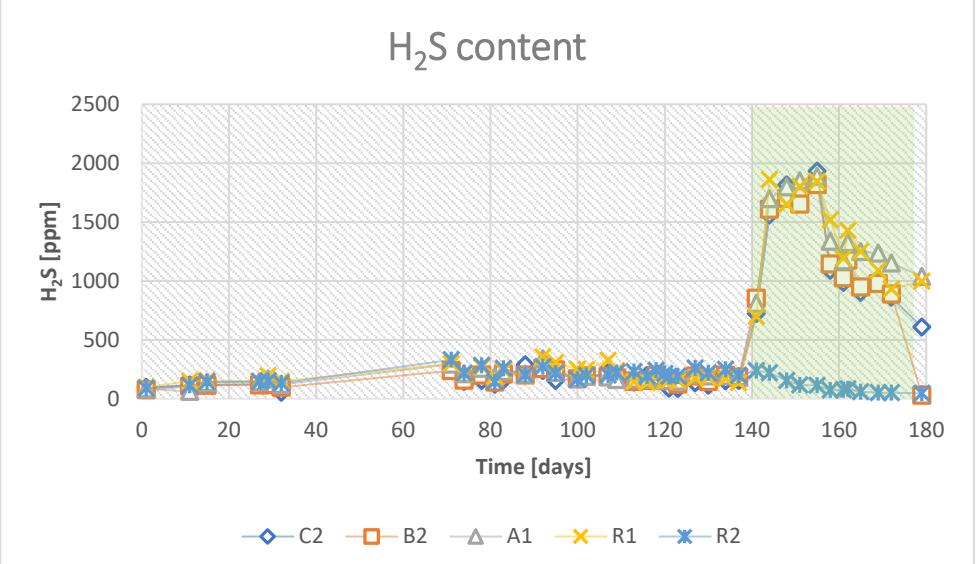

Figure 20. The H_2S content in the produced biogas from reactors C2, B2, A1, R1 and R2.

4.2.3 VFAs during the increasing ammonia and acetic acid pulsing

The VFAs showed a clear difference between the reactors, with the concentrations of both acetate and propionate remaining higher in reactors C2, B2, A1 and R1 while being low in reactor R2, the control (figure 22, 23). The concentration of acetate was constantly higher in reactors C2 and B2, pulsed with acetic acid. The zig-zag pattern which can be seen in figure 22 is not strange, as the data points where the acetate concentration was lowest represents the concentration on a Tuesday, in contrast to the data points where the concentration is higher, which were sampled on a Friday. During the week, acetate accumulate slightly in the pulsed reactors, but decreased again during the weekends when no pulsing was performed. The reactors were also fed only one of the two days of the weekend.

When looking at reactors A1 and R1, which were subjected to an increase in ammonia but not being pulsed, an increase in acetate could also be seen in both reactors during the time when the TAN was increased. The acetate concentration increased in reactors from 0.4 g/L to 1.6 g/L (A1) and 1 g/L (R1).

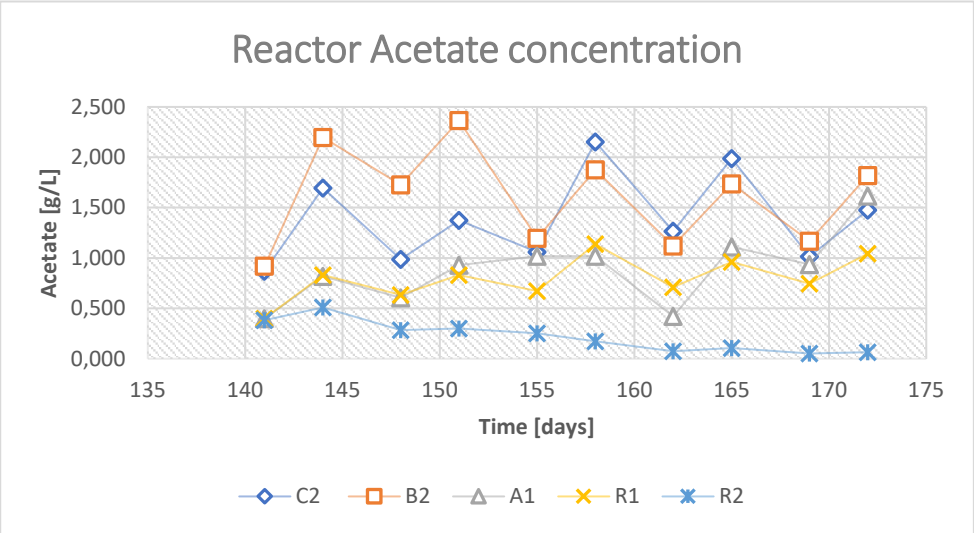

Figure 21. The acetate concentration in reactors C2, B2, A1, R1 and R2 during day 141 to 172. Reactors C2, B2, A1 and R1 were subjected to an increase in ammonia and reactor C2 and B2 were in addition pulsed with acetic acid.

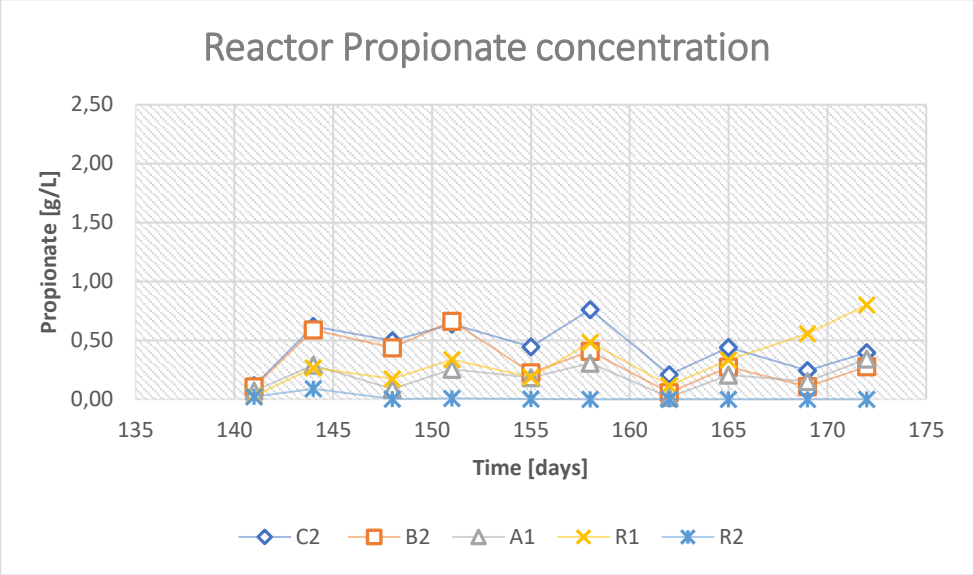

Figure 22. The propionate concentration in reactors C2, B2, A1, R1 and R2 during day 141 to 172. Reactors C2, B2, A1 and R1 were subjected to an increase in ammonia and reactor C2 and B2 were in addition pulsed with acetic acid.

4.3 Reactor kinetics after increasing ammonia test

After the increased ammonia test, a test similar to the initial kinetics test was done to investigate the difference VFA kinetics between the reactors. The reactors were pulsed after hour 0, then fed immediately before 2.7 hours and pulsed again after 7.2 hours. Samples for VFA were taken over one day and again the next morning.

The samples were taken during a day which proceeded like a normal weekday during the increased ammonia test. That is, reactors C2 and B2 were pulsed with 2.8 mL acetic acid in the morning and afternoon, and all reactors except R2 (control) was fed with substrate supplemented with albumin.

All reactors except R2 showed an increased acetate concentration after 24 hours compared to hour 0 (figure 24). An increase in acetate was seen until 8.2 hours (figure 24). The increase was highest in reactors C2 and B2, having acetate concentrations of 3.2 and 3.5 g/L respectively compared to A1 and R1, with acetate concentrations at 8.2 hours of 2.5 and 2.2 g/L (figure 24). This was likely a result of reactors C2 and B2 being pulsed with acetic acid, as compared to A1 and R1 which were not.

When it comes to the propionate concentrations in the reactors (figure 25), reactors C2 and B2 exhibited somewhat similar kinetics. A1 and R1 on the other

hand differed between one another as the concentrations of propionate in reactor R1 was constantly higher than the propionate concentration in A1.

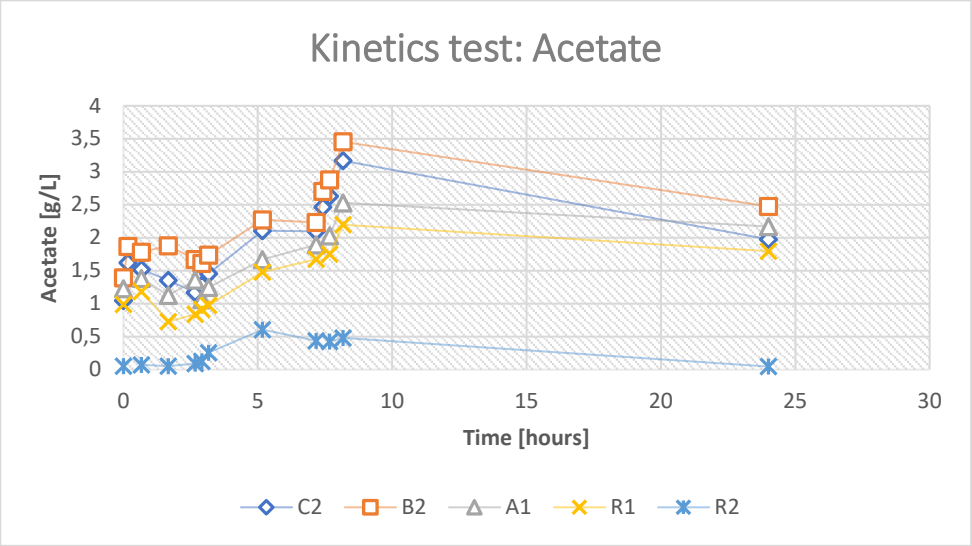

Figure 23. The figure shows the acetate concentration in reactors C2, B2, A1, R1 and R2 during the kinetics test.

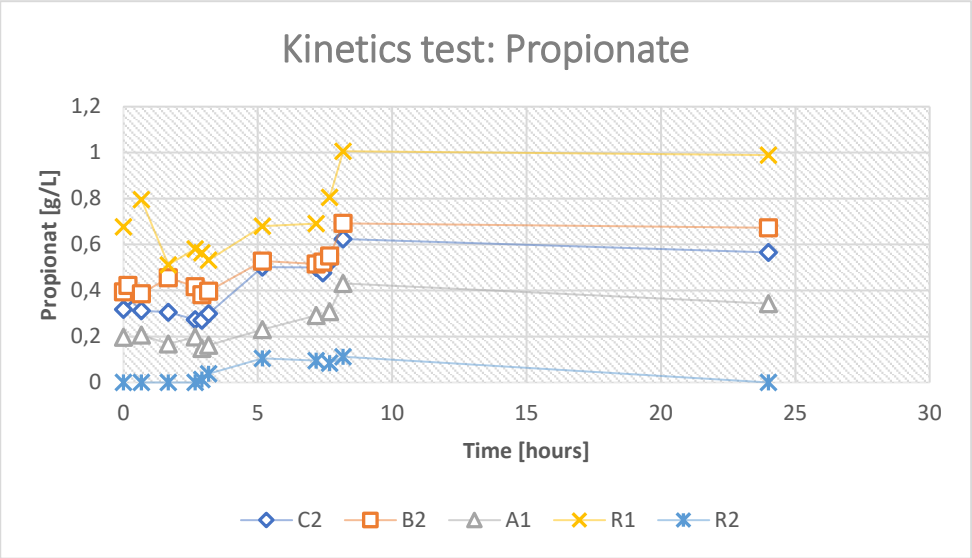

Figure 24. The figure shows the propionate concentration in reactors C2, B2, A1, R1 and R2 during the kinetics test.

4.4 Microbial composition

The microbial population was quantified using qPCR with samples taken once a week during the increased ammonia test and were compared to a sample taken the week before the test started (day 137).

The abundance of the Archaea and Bacteria at day 131 and 172 is presented in table 9. The samples from day 131 is before the total ammonium-nitrogen was increased in reactors C2, B2, A1 and R1 and C2 and B2 were pulsed with acetic acid. The samples from day 172 is after the HRT with increased ammonium-nitrogen and acetic acid pulsing was finished.

Table 9. *The average microbial abundance of analysed microorganism and microbial groups (qPCR) in reactors C2, B2, A1, R1 and R2 at the start and after the increased ammonia test. (Before: day 137, After: day 172). The table also shows the percental difference between the averages of the species between day 137 and 172.*

Species	**C2**	**B2**	**A1**	**R1**	**R2**
Tot. Methanogens	2.8±0.3E+09	3.2±1.1E+09	4.03±0.3E+09	3.3±0.3E+09	3.8±0.1E+09
Prevalence before/after	3.9±0.03E+09	5.1±1.1E+09	2.3±0.05E+09	3.3±0.2E+09	3.8±0.1E+09
Difference [%]	37	58	-42	-0.6	0.7
Methanobacteriales	-/-	-/-	-/-	-/-	-/-
Prevalence before/after	-	-	-	-	-
Difference [%]	-	-	-	-	-
Methanoculleus	1.6±0.1E+09	2.5±0.1E+09	1.7±0.05E+09	2.5±0.01E+09	1.6±1.3E+09
Prevalence before/after	2.8±0.3E+09	4.5±0.4E+09	1.7±0.1E+09	2.5±0.06E+09	3.2±0.3E+09
Difference [%]	81	84	2	-1.4	103
Methanomicrobiales	7.1±0.7E+08	1.5±0.005E+09	9±0.1E+08	1.3±0.02E+09	1.1±0.5E+09
Prevalence before/after	2.9±0.1E+09	4.1±0.6E+09	1.5±0.2E+09	2.2±0.03E+09	2.7±0.6E+09
Difference [%]	299	179	67	70	145
Methanosaeta	7.6±0.3E+07	1.2±0.05E+08	9.7±2.1E+07	1.6±0.09E+08	5.6±6.6E+07
Prevalence before/after	3.6±0.4E+07	5.4±0.4E+07	1.9±0.1E+07	4.6±0.1E+07	2.1±0.9E+07
Difference [%]	-53	-54	-81	-71	-63

Species	C2	B2	A1	R1	R2
Methanosarcina *Prevalence before/after*	2.2±0.2E+08 1.5±0.2E+08	6.4±0.7E+08 3.5±0.01E+08	2.3±0.1E+08 6.2±0.3E+07	2.0±0.1E+08 5.2±0.2E+07	1.4±1.3E+08 2.5E±0.2+08
Difference [%]	-32	-45	-73	-74	77
C.ultunense *Prevalence before/after*	-/- -	-/- -	-/- -	-/- -	-/- -
Difference [%]	-	-	-	-	-
S. schinkii *Prevalence before/after*	8.9±0.1E+08 2.3±0.1E+08	1±0.02E+09 4.6±0.2E+08	7.7±0.9E+08 1±0.3E+08	9.2±0.3E+08 1.4±0.8E+08	4.8±5.3E+08 2.1±0.04E+08
Difference [%]	-74	-57	-87	-85	-56
T.acetoxydans *Prevalence before/after*	5E+06 -	1.7E+06 [1] -	-	-	-
Difference [%]	-	-	-	-	-
T. phaeum *Prevalence before/after*	4±0.07E+06 1.7±0.3E+06	2.6±0.9E+06 2.5±1.1E+06	1.7±0.4E+06 1.5±1.4E+06	3±1.2E+06 1±1.1E+06	1.2±1.1E+06 5.7±2.01E+05
Difference [%]	-58	-3.2	-11	-66	-52

1. The data point for *T. acetoxydans* was from day 151 and not 172.

4.4.1 Methanogens

The methanogens quantified using qPCR included species of hydrogenic methanogens: Methanobacteriales, Methanomicrobiales, the Methanomicrobales species *Methanoculleus*, and acetoclastic methanogens: *Methanosarcina*, *Methanosaeta* as well as total methanogens.

The quantitative PCR results for these species except Methanobacteriales can be seen in figures 26-30.

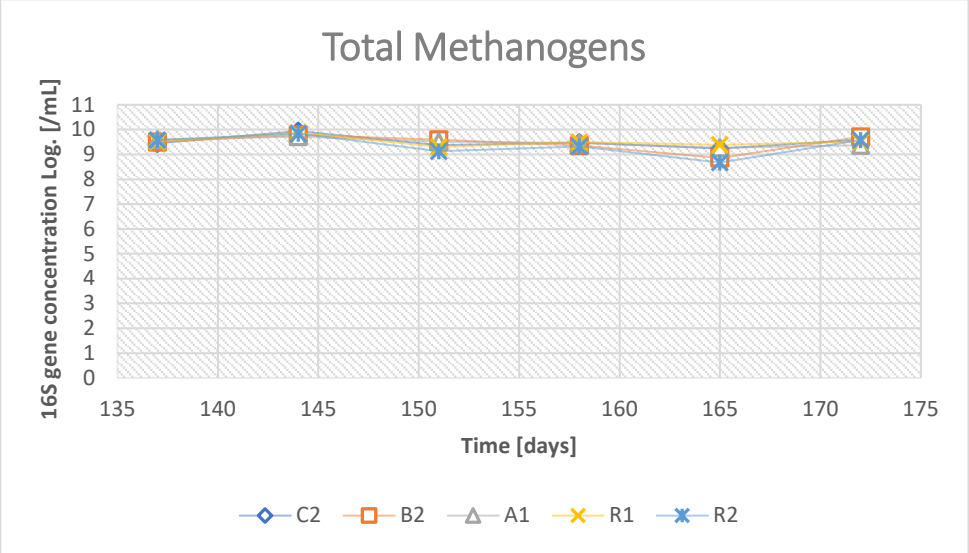

Figure 25. Total methanogen abundance in the reactors.

Hydrogenotrophic methanogens

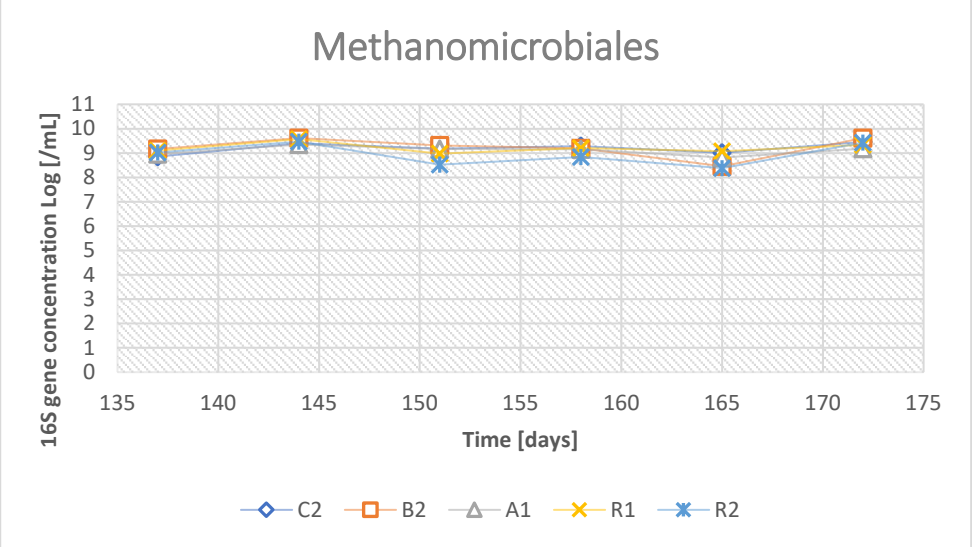

Figure 26. Methanomicrobiales abundance in the reactors.

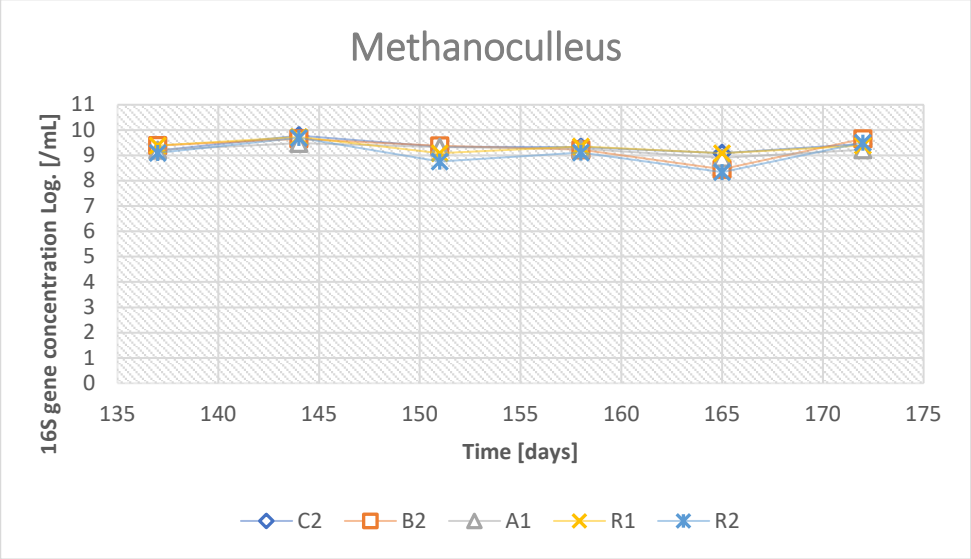

Figure 27. Methanoculleus abundance in the reactors.

The quantification of Methanobacteriales did not work as planned. The qPCR analyses showed a double peak in the melt curve, which was done as the last step of the qPCR. Double peaks indicate that the primers did not bind to one specific product, in this case the 16s gene sequence for Methanobacteriales, or the presence of primer dimers. Gel electrophoresis was done to investigate this issue and it showed that multiple DNA products had been bound to by the primers and amplified in the PCR. The results from that qPCR was therefore not reliable and could not be used.

Acetoclastic methanogens

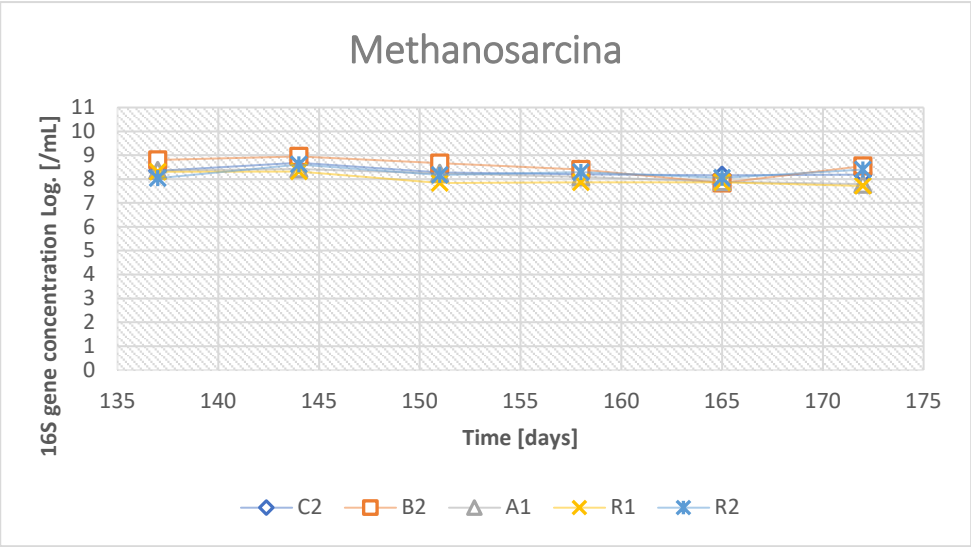

Figure 28. Methanosarcina abundance in the reactors.

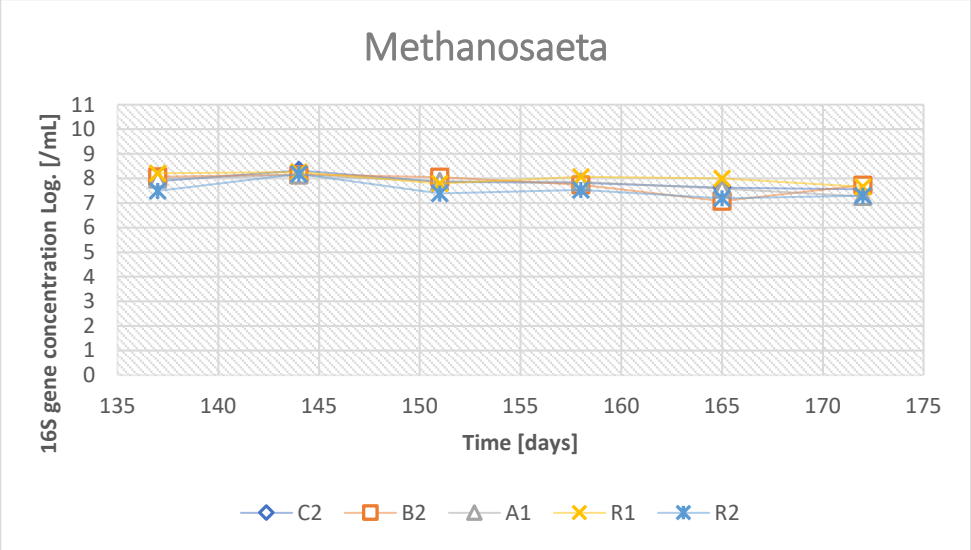

Figure 29. Methanosaeta abundance in the reactors.

4.4.2 SAOB

The qPCR results of the SAOB can be seen in figures 31-33. The quantification of *C. ultunense* produced results where the detected concentration of the species was lower in the samples than the negative control. The prevalence of *C. ultunense* in

the reactor samples was therefore too low to be reliably quantified and used as data in this study. The concentrations of *T. acetoxydans* were also lower than the negative control for all measurements than two (figure 32).

The abundance of *S. schinkii* and *T. phauem* did not change remarkably in any of the reactors throughout the HRT (figure 31, 33).

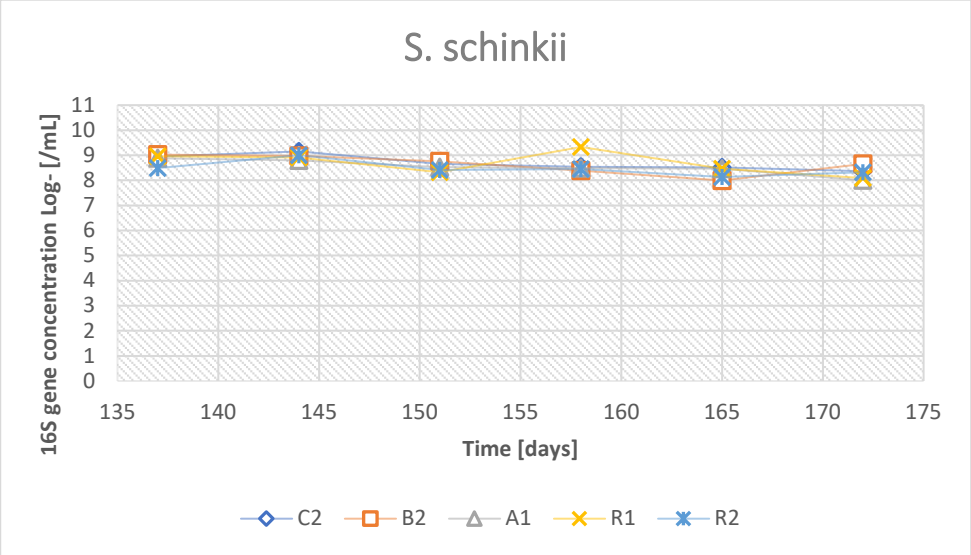

Figure 30. S. schinkii abundance in the reactors.

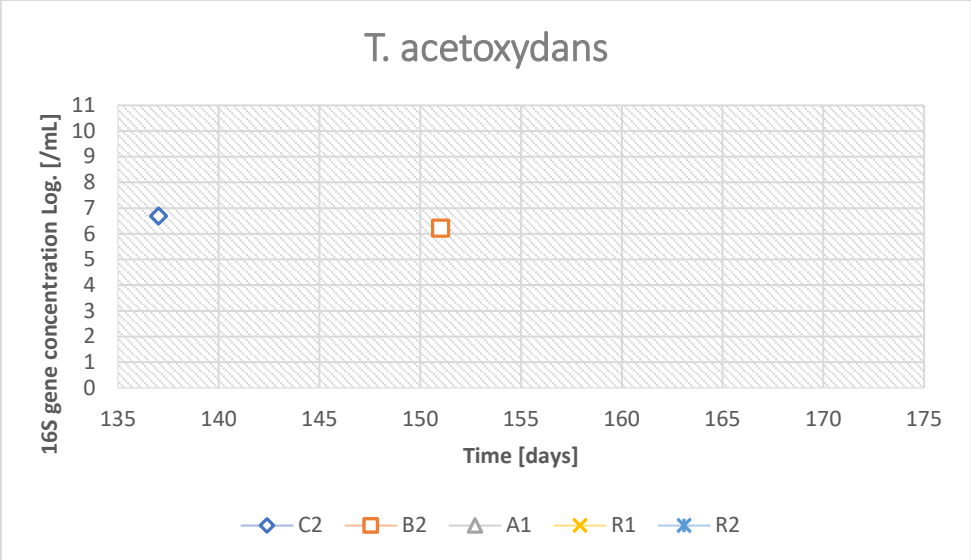

Figure 31. T. acetoxydans abundance in the reactors.

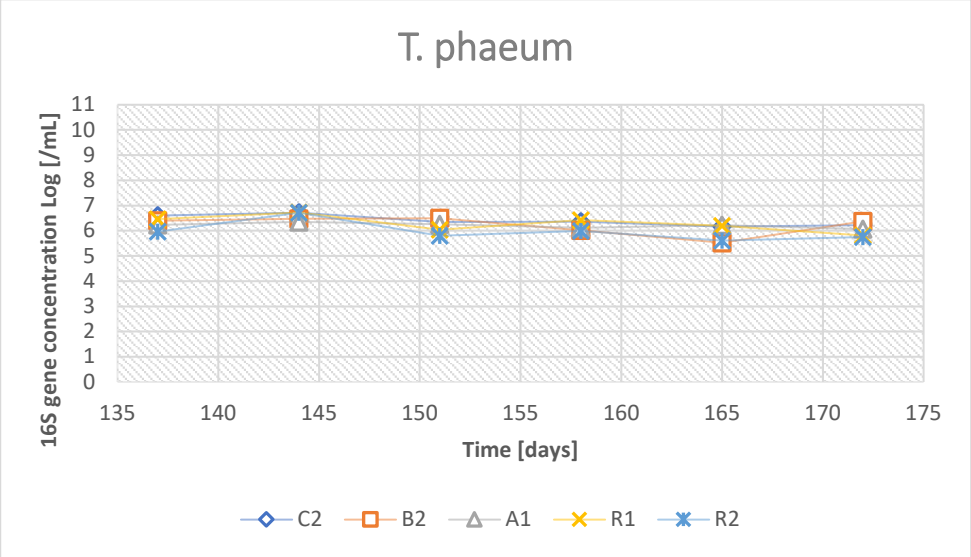

Figure 32. T. phaeum abundance in the reactors.

5 Discussion

In this chapter, the results of this study are discussed and analysed.

5.1 Total ammonium-nitrogen increase

Studies have found that inhibitory levels of TAN are around 3.0-5 g/L at mesophilic conditions (Schnürer and Nordberg, 2008) (Westerholm et al., 2016), (Rajagopal et al., 2013). 3.0 g TAN/L was reached in reactors C2, B2, A1 and R1 somewhere after day 155, which is less than halfway through the HRT of 30 days. That leaves half the HRT for signs of inhibition to show, such as VFA accumulation. However, even though the concentration of 3.0 g/L TAN was surpassed in the reactors no clear signs of process inhibition occurred.

Concentrations of TAN where SAO has been shown to be the dominant pathway are 2.8-4.6 g/L (Fotidis et al., 2014). One study (Westerholm et al., 2016) suggests that the threshold for SAO dominance and growth of SAOB and hydrogenotrophic methanogens occur around free ammonia concentrations of 0.14-0.28 g/L (37-38^{o}C, pH: 7.5-8.0) which is equivalent to a TAN of 3.0-6.0 in a reactor with pH 7.6 and temp: 37^{o}C. Another study found that high abundance of SAO, *C. ultunense*, *S. schinkii* and *T. acetoxydanse,* correlated with high ammonia concentrations (>160 mg/L, which would correlate to a TAN of 3.4 g/L in a reactor with pH 7.6 and temperature 37 C° (Sun et al., 2014). This approximate level was reached in reactors C2, B2, A1 and R1 at day 172. In the same study, acetoclastic methanogens had an abundance which correlated negatively to the same parameters.

The concentration of TAN achieved in reactors C2, B2, A1 and R1 was therefore most likely high enough for a shift in metabolic pathway to happen.

5.2 Reactor performance impact

The weekly average specific biogas production did not seem to be affected by the increase in TAN and/or the pulsing with acetic acid. There was however a clear effect on reactors C2 and B2 for the methane and CO_2 content of the produced biogas. This effect was clear from day 140 when the pulsing with acetic acid started. The methane and carbon dioxide content after day 140 was lower and higher, respectively, in reactors C2 and B2 as compared to the rest of the reactors. This suggests that the pulsing had a clear effect on the gas composition at the time when the gas measurements were taken. But as no apparent difference in weekly average biogas production/was seen from day 140, the pulsing with acetic acid did not seem to affect the actual biogas production from the anaerobic digestion. Instead the increase of CO_2 in the gas was likely a result of a chemical reaction caused by the addition of the acetic acid causing a local pH drop in the reactor liquid at the time of the gas content measurements.

Neither the acetic acid pulsing nor the increase in TAN seemed to have a significant effect on the weekly specific biogas production. However, the reactors subjected to acetate supplementation had, as a result of the pulsing, been given more substrate than the others from which also biogas can be produced.

The reason why no increase in biogas production is seen could be because a slight acetate accumulation occurred in those reactors. No clear increased activity in acetate consumption by SAOB or the acetoclastic methanogens seem to happen as this might otherwise lower the acetate concentrations. This is further supported by the results from the microbial analysis which does not indicate a change in the abundance of any of the acetate consumers.

When it comes to differences between the reactors due to their given substrates, replacing some of the household waste with albumin should have resulted in reactors C2, B2, A1 and R1 only producing 90% of the biogas as compared to reactor R2. This is because the albumin makes up a proportionally smaller part of the total gVS in the substrate used for R2 compared to the substrate for the other reactors during day 140-179. Albumin has a lower methane potential of 283 ml CH_4/gVS (data from the biomethane potential test), compared to the methane potential of the household waste alone which has a methane potential of 450-500 ml CH_4/gVS (Schnürer et al., 2019). Reactors C2, B2, A1 and R1 received 12.7 gVS from household waste and 6.44 gVS from albumin, compared to 17.3 gVS from household waste and 1.71 gVS from albumin for reactor R2 during day 140-179. The total gVS for all reactors were otherwise equivalent during day 140-179. The difference in outcome could be explained by the degree of degradation being higher in reactors C2, B2, A1 and R1 as compared to reactor R2, as the nitrogen mineralization is higher in those reactors (table 8).

The increase of H_2S is common when increasing the levels of protein added to a process. The increase in H_2S in this case is typically is related to the degradation of sulfur containing amino acids. (Schnürer and Jarvis, 2017). Increasing levels of H_2S is problematic as it is toxic to the microorganisms and results in inhibition of the methanogens, either by direct effect or indirect by an inhibition to the cellular function due to lack of trace metals (Chen et al., 2014). The inhibition occurs as the sulphides form complexes with essential metals, making them unavailable for the microorganisms. The additional supplementation of BDP was successful in lowering the H_2S concentrations as the iron in the BDP bind to the sulphide, resulting in precipitations, and thereby decreasing the H_2S in the reactors subject to an increase in TAN. Consequence the microbial cells were likely not experiencing a limitation of trace metals in the present study

The supplementation of trace elements could be a reason to why the process did not seem to be affected by the increasing TAN, as it could aid the methanogens, which otherwise are affected by the increasing H_2S concentrations in addition to the ammonia stress. This reasoning is further supported by (Westerholm et al., 2015) who found that addition of trace elements enhanced digester performance and influenced the microbial community in a high ammonia process operating under similar conditions as in the present study.

5.3 VFA concentrations

Concentrations of propionic acid of 0.9 g/L have been reported to cause significant inhibition of methanogens (Wang et al., 2009). The same study found that the optimal propionic acid concentration for methane yield was 0.3 g/L and the optimal acetic acid concentration was found to be 1.6 g/L. When it comes to acetic acid, the study found that concentrations of 2.4 g/L were not significant enough to cause inhibition. When comparing these results to the results from this study, this suggests that both the acetate and propionate concentrations were still within reasonable concentrations at the end of the HRT. However, comparing levels of VFAs between anaerobic digestion systems is often problematic, as different systems have their own “normal levels” (Angelidaki et al., 1993). A good reference is instead to compare the increase in acetate and propionate to previous system levels. Concentrations in the beginning of this study were for acetate: 0.89-2.05 g/L and propionate: 0.03-0.09 g/L. The acetate concentrations were thus not extremely high when comparing to levels for this system in the beginning of this study. When it comes to propionate the concentrations were higher at the end compared to the

beginning of this study and reaching levels previously suggested as inhibitory, e.g. close to 0.9 g/L, especially in reactor R1.

The total VFA levels were affected by the acetic acid pulsing, which probably was why reactors C2 and B2 had higher acetate concentrations than the other reactors. Towards the end of the HRT however, the concentrations of acetate increase in reactors A1 and R1 as well, who were not subject to pulsing with acetic acid, but only subject to an increase in TAN. This would indicate that reactors A1 and R1 were starting to experience reactor inhibition by increased ammonia, which often causes VFA accumulation.

The same signs of reactor disturbance can be distinguished when it comes to the propionate concentrations in reactor R1 which shows a significant increase in propionate concentration to reach 0.8 g/L at day 172. Another sign of VFA accumulation is the data from the kinetics test that was done in the end of the study. The kinetics test showed that the VFA concentrations (acetate and propionate) did not decrease during the course of a day, but instead remains high in all reactors except the control reactor R2.

However, the pulsed reactors seem to be better at consuming the acetate compared to A1 and R1, as the acetate concentrations after 24 hours had decreased comparatively more in the pulsed reactors, as illustrated in the kinetics test made after the increased ammonium-test.

5.4 Microbial population dynamics

An increase in gene abundance in SAOB or hydrogenotrophic methanogens would indicate that the metabolic shift to SAO had taken place, as would a decrease in acetoclastic methanogens. However, the results from the microbial analysis using qPCR did not show any considerable changes in gene abundance between day 137 and day 172.

Still, after the increase in TAN and pulsing with acetic acid, the 16S rDNA gene abundance changed somewhat in the reactors between day 137 and 172. An increase in hydrogenotrophic methanogens took place in *Methanoculleus* as well as Methanomicrobiales. The increase for *Methanoculleus* was highest in the control reactor R2, where the gene abundance increased 103% compared to reactors C2, B2 (81%, 84%) and A1, R1 (2.0%, -1.4%). The increase in gene abundance for Methanomicrobiales was also high in the control reactor R2 (145%), and even higher in the pulsed reactors C2, B2 (299%, 179%) as compared to A1, R1 (67%, 70%). When it comes to the gene abundance of SAOB, no changes indicating a metabolic shift took place during the investigated time period for neither *S. schinkii* nor *T. phaeum*. The gene abundance in *S. schinkii* decreased in all reactors: C2, B2

(-74%, -57%) as well as in A1, R1 (-87%, -85%), as well as the control reactor R2 (-56%). The decrease in gene abundance of *S. schinkii* was low compared to another study (Westerholm et al., 2018) where the gene abundance of *S. schinkii* increased 100 times (from 10^5 to 10^7 gene copies μg^{-1} DNA in a mesophilic, high ammonia (7.5 g/L) chemostat during six hydraulic retention times (HRT 28 days)). The gene abundance in *T. phaeum* also decreased slightly in all reactors including the control: C2, B2 (-58%, -3.2%) and A1, R1 (-11%, -66%) as well as the control reactor R2 (-52%). It is hence difficult to draw conclusions about whether a metabolic shift occurred or not when looking for an increase in the gene abundance in SAOB or the hydrogenotrophic methanogens in this study.

The gene abundance of the acetoclastic methanogens *Methanosaeta* and *Methanosarcina* decreased slightly in all reactors (-32% to -81%) except for the control reactor R2 for *Methanosarcina* (+77%). The decrease was not considerable compared to one study (Lü et al., 2013) where the gene abundance of the acetoclastic methanogen *Methanosarcinaceae* (family to which genus *Methanosarcina* belong), decreased 466 times under similar circumstances as in this study. It is therefore not possible to decide if a microbial shift happened based on the microbial analysis of the acetoclastic methanogens.

5.5 Acetic acid pulsing

The concentrations of acetate used when pulsing reactors C2 and B2 could have played a role in why there was no distinct indication of changes in gene abundance among the hydrogenotrophic methanogens, SAOB or acetoclastic methanogens. In this study, 20 mmol/L of acetic acid (equivalent of 1.2 g/L) was added to reactors C2 and B2 to stimulate the shift from acetoclastic methanogenesis to SAO and hydrogenotrophic methanogenesis. However, the pulsing did not result in clear effects to the process compared to the reactors that were not pulsed.

The reason could be because the acetic acid concentration was too low. In one study, (Westerholm et al., 2018) achieved a greater increase in both hydrogenotrophic methanogens and *S. schinkii* by enriching chemostats which were inoculated with sludge from SAO dominated systems with higher acetate (0.4 g/L or 7.5 g/L), at high ammonia for six HRTs of 28 days.

Another study by (Lü et al., 2013) investigated the effect of low and high acetate concentrations (50-250 mmol/L) on the degradation pathway of acetate between the acetoclastic methanogenesis and SAOB together with hydrogenotrophic methanogenesis. The conclusion of that study was that the hydrogenotrophic methanogen Methanomicrobiales increased and both the acetoclastic methanogens *Methanosaetaceae* and *Methanosarcinaceae* decreased at higher concentrations of

acetate >150 mmol/L, changes that did not happen in the same extent in this study with acetate concentrations of 20 mmol/L. A third study claimed that acetate concentrations of >100mmol was needed to inhibit the acetogenic methanogens in thermophilic conditions (Hao et al., 2013).
When comparing the results from this study with the results from previous work (Westerholm et al., 2018, Lü et al., 2013, Hao et al., 2013), one can suggest that the concentrations of acetate needed to be higher as used in the present study to promote growth of SAOB.

Another factor that could affect the relatively low increase and decrease in 16s gene abundance of the investigated microorganisms is the time of the study. The increase in TAN and pulsing with acetic acid only lasted for one HRT. Mesophilic syntrophs is said to have a generation time of 28-78 days (Hao et al., 2013), compared to *Methanosarcina* and *Methanosaeta* which have doubling times of 24 hours and 3.5-9.0 days (Anderson et al., 2003). The short duration of this study could be the reason why no clear changes in the microbial population took place. A longer study might have shown changes in the microbial population, as did the similar study by (Westerholm et al., 2018) which studied acetate enrichment for six HRT (28 days).

However, the reason why no significant population changes to the microbial population took place could be a result of the addition of trace elements, as discussed in 5.2.

5.6 Method improvements

The time for sampling the reactors was done at around 11:00 just before the feeding was done. This sampling time was the same for the whole study (day 0-179). When acetate pulsing started in reactors C2 and B2 and the TAN was increased in reactors C2, B2, A1 and R1 from day 140, the sampling time remained the same. One thing that might have affected the data is that the first pulsing with acetic acid was done at 9:00, which produced a lot of CO_2 gas when added to the reactors. The fact that the gas content measurements were done just two hours later is source of error that could affect the reliability of the methane content data and CO_2, data figures 18, 19, 20. This is also the reason why biogas production/OLR is presented instead of specific methane production/OLR.

If the study was to be redone, gas measurements would have been taken before the daily pulsing at 9:00 which in a better way would have shown the actual gas composition and potential differences in gas composition between the reactors.

A better way of determining the methane content of the gas would have been to collect the gas produced during a day and then do a methane analysis of the in a gas chromatograph.

Two data points from day 120 from the biogas content measurements using the Geotech Biogas 5000 instrument from Scantec Nordic were omitted which showed abnormal values that deviated from the rest of the measurements. The deviation was probably due to equipment malfunction.

The total ammonium-nitrogen measurements showed a slight decrease in the total ammonium-nitrogen concentrations during days 151 and 155. The decrease in concentration happens in all reactors, which suggests that a mistake could have been made when preparing the samples. Apart from the decrease in total ammonium-nitrogen concentrations in the reactors, the concentrations of total ammonium-nitrogen increased as expected.

When it comes to the increases in BDP-865, no notations were made about the specific dates which the BDP-865 concentrations were made to increase the concentrations in the substrate. This data would have been interesting to have as to further enable the understanding of the correlation between the increase in BDP-865 and its effect on the H_2S content of the biogas. With the data with the exact dates of when the BDP-865 was increased, the correlation could have been more decisively established, e.g. the time between the increase in BDP-865 and the decrease in H_2S.

5.7 Further recommendations

One issue with measuring the gene abundance of the species in the reactors is that even if microorganisms were to be inhibited or die, the qPCR could still detect free DNA or DNA still in inhibited cells. The results from the qPCR analysis could therefore show an abundance which includes free DNA or DNA from inhibited cell. One HRT might be too short time to flush out dead cells and free DNA from the acetoclastic methanogens. However, an effect can still be seen in the process from VFA accumulation.

Another issue is that even though abundance of a specific species is estimated using qPCR, this does not show which pathway that is dominant. A way of tracking this is to label C-atoms in acetate and then detect pathway activity (Jiang et al., 2018).

6 Conclusion

- The TAN in the reactors were successfully increased, surpassing 3 g/L after half HRT (15 days) and reaching 4.0 g/L towards the end of the HRT. These are concentrations of TAN that have been documented to cause reactor disturbances in form VFA accumulation, decreased biogas production and methane content and a shift in the metabolic pathway from acetoclastic methanogenesis to SAO-hydrogenic methanogenesis. The increase in TAN correlated with increased H_2S concentrations in the reactors and possibly an increase in VFAs towards the end of the HRT, which indicates ammonia inhibition. The H_2S increase was countered with added supplementation of BDP-865 containing trace elements.

- The pulsing with acetic acid caused the acetate concentrations in reactors C2 and B2 to remain at a higher concentration than the acetate concentrations in A1 and R1, still without acetate accumulation when the TAN increased.

- Among the methanogens the largest increase in abundance seemed to be within the group of hydrogenotrophic methanogens; Methanomicrobiales and *Methanoculleus* and where the largest difference was measured between the reactors that were pulsed with acetic acid and not. However, the changes were low compared to other studies of acetate enriched reactors at higher concentrations (from 125 mmol/L). No significant difference could be distinguished in gene abundance of acetoclastic methanogens or SAOB during the study. Enriching the reactors with more acetic acid could have had a greater effect on the microbial population, according to other studies.

- The supplementation of trace elements could be a reason to why the process does not seem affected by the increasing TAN, as it could aid the methanogens which are otherwise affected by increasing H_2S concentrations.

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