Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Comparison of anaerobic sludge granules from different wastewater plants with respect to granule size, substrate degradation and methanogenic community

Jonathan Andrén

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Comparison of anaerobic sludge granules from different wastewater plants with respect to granule size, substrate degradation and methanogenic community

Jonathan Andrén

Supervisor:	Maria Westerholm, Swedish University of Agricultural Sciences, Molecular Sciences
External supervisor:	Sabine Keinstuber, Helmholtz- Center for Environmental Research
Examiner:	Anna Schnürer, Swedish University of Agricultural Sciences, Molecular Sciences

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Abstract

Within the objective to replace fossil fuel with sustainable sources there is a growing pressure to advance such development. Anaerobic digestion (AD) is beneficial not only as a waste management technology, but also to recycle organic waste into methane which can be used for heating, production of electricity or vehicle fuel. Although the anaerobic digestion for biogas production has been used and refined at a larger scale for several decades the system is susceptible to perturbations due to sensitivity of the degradation process. The process of degrading organic waste anaerobically is a complex process involving many trophic groups of microorganisms in a series of degradation steps, each step decomposing the substrate further. The most sensitive group are the methanogens, which come exclusively from the domain *Archaea* and produce methane. Because they are easily inhibited they are often rate limiting in the degradation process.

Common digesters for AD of sludge from wastewater treatment plants are the Up-flow Anaerobic Sludge Blanket (UASB) digester and the Expanded Granule Sludge Bed (EGSB) digester, which both utilize granular suspended solids that form a thick sludge blanket at the bottom of the digester tank. The major difference between the digesters is that the EGSB operates with higher up-flow velocity, enabling treatment of higher organic load. The size and shape and the microbial community composition in the granules impact on the operational performance of the digester.

The focus of this project was to investigate granules in respect to size distribution, their fraction of volatile solids, monitoring the consumption hydrogen, acetate, propionate and *n*-butyrate in respect to gas production, gas composition and volatile fatty acid (VFA) consumption. The methanogenic community of granules was measured using Terminal Restriction Fragment Length Polymorphism (T-RFLP) fingerprinting with the restriction endonucleases *Bst*NI and *Mwo*I. Granules was sampled from mesophilic UASB and EGSB digesters feed with wastewater from sugar industry, paper industry and methylcellulose.

The dominating methanogens found in the granules studied, ranked in order of prevalence include: *Methanosaeta* (6-63%), *Methanobacterium* (0-45%), *Methanosarcinaceae* (0-42%), *Methanocaldococcus* (0-17%), *Methanomicrobiales* (0-19%), *Methanomassiliicoccaceae* (0-13%) and *Methanoculleus* (0-29%). The results show that there can be a significant difference in the consumption of acetate, propionate and *n*-butyrate between reactor granules. Granules dominated by *Methanosaeta* had high VFA degradation rates, while granules dominated by *Methanosarcinaceae* had greater hydrogenotrophic activity. Granules from investigated paper waste reactors were predominantly 3 mm or larger, whereas reactors treating methylcellulose had granules that were more evenly distributed. In general, larger granules had slightly higher VS content than average. The methanogenic community varied between granules of different size but seems to be more influenced by reactor parameters such as high salt concentration in methylcellulose waste, which favoured *Methanosarcinaceae*. While other reactor granules treating paper and sugar waste was dominated by *Methanosaeta*.

Keywords: Anaerobic digestion, Granule, UASB, EGSB, VFA, Methanogen, Acetoclastic, Hydrogenotrophic, Acetogenic, mcrA, T-RFLP, *Methanosarcina*, *Methanosaeta*

Degradation by methanogens have never gone out of fashion

Human activity produces waste in large quantities, predicted to become even larger in the future, mainly because of a growing world population. Organic waste, such as food waste, agricultural plant material, animal manure, municipal wastewater and others. These threatens to pollute the environment if not handled properly, by leaching nutrients and diseases into the environment, causing shifts and disturbance in sensitive aquatic ecosystems. Or release greenhouse gases, contributing to the warming of the planet. It is becoming increasingly clear that for humans to develop sustainably, not causing harm to the environment, the production of waste and recycling of it needs to go hand in hand.

Anaerobic digestion is a growing waste management technology. With the help of bacteria, archaea and fungi complex organic matter can be degraded in the absence of oxygen. Many specialized microorganisms are involved, degrading large carbon molecules in succession into smaller ones, eventually leaving as gas or in a form that the microorganisms cannot degrade efficiently. The last step in the degradation chain produces methane which is the most reduced form of carbon and contain chemically stored energy that can be released for heat, electricity and even as vehicle fuel when processed. Also, the residue of anaerobic digestion can be used and returned to the farmer as a fertilizer or to improve the soil structure.

One type of anaerobic digester utilizes aggregated biofilms called granules, on which the microorganisms grow. Each granule has a certain lifespan in which they form, grow and eventually disintegrate or is washed out. Because they are dense and sink to the bottom of the digester tank, they remain within the reactor. Such reactors can harbour a high density of active microorganisms, degrading waste with high efficiency compared to non-granular digester. Another benefit is that inhibitory molecules need to diffuse into the granule before they come in contact with microorganisms, protecting against quick changes in environment.

Methane is produced exclusively by members of archaea, collectively referred to as methanogens which are ancient inhabitants on planet earth, thought to have thrived in the time before atmospheric oxygen. They are sensitive compared to bacteria because of a thinner cell membrane and are often inhibited in environments of high stresses, such as high concentration of salt or ammonia etc. In anaerobic digestion the growth requirement of all involved microorganisms needs to be met. If one group fail, the hole process suffers. In case that the methanogenic community is inhibited, accumulation of organic acids follows causing acidic conditions, which reduces the performance of the digester. The community of methanogens is continuously adapting to the environmental conditions, which is why it has been proposed that monitoring of methanogenic community structure would allow operation giving increased reactor stability.

In this study screening of methanogenic community in reactor granules from five full-scale anaerobic digesters was done with molecular fingerprinting. Also, the rate at which common substrates are degraded was investigated as well as the size distribution of granules from each reactor. In conclusion

the methanogenic community structure was mainly influenced by the type of waste and environmental conditions of the reactor. In reactors with high salinity the methanogen *Methanosarcinaceae* dominated, suggesting tolerance in such environments. Further development of screening methods of methanogenic community will help to diagnose anaerobic reactors and increase the understanding of shifts in community when process parameters change.

Nedbrytning med hjälp av metanogener har aldrig gått ur mode

I takt med en stigande global världsbefolkning, ökar trycket att ta hand om en växande mängd avfall. Organiskt avfall från boskap, växtodling, avfallsvatten och industri riskerar att skada vår miljö, genom att läcka näringsämnen, sjukdomar eller att släppa ut växthusgaser. För att tackla dessa utmaningar krävs bättre kunskap och lösningar för att ta hand om detta avfall.

Anaerob nedbrytning av organiskt material utnyttjar mikroorganismers förmåga, som har utvecklats och finslipats under årmiljoner av evolution, att bryta ner stora komplexa kolkedjor till den mest reducerade formen av kol, metan. Detta sker genom en stegvis nedbrytningsprocess, med flera olika grupper av mikroorganismer involverade, både bakterier och arkéer. Det nära samspel kan delvis förklaras av att nedbrytningen sker utan tillgång av syre, vilket försvårar och gör nedbrytningen ”mindre lönsam” vilket motiverar organismer att samarbeta. Slutprodukten metan innehåller kemiskt lagrad energi som kan frigöras till värme, elektricitet eller uppgraderas till fordonsbränsle.

Det finns flera typer av teknologier för anaerob nedbrytning. En variant som kallas Upflow Anaerobic Sludge Blanket (UASB) låter mikroorganismerna växa på centimeterstora korn, eller granuler som de kallas. Dessa granuler cirkulerar i en tank som matas kontinuerligt. En fördel med denna teknik är att nedbrytarna växer på de sjunkande granulerna, därav behålls den största delen av biomassan i tanken istället för att sköljas ut, vilket möjliggör snabb och effektiv nedbrytning.

Metan bildas endast av arkéer, de är känsliga för olika typer av stress som hög koncentration av salt eller ammoniak. Om metanogenerna, som de kallas, inte kan bilda methan för att de inte tolererar miljön kan hela processen haverera. Istället ackumuleras då organiska syror som försurar reaktorn. Vissa metanogener tolererar emellertid stress i större utsträckning än andra. Det är därför viktigt med kunskap om hur det metanogena samhället är uppbyggt i olika granuler.

I denna studien har granuler från fem olika fullskaliga anaeroba reaktorer jämförts dels med hänsyn till substratnedbrytning, storlek på granuler och relativ distribution av metanogener. Resultaten visar att den metanogena strukturen främst är influerad av typ av avfall och den rådande miljön i reaktorn. I reaktorer med hög salinitet var t ex metanogenen *Methanosarcinaceae* dominerande. Framtida utveckling av undersökningsmetoder av metanogener kan hjälpa att diagnostisera anaeroba reaktorer och öka förståelsen för hur skiften av samhällen sker till följd av förändring av process parametrar.

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Abbreviations

AD	Anaerobic digestion
UASB	Up-flow anaerobic sludge blanket
EGSB	Expanded granular sludge bed
OLR	Organic loading rate
HRT	Hydraulic retention time
VFA	Volatile fatty acids
SAO	Syntrophic acetate oxidation
SRB	Sulfate reducing bacteria
TS	Total solid
VS	Volatile solid
GC	Gas chromatography
HPLC	High-performance liquid chromatographer
T-RFLP	Terminal Restriction Fragment Length Polymorphism

1 Introduction

1.1 Background

This master thesis work is a collaboration between the Swedish agricultural university (SLU) in Uppsala, Helmholtz Zentrum für Umweltforschung (UFZ) and Deutsches Biomasseforschungszentrum (DBFZ) in Leipzig, Germany.

1.2 Anaerobic Digestion

Anaerobic digestion (AD) is a microbiologically mediated process and refers do the degradation of complex organic matter under anaerobic conditions (without oxygen). Often the term involves formation of gaseous carbon in its most reduced form (methane) and most oxidized form (carbon dioxide), together referred to as biogas (Madsen, 2011). This is a highly complex process that involves several microbial trophic groups that have evolved close relationships to sustain themselves in the digester and be competitive (Schink, 1997). Many types of organic waste can be utilized in AD because of their high levels of easily degradable materials, such as municipal food waste and slaughter waste. Other waste such as agricultural residues, paper industry, breweries, and animal manure can also be used (Chen et al., 2008). If neglected, organic waste can become a major source of environmental air and water pollution due to leaching of nutrients (nitrogen and phosphorous) and pathogens into sensitive environments. Furthermore, unmanaged degradation of organic waste might produce greenhouse gases, such as carbon dioxide, methane and nitrous oxide, that is emitted to the atmosphere (Holm-Nielsen et al., 2008).

Anaerobic digestion is a multi-purpose technology with the potential to fulfil several national and European environmental, agricultural and energy policy objectives. By using AD as waste management two main products are produced which have a monetary value (Holm-Nielsen et al., 2008).
(i) Not all organic matter is degraded in the process and the residue may be returned to the farmer as a renewable pathogen free fertilizer and for soil amendment, which is very valuable in soils with low organic content. This will become more valuable in the future as inorganic fertilizers become less feasible (Nkoa, 2014; Holm-Nielsen et al., 2008).
(ii) AD produces biogas which is a renewable energy source and can be used to provide heat, electricity and if upgraded also as vehicle fuel as is done in several municipalities in Sweden (Holm-Nielsen et al., 2008).

With a growing human population, organic waste is plentiful (Chen et al., 2007). Currently the European biogas production is on the rise, among them Germany stands out in the top. In 2016,

more than half of the total biogas produced in Europe, was generated in Germany alone (Biogas barometer, 2018). However, the fact that AD is associated with process instability, forces operators to use lower organic loading rates (OLR) with less methane yield as a result, prohibiting the technology to be fully exploited. This instability is derived from the fact that AD is a complex biological process and different reactors may have very different environmental conditions for the microorganisms to grow in.

1.2.1 Microbial interactions

Anaerobic degradation of organic matter is widely found in natural environments such as organic sediments, waterlogged soils and in the gut of animals, especially ruminants (Schnürer & Jarvis, 2018). In these environments oxygen is consumed faster than it can diffuse, as a result other electron acceptor are favoured such as nitrate, oxidized iron or manganese, sulfate and eventually carbon dioxide. For thermodynamic reasons the most favourable electron acceptor is consumed first, as exploiting microorganisms will have more available energy for growth. In comparison, aerobic respiration produces many times more energy than using carbon dioxide. When converting hexose anaerobically (1) into methane and carbon dioxide only 15% of the energy is made available compared to aerobic degradation (2) (Schink, 1997).

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \qquad \Delta G^{\circ\prime} = -390 \text{ kJ/mol} \qquad (1)$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta G^{\circ\prime} = -2870 \text{ kJ/mol} \qquad (2)$$

Because of the small energy gain for the methanogens themselves, chemical energy is instead stored in methane. These unfavourable conditions have forced anaerobic microorganisms to form very efficient cooperation between themselves in a special type of symbiotic relationship called syntrophy. Syntrophic relationships form between two metabolically different organisms which are dependent on each other to degrade a specific substrate, often because of energetic advantages (Schink, 1997).
In the AD process the degradation of large organic molecules (sugars, proteins and fats) are divided into four major stages, hydrolysis, fermentation, anaerobic oxidation and methanogenesis (figure 1). In this process many different microorganisms are active and close cooperation between different groups is required, each with different nutritional and environmental requirements (Schnürer & Jarvis, 2018). Consequently, in this type of pipeline degradation every downstream degradation step is dependent on the preceding steps to be completed. This makes the system vulnerable and the performance of the whole may very well be haltered by inhibition of one reaction step (Alvarado et al., 2014).

(1) *Hydrolysis* is the first step in which insoluble organic macromolecules (polysaccharides, proteins and lipids) are hydrolysed into simple and soluble products (Alvarado et al., 2014). This is very important because these polymers are simply too large for direct use by the microorganisms. To degrade these large compounds extracellular enzymes are used, such as cellulases, protease and lipase, specialized in degradation of a specific type of macromolecule. Some hydrolytic microorganisms may be able to break down several or only one type of polymer. The nature of the substrates determines the rate of degradation, cellulose and hemicellulose are degraded more slowly than proteins (Schnürer & Jarvis, 2018). It is well known that the genus *Clostridium* dominates in

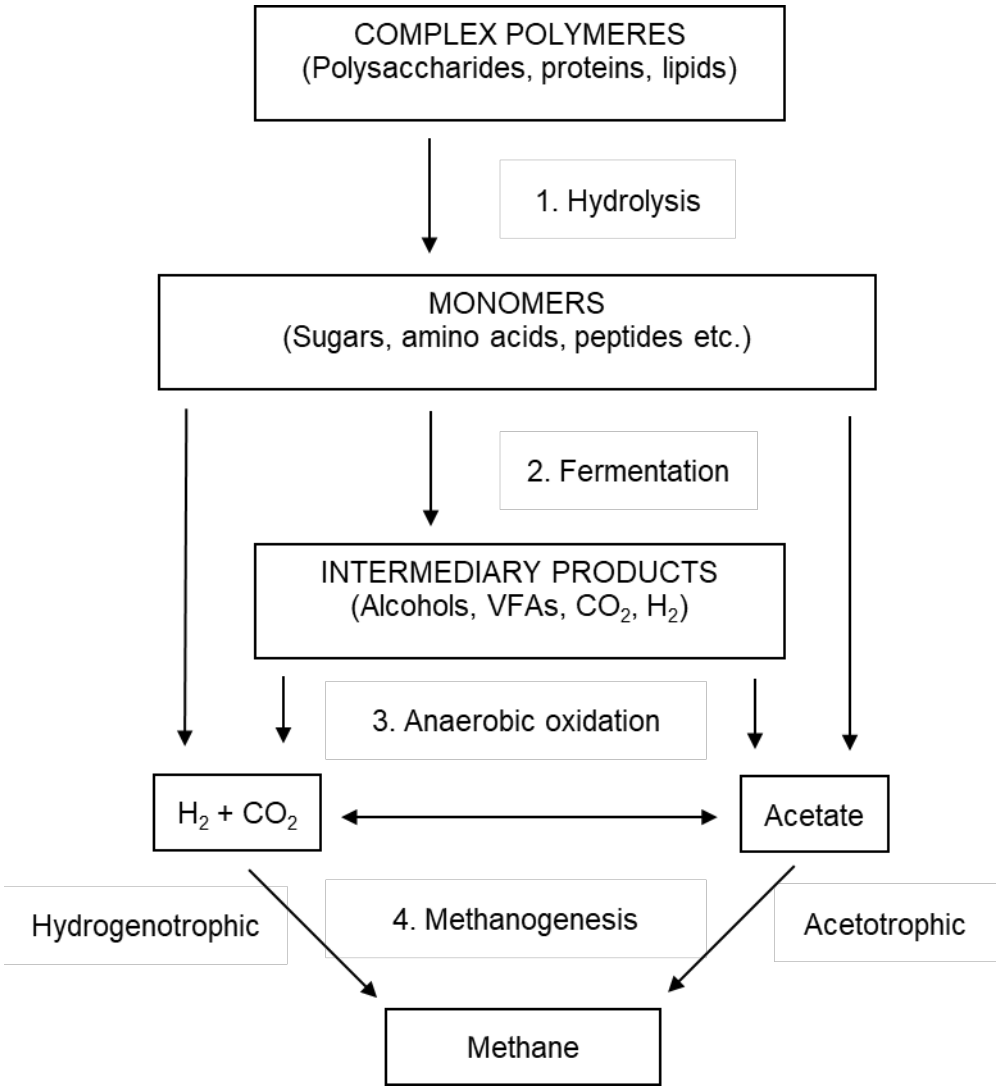

Figure 1. Four major degradation steps in AD (modified from Schnürer & Jarvis, 2018; Alvarado et al., 2014; Schink, 1997)

cellulolytic environments, other common hydrolytic bacteria in AD are Ruminococcus, Caldicellulosiruptor, Caldanaerobacter, Butyrivibrio, Acetivibrio, Halocella, Eubacterium, Bacteroidetes, Fibrobacter, Spirochaetes and Thermotogae (Azman et al., 2015).

(2) The second step is *fermentation* of the hydrolysed products such as monomers (sugar, amino acids, peptides etc.), which are catabolized into alcohols, organic acids (acetate, propionate, butyrate, valerate, caproate, succinic acid, lactic acid etc.), carbon dioxide and hydrogen (Alvarado et al., 2014). What reactions that occur and what products that are produced is dependent on presence and abundance of different microorganisms i.e. the same substrate may be degraded into different compounds, depending on who is responsible. The prevalence of different fermentative bacteria is tightly connected to the environmental conditions, which may also change the fermentation process within the same organism. Fermenting bacteria are a very diverse group and many of them are also active during the hydrolysis stage. Common fermentative bacteria include *Clostridium*, *Ruminococcus*, *Enterobacterium*, *Bacteriodes*, *Acetobacterium* and *Eubacterium* (Schnürer & Jarvis, 2018). Others like *Lactobacillus* sp. are also common in biodigesters, producing lactate (Alvarado et al., 2014).

Table 1. Gibbs free energy of common anaerobic oxidation reactions, releasing hydrogen and acetic acid (modified based on Schink, 1997)

Substrate	Reactions	ΔG°'(kJ/mol)

n-Butyrate	$CH_3CH_2CH_2COO^- + 2H_2O$	→	$2H_2O + 2CH_3COO^- + 2H^+ + 2H_2$	+48.3
Propionate	$CH_3CH_2COO^- + 2H_2O$	→	$2H_2O + 2CH_3COO^- + 2H^+ + 2H_2$	+76.0
Acetate	$CH_3COO^- + H_2 + 2H_2O$	→	$2H_2O + 2H^+ + 2H_2$	+94.9
Alcohols	$CH_3CH_2OH + H_2O$	→	$CH_3COO^- + H^+ + 2H_2$	+9.6

(3) The third step is *anaerobic oxidation* or sometimes more specifically called acetogenesis, in which the products of fermentation are used by acetogenic bacteria to produce acetate and hydrogen (Alvarado et al., 2014). These conversions are extremely unfavourable from a thermodynamically point of view and the reactions results in the smallest energy quantum that can be exploited by a living cell. As can be seen in table 1 all the oxidation reactions are endergonic at standard conditions i.e. they are not spontaneous ($\Delta G \geq 0$) (Schink, 1997).

The key for these reactions to occur lies with hydrogen, which is the predominant electron carrier between oxidative and reductive metabolic processes. Its small size and effective ability to diffuse makes it ideal in most circumstances. These anaerobic oxidation reactions can only proceed when hydrogen pressure is low, which will tilt the reactions to the right. In other words, for anaerobic oxidation to occur at all hydrogen needs to be consumed by another group of microorganisms, such as methanogens. This process is referred to as "Inter-species Hydrogen Transfer" and reveals a syntrophic relationship between anaerobic oxidizing and methanogenic microorganisms (Schink, 1997). A number of strict anaerobes participate in acidogenesis such as *Clostridium*, *Bacteroides* and *Butyrivibrio*, but also facultative anaerobes such as *Bacillus* and *Enterobacter* (Nishio & Nakashimada, 2013).

(4) The last degradation step is called *methanogenesis*, during which methane and carbon dioxide are produced (Alvarado et al., 2014). Methanogenic microorganisms performing this conversion belong exclusively to the domain *Archaea* and represent some of the most ancient organism known. They thrived and may have been major producers of organic matter in the time before atmospheric oxygen (Kasting, 2002). Methanogens are compared with other microorganisms slow growing and easily inhibited, which may be rate limiting in the biogas process (Schnürer & Jarvis, 2018). Today there are seven orders of methanogens discovered. *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* are common in anaerobic biodigesters, *Methanococcales* are not common but have been found in granular sludge (Alvarado et al., 2014). *Methanomassiliicoccales*, which has been identified most recently is encountered in anaerobic digesters (Bühligen et al., 2016), derived from the guts of termites (Iino et al., 2013). Others include *Methanocellales*, which was obtained in a culture from rice paddy soils. *Methanopyrales* contain only one hyperthermophilic species, not likely to be found in a reactor (Alvarado et al., 2014).

There are two major groups of methanogens based on their preferred substrates, either hydrogenotrophic or methylotrophic. In the hydrogenotrophic pathway hydrogen and carbon dioxide are mostly used, but some hydrogenotrophs also use formate instead of hydrogen. Other utilize hydrogen and methanol together. The methylotrophic pathway is broader still, commonly using substrates such as hydrogen, acetate, methylamines, methanol and carbon monoxide. Acetate degrading methylotrophs are called acetotrophic, or acetoclastic methanogens (table 2). In AD with low

levels of inhibiting substances and relative low temperature the acetoclastic pathway dominates and represent roughly 70% of the methane production (Schnürer & Jarvis, 2018).

Table 2. Two common methanogenic reactions (modified from Nishio & Nakashimada, 2013)

Pathway	Reaction	ΔG°'(kJ/reaction)
Hydrogen-otrophic	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-136
Acetoclastic	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31

There are only two known groups of acetoclastic methanogens, both belonging to the order Methanosarcinales: Methanosarcina is flexible in its choice of substrate, able to use the hydrogenotrophic pathway and some methylated compounds, while Methanosaeta is strictly acetoclastic (Alvarado et al., 2014). At high acetate concentration and in environment with stresses Methanosarcina will dominate because of faster growth rate and higher tolerance to toxic compounds. When the environment is not causing inhibition for Methanosaeta it usually dominates because of higher affinity to acetate, causing low acetate concentration that becomes unavailable to Methanosarcina (Schnürer & Jarvis, 2018). Members of Methanobacteriales, Methanococcales and Methanomicrobiales are mostly hydrogenotrophs, sometimes able to use formate and/or some alcohols (Alvarado et al., 2014). During certain adverse environmental conditions, such as high ammonia and temperature or long retention times, syntrophic acetate oxidation (SAO) may be more favourable over the acetoclastic pathway (Westerholm et al., 2016). In SAO, acetate is first oxidized by non-methanogens into hydrogen and carbon dioxide and later converted into methane by hydrogenotrophic methanogens (Schnürer & Jarvis, 2018).

In biodigesters the formation of methane is known to be a sensitive process, especially to low pH and temperature, in which the process will either be completely inhibited or proceed at a very slow pace (Alvarado et al., 2014). Issues related to inhibition will be further explained below.

1.2.2 Inhibition of AD processes

A wide array of inhibitory substances can cause upsets and failure which include ammonia, sulphide, light metal ions and heavy metals and organics (Chen et al., 2007). In AD processes the first (hydrolysis) or the last (methanogenesis) degradation step are usually rate limiting. If the limiting microorganisms performing these functions are inhibited the performance of the reactor will suffer. Inhibition of the hydrolytic step slows down the process since downstream degradation are "waiting" for hydrolysed degradation products. Methanogens are the least tolerant out of the anaerobic microorganisms. If they are inhibited the degradation of volatile fatty acids (VFA) stops, followed by accumulation of organic acids and finally pH drop, with resulting decrease in methane production (Alvarado et al., 2014). This is a major concern for reactors with high concentration of ammonia and high pH. The interplay between ammonia, pH, temperature and VFAs may lead to an inhibited steady state i.e. the process is running stably but with lower methane yield. It is reported that methanogens can acclimate to an inhibition over time and produce more methane. This can be explained by a shift methanogenic structure (Chen et al., 2008).

Inorganic ammonia may take two principal forms. The ammonium ion (NH_4^+) is favoured at low pH and free ammonia (NH_3) at high pH, increasing temperature also favours NH_3. Free ammonia

has been suggested to be inhibitory because it is membrane-permeable and may diffuse into the cell, causing proton imbalance and potassium deficiency. Sulfate is common in wastewaters, in AD it is reduced into sulfide by sulfate reducing bacteria (SRB). This causes competition for substrates between SRB and methanogens, which reduces the methane production (Chen et al., 2008). Sulfide can also form complex with important trace elements, becoming inaccessible for microorganisms (Schnürer & Jarvis, 2018). Influent of digesters always contain light metal ions including sodium, potassium, calcium and magnesium, which may have been added as pH adjustment or released in the degradation of organic matter (Chen et al., 2008). They are essential elements and can in moderate concentration stimulate growth. In higher concentration growth is impaired and may even cause severe inhibition in extreme environments (Soto et al., 1993). Heavy metals are never degraded and may accumulate in the reactor. Especially chromium, iron, cobalt, copper, zinc, cadmium and nickel are of concern and may be one of the major causes of digester failures (Chen et al., 2008). Organic pollutants, which are toxic for AD are a diverse group including benzenes, phenols, alkanes, alcohols, aldehyds, ketons, surfactants and more. Some toxicants are degradable (Chen et al., 2008).

Commonly AD is operated in one of either the psychrophilic (4-15°C), mesophilic (20-40 °C) or thermophilic (45-70 °C) temperature range, out of the three the mesophilic is most in use (Nishio & Nakashimada, 2013). Colder temperature means slower microbial growth rates, but also more stable performance. High temperature on the other hand may achieve very effective degradation but such processes are also more susceptible to upsets and failures (Mao et al., 2015).

Many of the inhibitions that may occur in AD originate from using only one type of feedstock with unbalanced composition. Cellulose based influents can cause poor performance if hydrolysis becomes rate limiting. Fat and protein rich feedstock may cause ammonia inhibition, with process upsets or failures. Municipal sewage waste commonly contains heavy metals. One way of dealing with these negative aspects is to co-digest i.e. to digest mixed substrates. There are several benefits of applying this method as increased biogas production, improved fertilizer value of digestate and more stable reactor performance. Although it often requires more centralized facilities and longer transport (Holm-Nielsen et al., 2008).

1.2.3 Development and current design of biogas reactors

Products and process of AD have been utilized for many centuries. As early as tenth century BCE in Assyria biogas was used to heat bath water (Auer et al., 2017). By the 17th-century scientific interest arose when Robert Boyle and Stephen Hales noted that when disturbing aquatic sediments,

a flammable gas was released (Fergusen & Mah, 2006). In 1778, Alessandro Volta an Italian physicist identified the flammable gas to be methane. After the discovery it took about 100 years before the anaerobic degradation process was applied to treat wastewater. High-rate digesters for sewage and industrial wastewater took until the 1970s and solid waste were not used until the 1980s (Gijzen, 2002). The development is going forward and today there are many different types of anaerobic reactors, the simplest ones being the anaerobic sequencing batch reactor (ASBR) which is a single tank without mixing and are fed in batches. Other systems like the continuous stirred tank reactor (CSTR) are fed continuously and also suspend the sludge which gives better contact with microorganisms. However due to the repeated washout of effluent it is difficult to retain high microorganism concentration within the reactor i.e. the washout of microorganisms is faster than the growth in the reactor. The up-flow anaerobic sludge blanket (UASB) reactor is a common type of AD which retains a thick granular sludge blanket in the bottom, this facilitates good wastewater-biomass contact (Mao et al., 2015). The expanded granular sludge bed (EGSB) is very similar to the UASB but have a higher up-flow velocity, which more intensively mixes the substrates (figure 2). As an effect the biomass activity is improved (Li et al., 2018).

Figure 3. Anaerobic digester granules. Photo: Jonathan Andrén

1.2.4 High-rate reactors and formation of granules

Both UASB and EGSB are high-rate reactors, to operate these reactors at high OLR and short hydraulic retention times (HRT) one of the most vital component is the nature of the active biomass and the prevention of washout of degrading microorganisms (Quarmby

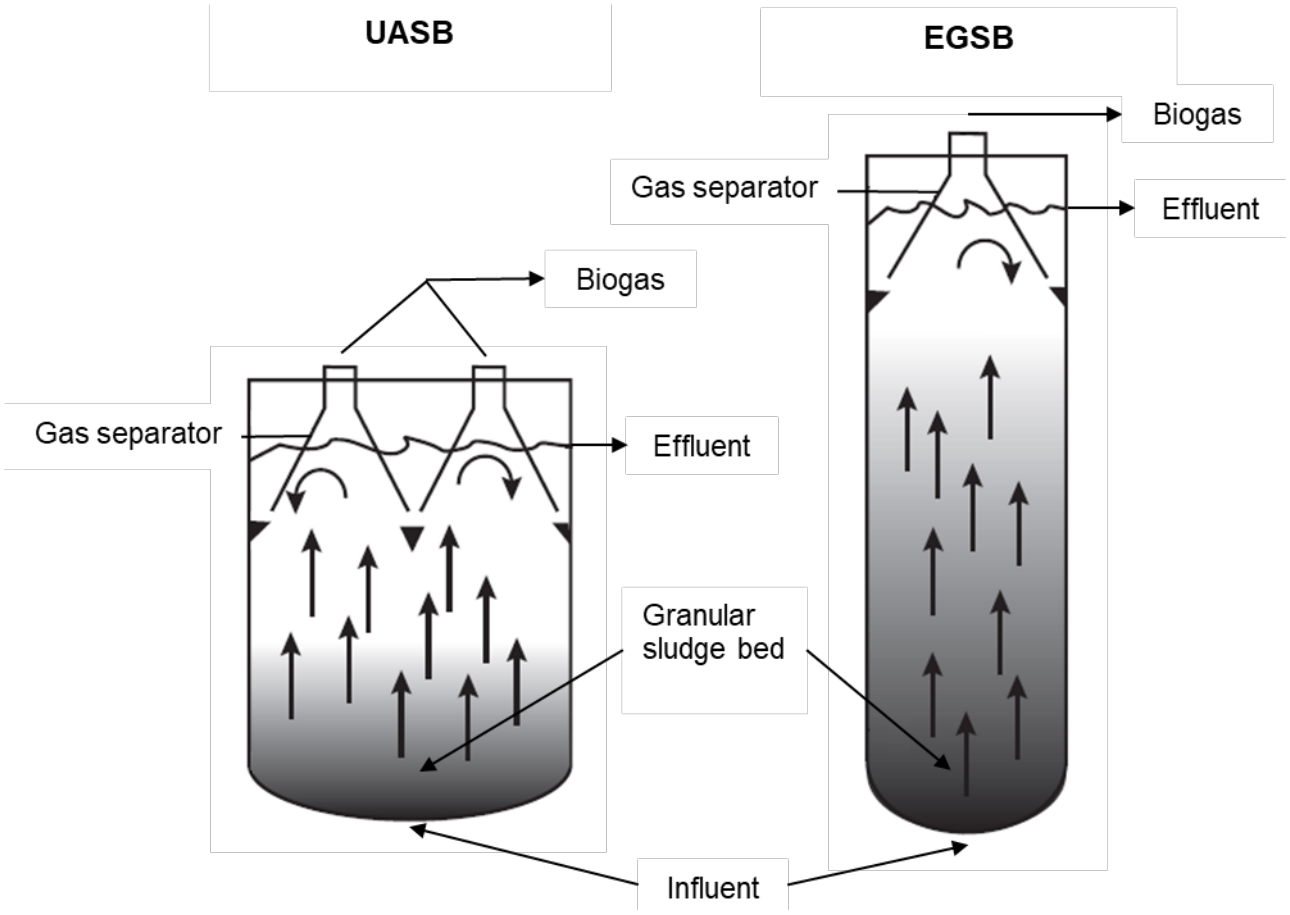

Figure 2. Schematic picture of UASB and EGSB reactors adopted from (Nishio & Nakashimada, 2013)

& Forster, 1995). In these reactors the microbial consortium is often aggregated into dense granular biofilms (figure 3), they are essentially circulating anchor points on which the microbes can grow. Because of high settling velocity of granules which negates washout, a longer solid retention time (SRT) is achieved with high concentration of microbes retained within the reactor. In fact, the formation of granular sludge may be considered as the major indicator of successful UASB and EGSB operation and allows OLR far beyond conventional activated sludge treatment (Pol et al., 2004; Mao et al., 2015).

Granules have a certain length of life, a granulation process in which they are formed, grow, age and eventually either leave the reactor with the effluent or are disintegrated (Pol et al., 2004). In the early formation of a granule the acetoclastic methanogen *Methanosaeta* plays a key role. The growth of this filamentous microorganism forms a nucleus on which other microbes can grow, forming a layered granular structure. The distribution of microorganisms within a single granule is well organized, with close forming micro-colonies between cooperating organisms (O'Flaherty et. al., 2006).

Every reactor has a unique size distribution of granules that is continuously changing. Some granules are reported to be up to 8 mm in diameter, although the most prevalent size seems to be between 1-2 mm (Batstone & Keller, 2001). Earlier findings suggest that small granules are less dense than large ones (Wu et al., 2016). It is known that the development and quality of the granules is strongly connected to what feedstock that is used in the reactor. Some waste readily forms intact granules while others produce granules more slowly or with poor integrity that disintegrate, which may be detrimental for slow growing microorganisms such as methanogens (Batstone & Keller, 2001).

1.3 Aim

Granular digesters, with a superior ability to concentrate and retain active microorganisms offer degradation at very high rates and are at the forefront of AD technologies. To make renewable energy sources more attractive, a prime concern is to make the system efficient and stable. In anaerobic digestion the last step of degradation is done by methanogens, they are sensitive to changes in the environment and are often responsible for failure in the process. Earlier research proposes *Methanosarcina* as an important player and that a diverse and even methanogenic community structure is resilient when subjected to environmental stresses. Knowledge about methanogenic community of granules may give valuable insights into community-based strategies to increase reactor stability.

The objective of this master thesis was to:
Compare granules sampled from five full-scale anaerobic digesters with respect to granular size distribution, volatile solid content, degradation of hydrogen, acetate, propionate, *n*-butyrate into methane and methanogenic community structure.

2 Method

2.1 Sampling of granules

Granule based anaerobic reactors were sampled on the 5th and 7th of January 2018 from four different sources around Leipzig, Germany.
(R1) Originate from Julius Schulte Trebsen GmbH & Co. KG, Trebsen. This reactor is of EGSB type, hold a volume of 110 m^3 and is fed with paper waste.
(R2) Originate from Stora Enso, Eilenburg and is fed with paper waste.
(R3) Originate from Südzucker AG, Zeitz & Brottewitz and is fed with waste from sugar industry.
(R4) and (R5) are two separate reactors that both originate from Bitterfeld-Wolfen GmbH, Bitterfeld. They are of UASB reactor type and are fed with methylcellulose waste water. Of note is the high salt concentration and salinity in the digestate, measured to be 2.5% NaCl, measuring up to about 39 mS/cm in conductivity (Bitterfeld-Wolfen, 2018).

From each reactor the granules were collected at different heights (appendix 1). Equal volumes from each height were mixed together to get a combined sample for each reactor. Granules were stored at 4 °C in airtight containers.

2.2 Sieving of granules

Sieving of reactor granules from R1-5 was performed as follows. A volume of 900 ml reactor sludge was sieved inside of an anaerobic chamber. The following sizes of granules were sieved: > 3 mm, 1.6 mm, 0.8 mm, 0.4 mm, 0.25 mm and < 0.25 mm. Phosphate buffered saline (PBS) was generously flushed during sieving to improve the flow through and the granules were carefully moved with a spatula across the siew. Flow through was collected from underneath and put on the consecutive siew. Granules on top of siew were collected and stored in falcon tubes, the obtained volumes were noted respectively for each size range. After using the smallest siew (0.25 mm) the remaining granules were centrifuged for 10 min at 10000 rpm. The pellet was collected by discarding the supernatant and counted as < 0.25 mm fraction.

2.3 Organic and inorganic fractions

Total solids (TS, organic and inorganic fraction) and volatile solids (VS, organic fraction) and ash (inorganic fraction) were measured in duplicates both of combined samples and of sieved granules.

Crucibles of appropriate size were weighted (empty) using a four-decimal scale, pliers and a desiccator to prevent samples from rehydrating. To determine the TS and VS contents of the granules, samples (between 0.4-7 g, average 2.2 g) were dried at 105 °C for at least 24 h. The TS value was calculated from the difference in weight of the wet and dried sample. The VS value was measured as the loss of ignition when treating the dried samples in a muffle furnace at 550 °C for 2 h, what remains after ignition is the ash fraction.

2.4 Substrate degradation assay

In total, five sets of degradation assays (reactor R1-5) were conducted, each measuring the rate at which (A) acetate, (B) acetate, propionate and *n*-butyrate, and (C) hydrogen are consumed by each digester granules. This was done in airtight bottles containing granules and different substrates (figure 4). The degradation in the bottles was monitored in respect to biogas production, gas composition and VFA concentration. During a pre-test initial substrate concentration were adjusted to fit a reasonable timeframe of 10-14 days before complete consumption of substrates (data not presented).

Figure 4. Assay bottle with granules. Photo: Jonathan Andrén

In the assay bottle (59.5 ml), basic mineral media (20 ml, appendix 1), filtered granules (1.18 g VS, table 4) and substrate (A-bottles with 30 mM acetate, B-bottles with 10 mM acetate, 10 mM propionate and 10 mM *n*-butyrate) were mixed inside of an anaerobic chamber (initial O_2 < 26 ppm, H_2 < 3%). The bottles were sealed with butyl rubber stopper and an aluminium cap to become airtight. C-bottles were flushed with N_2 for 5 minutes and later pressurized with H_2:CO_2 (80:20) to 1 bar (+/- 0.2 bar). The degradation assay for each substrate were carried out in two replicates (table 3), including blanks and controls with granules but no substrate (D-bottles). The bottles were incubated stationary at 37°C (day 0) and monitored during the subsequent days.

Table 3. Substrate degradation assay setup. Bottle 1 and 2 contain substrate and granules, 3 and 4 contain substrate but without granules. Mixed refers to (acetate, propionate and n-butyrate). D-bottles are without substrates but contain granules

		Substrates		
		Liquid phase		Gas phase
Bottle	Granules	Acetate	Mixed	H_2:CO_2
A1-2	x	x		
A3-4		x		
B1-2	x		x	
B3-4			x	
C1-2	x			x
C3-4				x
D1-2	x			

The utilized basic mineral media contain several important minerals and vitamins for optimal function of anaerobic microorganisms and to avoid limiting circumstances. Up until inoculation the media was kept in two separate components (A (low pH) and B (high pH), later mixed in equal

volume), this was done to avoid excessive precipitation of salts (appendix 1). Further, the media was adjusted with HCl and NaOH to get pH 7, prior to inoculation. Na_2S was used as a reducing agent.

Table 4. Weight of granules used in degradation assays for each reactor (R1-5)

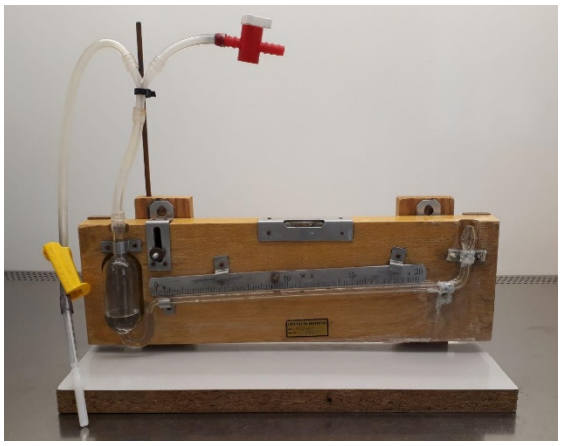

Figure 5. U-bend device. Photo: Jonathan Andrén

Reactor	Granules (g)	VS (g)
R1	1.91	1.18
R2	1.58	1.18
R3	2.88	1.18
R4	2.10	1.18
R5	2.08	1.18

2.4.1 Determination of gas volume

On day 1, 2, 4, 7, 10 and 14 of the degradation assay the biogas volume was measured by releasing the overpressure within the bottle into a U-bend device (figure 5), which measures the volumetric displacement of a NaCl/citric acid solution. This was done for assay bottles A and B. C-bottles were measured with a pressure meter on day 0, 1, 2, 4, 7 and 10. Blank bottles (3, 4) without granules were sampled less frequent but at least on 3 occasions.

The protocol for degassing were as follows:
(1) Initially the tubes were flushed with N2 for a few seconds using a Ø 0.4 mm x 20 mm needle attached at one end of the U-bend device (yellow) and opening the other valve (red). (2) The red

and yellow valve is then closed. (3) Assay bottle cap is soaked with 99% ethanol and lit for sterilization. (4) The needle is inserted into the bottle cap and the yellow valve is slowly opened to allow gas to escape. (5) Volumetric liquid displacement is noted until all over pressured gas is released, also the ambient temperature and pressure is monitored. (6) U-bend device is reset by opening both the yellow and red valve. (7) Needle is replaced after every bottle.

The protocol for using the pressure meter were as follows:
The pressure meter was fitted with a Ø 0.4 mm x 20 mm needle and inserted into the assay bottle. Pressure and temperature were noted. Needles were replaced between every bottle.

Measured displacement in the U-bend device were converted into volume by dividing observed length by coefficient of 6.67 to get volume degassed. This volume was normalized according to equation 3 where, V_N is the gas volume at standard pressure (101.325 kPa) and standard temperature (273.15 K) (mLN), P is the ambient pressure (kPa), T is the ambient temperature (°C), V_{gas} is the volume of raw biogas (mL) (FNR, 2006).

$$V_N = \frac{(P - 10^{(7.19621 - \frac{1730.63}{233.426 + T})} + 0.2\ kPa) \times 273.15\ K}{101.325 \times (273.15 + T)\ K} \times V_{gas} \quad (3)$$

Headspace volume was calculated based on the total volume of the bottle, the volume of liquid at day 0 and the volume of granules. Both floating and sinking granules were observed, hence a granule density was assumed to be roughly 1 g/cm^3 on average.

Figure 6. GC-vial. Photo: Jonathan Andrén

Degassed and headspace volume of gas were combined and calculated into mol using the ideal gas law in equation 4, were n is the amount of substance (mol), P is the pressure (pascal), V is the volume (m^3), R is the ideal gas constant (8.314) and T is the temperature (kelvin).

$$n = \frac{PV}{RT} \quad (4)$$

Methane production in D-bottles was subtracted from the production in assay tests A and B. In the C-bottles pressure loss was measured also in the blanks (3,4), this was accounted for in the calculation assuming that the same pressure was lost also in the experiment (1,2). This type of pressure loss is thought to be caused by inaccurate needle entry and exit of over pressured bottles. To avoid unnecessary pressure losses when sampling, the needle should be pulled out slowly, allowing the rubber stopper to properly consolidate.

2.4.2 Determination of gas composition

On day 0, 1, 2, 4, 7 and 10 of the degradation assay the gas composition in the headspace was measured using CP-2002 P micro gas chromatograph (GC) for the gases H_2, CO_2, O2, N_2 and CH_4.

This was done for all assay bottles and in duplicates. Blank bottles (3, 4) without granules were sampled less frequent but no less than 4 occasions.

The protocol for GC sampling were as follows:
GC-vials (21 ml, figure 6) were sealed with a rubber and aluminium cap and flushed with argon for 20 minutes at 1 bar overpressure. A 1 ml syringe with a Ø 0.4 mm x 20 mm needle were flushed with N_2 (5x pumping motions). By inserting a syringe into the assay bottle, a gas sample were extracted, filling the whole syringe. 1 ml gas volume is then injected into the GC-vial, now ready for GC analysis.

GC data was analysed using peak integration and calculated into relative percentage based on the peak area. By multiplying the percentage of gases with moles in headspace and degassed together the production of CH_4 and consumption of H_2 were calculated.

2.4.3 Determination of organic acids

On day 0, 1, 2, 4, 7 and 10 of the degradation assay the concentration of organic acid in the media was investigated by using High-Performance Liquid Chromatographer (HPLC, Shimadzu Corporation, Nakagyoku, Japan) equipped with a refractive index detector RID-6A and a Nukleogel ION 300 OA column with a precolumn (Macherey-Nagel GmbH & Co. KG, Germany). Together with the added substrates (acetic, propionic and n-butyric acid) other common organic acids were also monitored.

The protocol for HPLC sampling were as follows: By using a Ø 0,4 mm x 20 mm needle and 1 ml syringe a volume of 0,25 ml liquid were extracted from the assay bottle. Liquid were inserted into Eppendorf tubes and centrifuged (Centrifuge 5417R, Eppendorf) at 21817 rpm for 10 min at 4°C. Supernatant were extracted and put in new tubes. Samples were stored frozen until measured, no longer than 2 months. Upon measuring the samples were centrifuged again. 100 µl supernatant were transferred into a HPLC-vial (figure 7), now ready for HPLC analysis.

HPLC data was analysed using peak integration, on average the calculated ratio between experimental measured value and a standard solution was for acetic acid 0.876, propionic acid 0.950 and n-butyric acid 0.985.

2.5 Molecular methods

When studying methanogenic *Archaea*, the enzyme complex methyl-coenzyme M reductase (MCR) which catalyses the last step in CH_4 formation is proven to be ubiquitous as molecular marker. For this purpose, the *mcrA* gene that codes for the MCR α-subunit if frequently used (Borrel et al., 2013). In this study profiling of the methanogenic community were based on Terminal Restriction Fragment Length Polymorphism (T-RFLP) fingerprinting of *mcrA* amplicons as described by Bühligen et al. (2016) and Steinberg and Regan (2008).

Figure 7. HPLC-vial. Photo: Jonathan Andrén

2.5.1 DNA-extraction

Granule samples of 400 µl were stored frozen maximum 2 month after being sampled from full-scale reactor. One DNA-extraction were performed per sample on all combined granular samples (mixed reactor representative) and of sieved fractions from R1, R2, R3 and R5. DNA was isolated using NucleoSpin® soil kit (Macherey-Nagel) with SL1 buffer and enhancer solution SX as described by manufacturer. In addition, the granules were removed from liquid and smudged with a pipet tip before following protocol, this was proven necessary for the larger granules to break during ceramic bead shaking. DNA concentration (100-200 ng/µl) was quantified using NanoDrop® ND-1000 UV-Vis spectral photometer (Thermo Fisher Scientific Inc., USA) and by agarose gel (0.8%) electrophoresis.

2.5.2 PCR of the *mcrA* gene and T-RFLP

T-RFLP fingerprinting of methanogenic composition was done on all reactor granules of which the DNA had been extracted. Amplification of *mcrA* gene fragments was done using polymerase chain reaction (PCR) with forward primer mlas (5′-GGT GGT GTM GGD TTC ACM CAR TA-3′, Eurofins MWG Operon, Germany) and the reverse primer mcrA-rev (5′-CGT TCA TBG CGT AGT TVG GRT AGT-3′, Eurofins MWG Operon, Germany). The reaction was performed in 12.5 µl mixtures containing 15 ng of template DNA, 5 pmol of each primer, 6.25 µl of My Taq HS RED 2x Mastermix (Bioline GMBH, Germany) and H_2O (PCR). During PCR the following cycle parameters were used: denaturation at 95 °C for 3 min, 5 cycles of 20 s at 95 °C, 20 s at 48 °C, ramp rate of 0.1 K/s to 72 °C, 20 s elongation at 72 °C, followed by 25 cycles of 20 s at 95 °C, 20 s at 55 °C, 15 s at 72 °C and a final elongation at 72 °C for 10 min (Steinberg & Regan, 2008). The primer mcrA-rev were labelled with phosphoramidite fluorochrome 5-carboxyfluorescein for subsequent T-RFLP analysis. PCR product were cleaned using SureClean Plus according to manufacturer's protocol (Bioline, Germany). Purified products were determined using agarose gel (1.5%) electrophoresis and NanoDrop® ND-1000 UV-Vis spectral photometer (Thermo Fisher Scientific Inc., USA).

The T-RFLP was performed with 40 ng of PCR product, digested with 2 U of restriction endonucleases *Bst*NI and *Mwo*I respectively (New England Biolabs, Germany), 1 µl of buffer and H_2O (HPLC) up to a total volume of 10 µl. The *Bst*NI samples were incubated at 60 °C for 2 h and *Mwo*l at 37 °C overnight as described by (Bühligen et al., 2016). The restriction fragments were purified by adding 2,5 µl EDTA (125 mM) and 30 µl ethanol (100%) and incubated at room temperature for 5 min. Then centrifuged at 11800 rpm for 30 min and carefully removing supernatant. Pellet was resuspended in 90 µl ethanol (70%) and centrifuged at 11800 rpm for 25 min. Supernatant was removed and pellet dried in a desiccator for 5 min. Pellet was resuspended in 9.75 µl Hi-Di (Applied Biosystems, Germany) and 0.25 µl GeneScan-500 ROX (Applied Biosystems, Germany) and denaturated for 10 min at 95 °C. The analysis was carried out by capillary electrophoresis (ABI PRISM 3130xl) and T-RFLP peaks in the range of 50–500 bp were included. Data was digested with R using a deviation of 7 bp. Taxonomic assignment of *mcrA*-derived T-RF was done using Table S1 from (Bühligen et al., 2016). Multivariate statistical analysis of normalized sample-peaks was performed with R package "vegan" by non-metrical multidimensional scaling (NMDS) using Bray-Curtis index. Single T-RF vectors was fitted using "envfit" algorithm provided within the "vegan" package as described by (Sträuber et al. 2016). Vectors without similarity between the two restriction enzymes was removed for the sake of clarity. The significance of NMDS results were tested using a Monte-Carlo test with 1,000 permutations.

3 Results

3.1 Shape and sizes of granules

In R1-R3, granules with size fraction > 3 mm in diameter were the most common (56-78%, figure 8). The medium from the degradation tests became murky in R1, suggesting many small granules (< 0.25 mm), but also very large floating granules up to 1 cm of oval shape were distinguished. Granules from R2 had a very defined and compact oval shape, with little fragmentation. About 95% of the granules were above 1.6 mm in diameter. In R3 the dominating fraction were represented by very large granules (> 3 mm), but also smaller sizes with intact oval shape or to a minor extent fragmented were found. In contrast granules from R4 and R5 had an more even size distribution, with larger fraction of small granules compared to R1-R3. Both R4 and R5 have similar

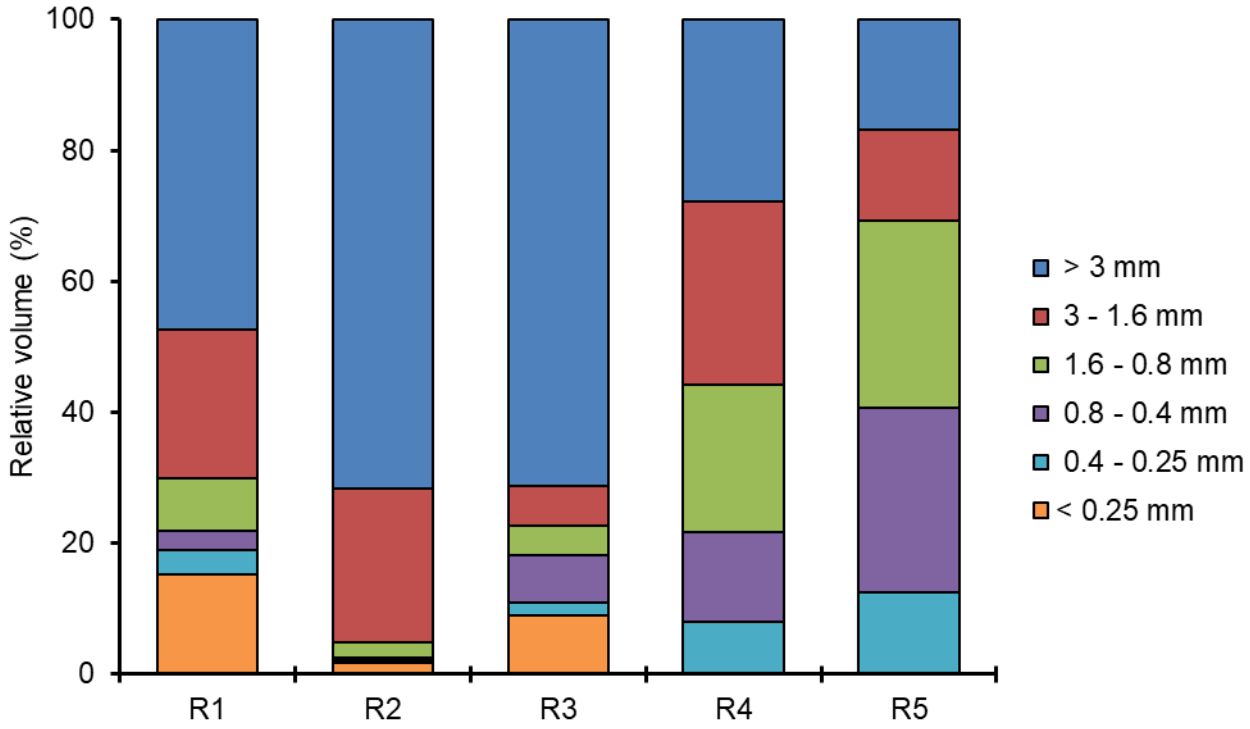

Figure 8. Relative volume of granules with different size fractions obtained in reactors R1-5. Calculated average of duplicates. R4 and R5 were calculated without accounting for the < 0.25 mm fraction

appearance with a rough shape, fragmented and broken, non-intact.

3.2 Organic and inorganic fractions

The VS content of the granules obtained from R1-R3 was roughly the same (about 80% of TS), whereas the VS content of granules from R4 and R5 was considerably lower (figure 9).

The result from VS measurements in granules of different sizes showed no general trend between

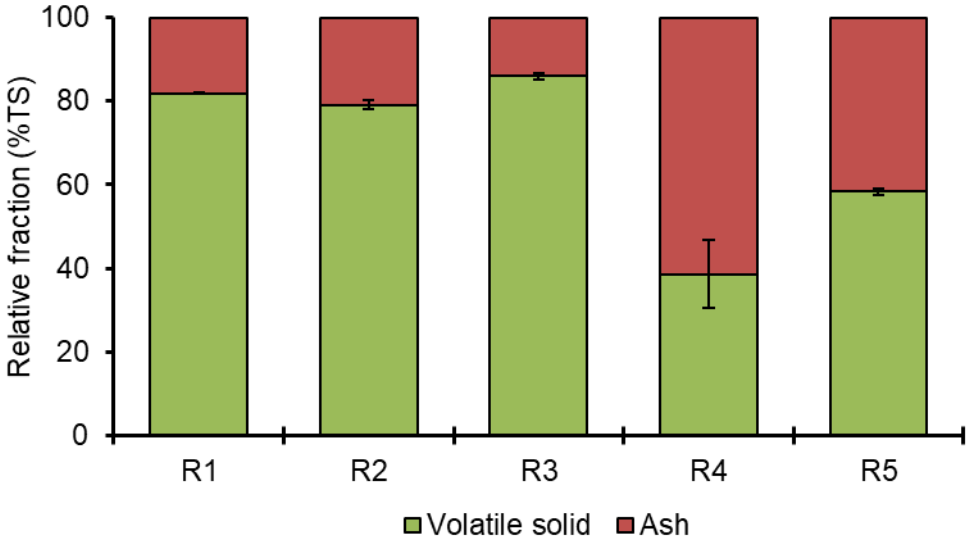

Figure 10. Volatile solid and ash fractions of granule samples from reactor R1-R5. Calculated average of duplicates, vertical bar represents maximum and minimum values. If not visible, it is less than the size of the marker

the reactors (figure 10). In general, granules < 0.25 mm and > 3 mm had the highest VS content. Variation of VS is reactor specific, R3 showed low variation, while R4 and R5 had large variations. In R4 and R5 the granules between 0.25-0.8 mm had the lowest VS content. In all reactors the largest granules (> 3 mm) had the highest VS content.

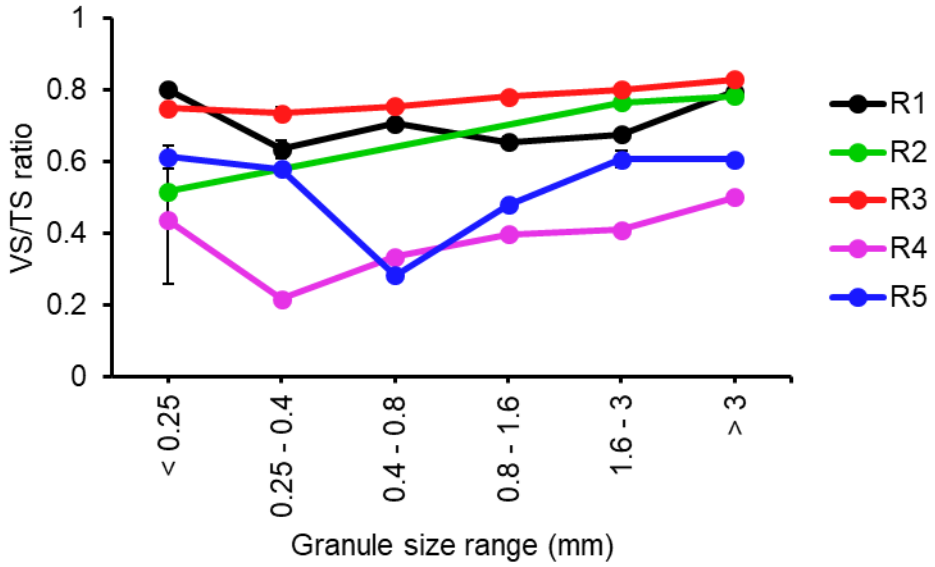

Figure 9. Ratio of volatile solid (VS) to total solid (TS) belonging to different sizes of granules. Measured on filtered granules. R5 (0.4 -0.25) represent only one sample. Calculated average of duplicates, vertical bar represents maximum and minimum values. If not visible, it is less than the size of the marker

3.3 Substrate degradation assay

The specific methane production (SMP) i.e. total production of methane related to the total consumption of COD during the whole degradation test can be seen in table 5. Between all reactors the SMP was higher in

Table 5. Specific methane production reported in volumetric CH_4 (Nm^3) per mass of COD (kg) for A (acetate) and B (acetate, propionate, n-butyrate) degradation test

	CH_4 (Nm^3) / COD (kg)	
Reactor	A	B
R1	1.72	2.33
R2	1.11	1.34
R3	1.36	2.13
R4	1.14	1.58
R5	1.51	1.64

the (B) degradation tests than in (A), suggesting higher stimulation of methanogenesis using a mix of substrates acetate, propionate, *n*-butyrate compared with only acetate as substrate. SMP was expected to be similar between the reactors, given that the same moles of substrate were consumed. This was found not to be the case with R1 having 55% and 74% higher SMP compared to R2 in A and B assays, respectively.

Degradation tests performed with acetate substrate (A-bottles) can be seen in figure 11. Acetate was expected to be readily consumed by either acetoclastic methanogens or other acetate degrading organisms, which was the case for all reactors. Total consumption was almost achieved in R1 and R3 after one day. R2 had the slowest rate with most of the acetate consumed between day 2-4. The production of methane clearly reaches a plateau in all reactors, which were in accordance to the total consumption of acetate. Although there is a significant difference in methane production between R1, R5 and R2, R3, R4.

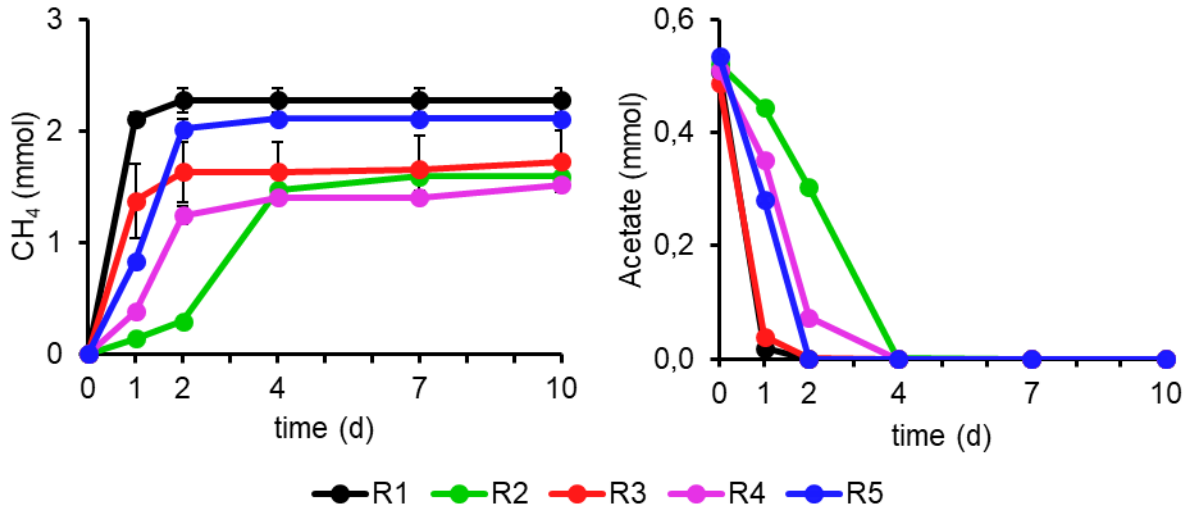

Figure 11. Cumulative methane production (left) and acetate consumption (right) in degradation bottles (A) over 10 days. Calculated average of duplicates, vertical bar represents maximum and minimum values. If not visible, it is less than the size of the marker.

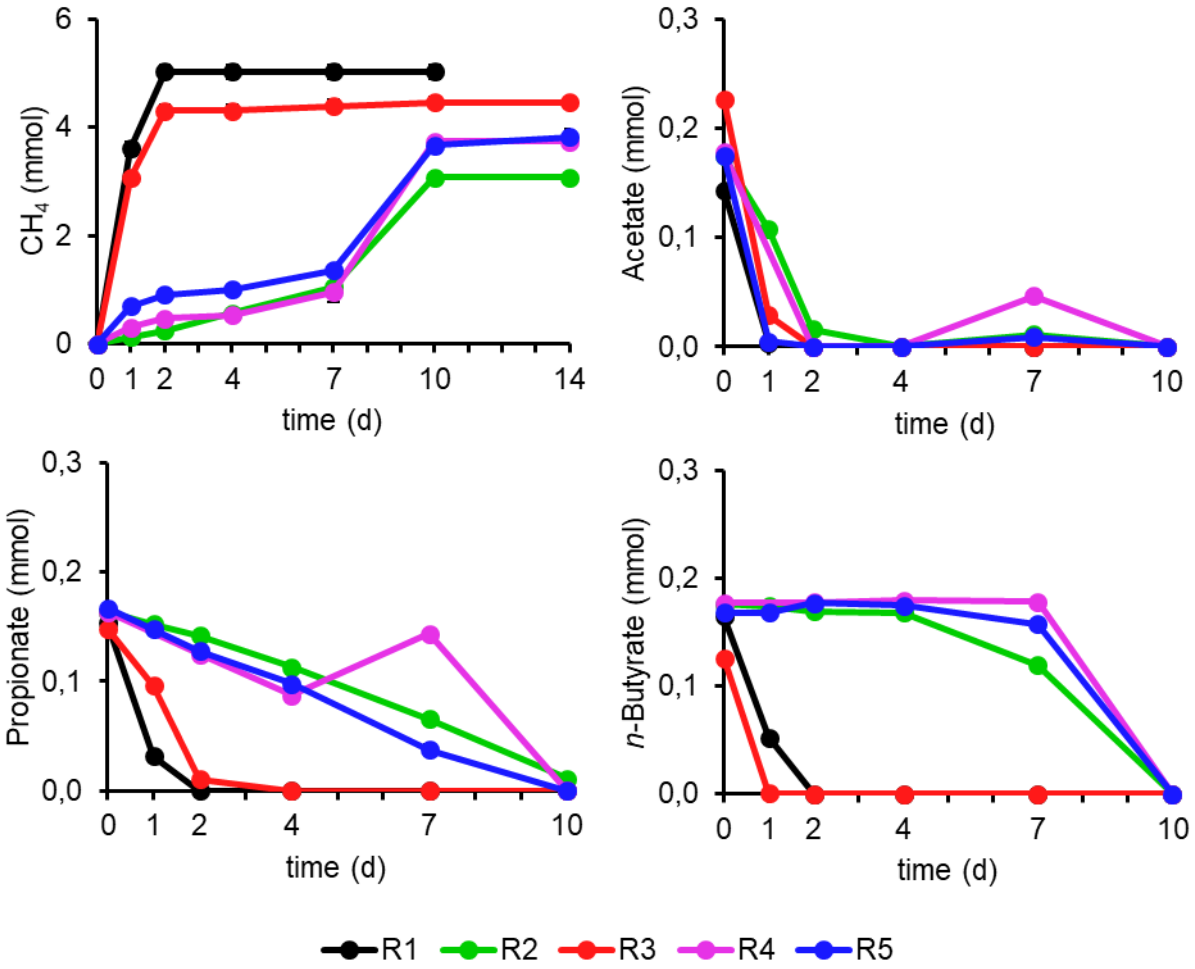

Figure 12. Cumulative methane production (top left), acetate consumption (top right), propionate acid consumption (bottom left), n*-butyrate consumption (bottom right) in degradation bottles (B) over maximum 14 days. Calculated average of duplicates, vertical bar represents maximum and minimum values. If not visible, it is less than the size of the marker.*

Result of degradation tests performed with mixed substrates (B-bottles) can be seen in figure 12. The VFA degradation rate seemed to be related to the size of the compound, with acetate (C2) being consumed faster than both propionate (C3) and *n*-butyrate (C4). This was the case except for R3, in which *n*-butyrate was degraded faster than propionate. The production of methane clearly reaches a plateau in all reactors, indicating the total consumption of VFAs. R1 and R3 both had a very rapid degradation, with total consumption of all VFAs on day 2 and 4 respectively, suggesting an active acetogenic community. In both reactors the methane production reached its maximum on day 2, indicating effective VFAs conversion to methane. R2, R4 and R5 were similar in degradation and methane production rates. Total degradation of all VFAs occur at day 10. The methane production increases between day 7-10, in the same time at which *n*-butyrate were degraded. Indicating initial low precence of *n*-butyrate degrading microorganisms, which by day 7

have grown substantially, which was followed by rapid degradation. On day 7 in R4 a production of both acetate and propionate was measured.

For degradation test performed with H_2:CO_2 (C-bottles), the consumption of hydrogen gas was similar between the reactors, with R2 having the slowest initial rate (figure 13). The two reactors with largest cumulative methane production was R4 and R5, while R3 and R1 produced the lowest amounts.

Because of using invasive sampling methods with multiple needles (gas volume, GC and HPLC), low concentrations of O_2 were measured in the headspace at day 7 and 10 of the degradation tests. Especially under-pressure within the headspace is thought to increase the risk of oxygen contamination when atmospheric gas is "sucked" into the bottle upon needle entry or exit. Oxygen contamination is believed to not have interfered significantly with any of the degradation test since most/all substrates by that time was consumed.

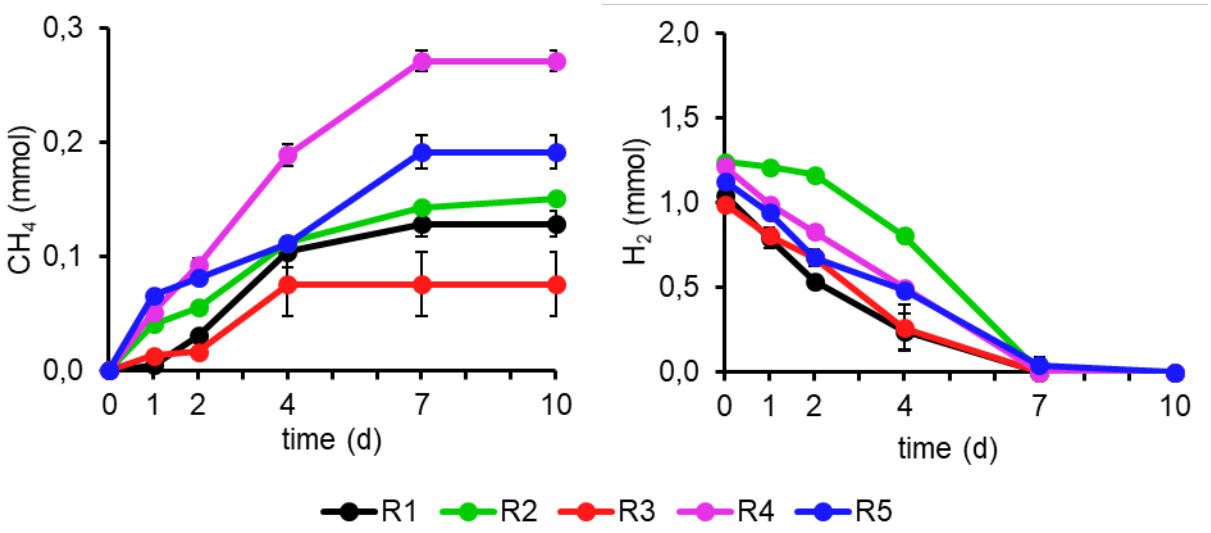

Figure 13. Cumulative methane production (left) and hydrogen consumption (right) in degradation bottles (C) over 10 days. Calculated average of duplicates, vertical bar represents maximum and minimum values. If not visible, it is less than the size of the marker.

3.4 Methanogenic community structure

T-RFLP fingerprinting of *mcrA* gives an overview of the relative abundance of methanogens. On average using the enzyme *Bst*NI about 77% and for *Mwo*I 94% of all T-RFs could be assigned to a taxa (figure 14), on family but also on genus level. T-RF 60 (*Bst*NI) and 268 (*Mwo*I) are the two major unclassified T-RFs.

The dominating methanogens found in all the granules studied were (ranked in order of overall prevalence): *Methanosaeta* (6-63%), *Methanobacterium* (0-45%), *Methanosarcinaceae* (0-42%), *Methanocaldococcus* (0-17%), *Methanomicrobiales* (0-19%), *Methanomassiliicoccaceae* (0-13%) and *Methanoculleus* (0-29%). Of the acetoclastic methanogens i.e. *Methanosarcinaceae* and *Methanosaetaceae*, R1 and R3 showed dominance of *Methanosaetaceae* while R2, R4 and R5 contained both families but predominantly from *Methanosarcinaceae*.

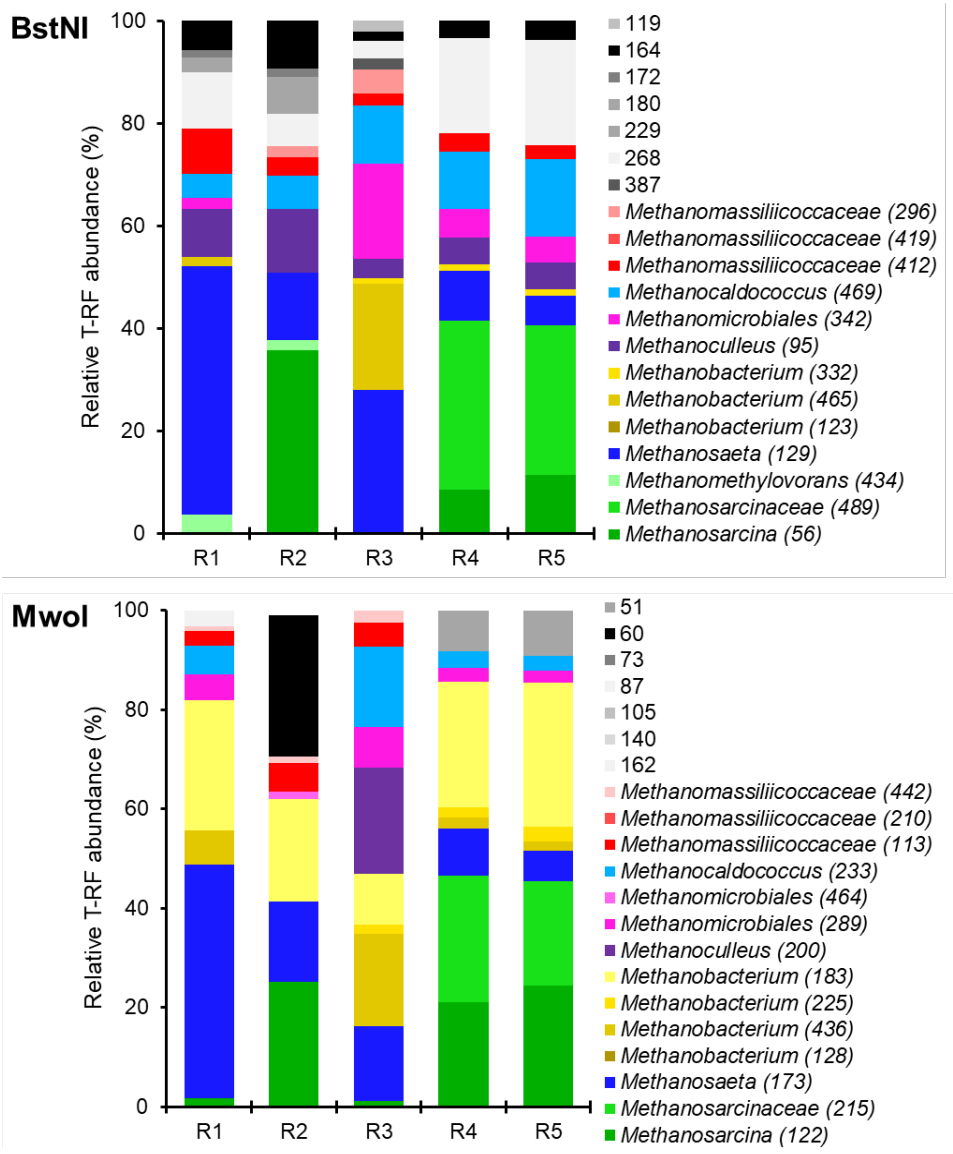

Figure 14. Relative abundance and taxonomic assignment of mcrA*-derived T-RFs retrieved from samples that were collected at different heights from reactors R1, R2, R3, R4, R5. The restriction enzyme BstNI (top) and Mwol (bottom) was used in the T-RFLP, calculated from a single extraction. Number in brackets after assignments refers to the number of base pair (bp) of the specific T-RF. Grey and black coloured sections are T-RFs that were not assigned to a taxa*

When comparing the T-RFLP profile of assignments made with *Bst*NI and *Mwo*I, they are in general similar (figure 14). Especially members of *Methanosaeta* and *Methanosarcinaceae* seems to correspond well. However, the overall assignment of *Methanobacterium* is much greater in the *Mwo*I data, but not in *Bst*NI. Also, *Methanoculleus* is assigned more dominantly in the *Bst*NI profiles.

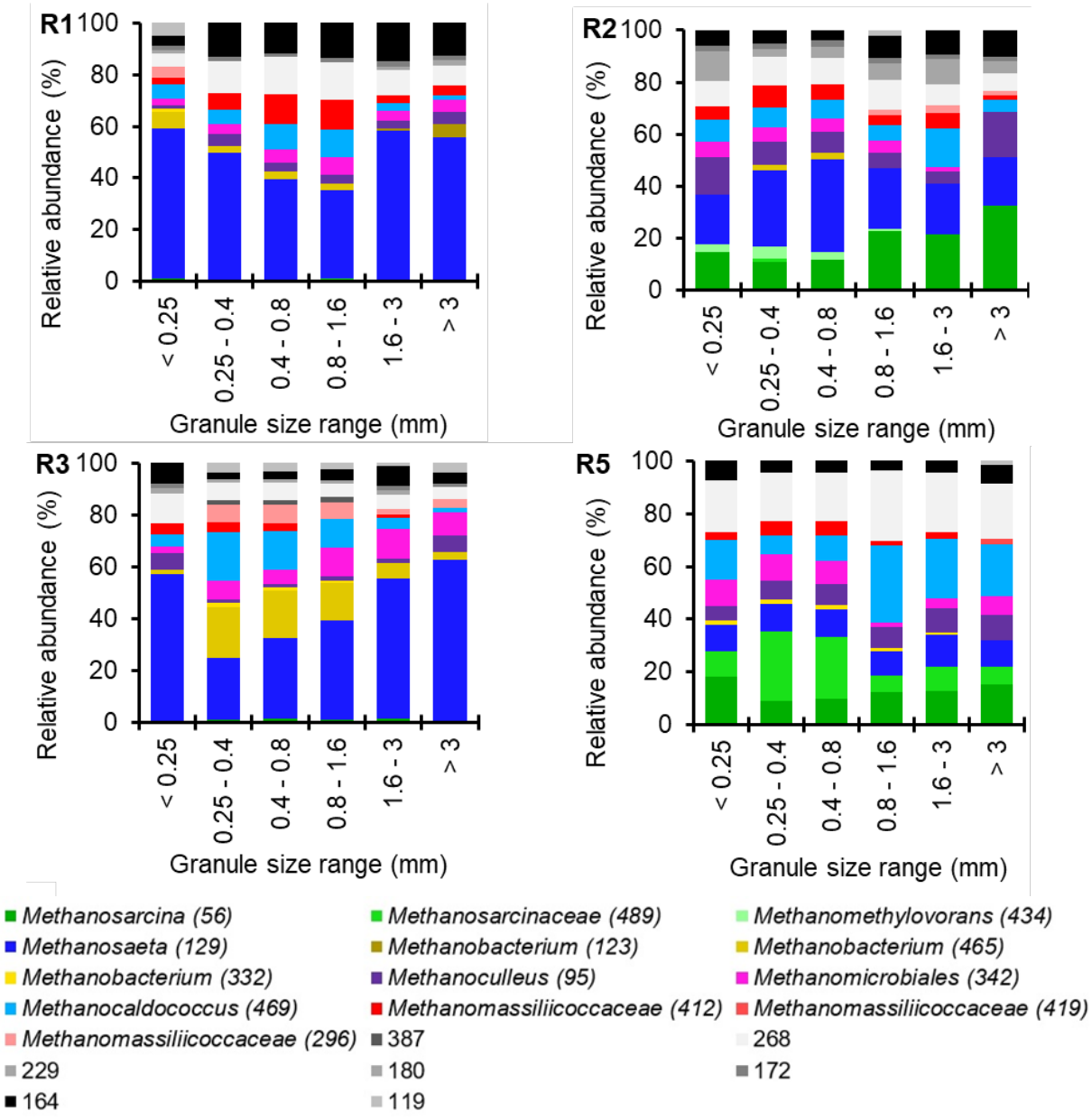

Figure 15. Relative T-RF abundance of mcrA *amplicons digested with enzyme BstNI of sieved granule fractions from R1, R2, R3 and R5, calculated from a single extraction. Number in brackets after assignments refers to the number of base pair (bp) of the specific T-RF. Grey and black coloured sections are T-RFs that were not assigned to a taxa*

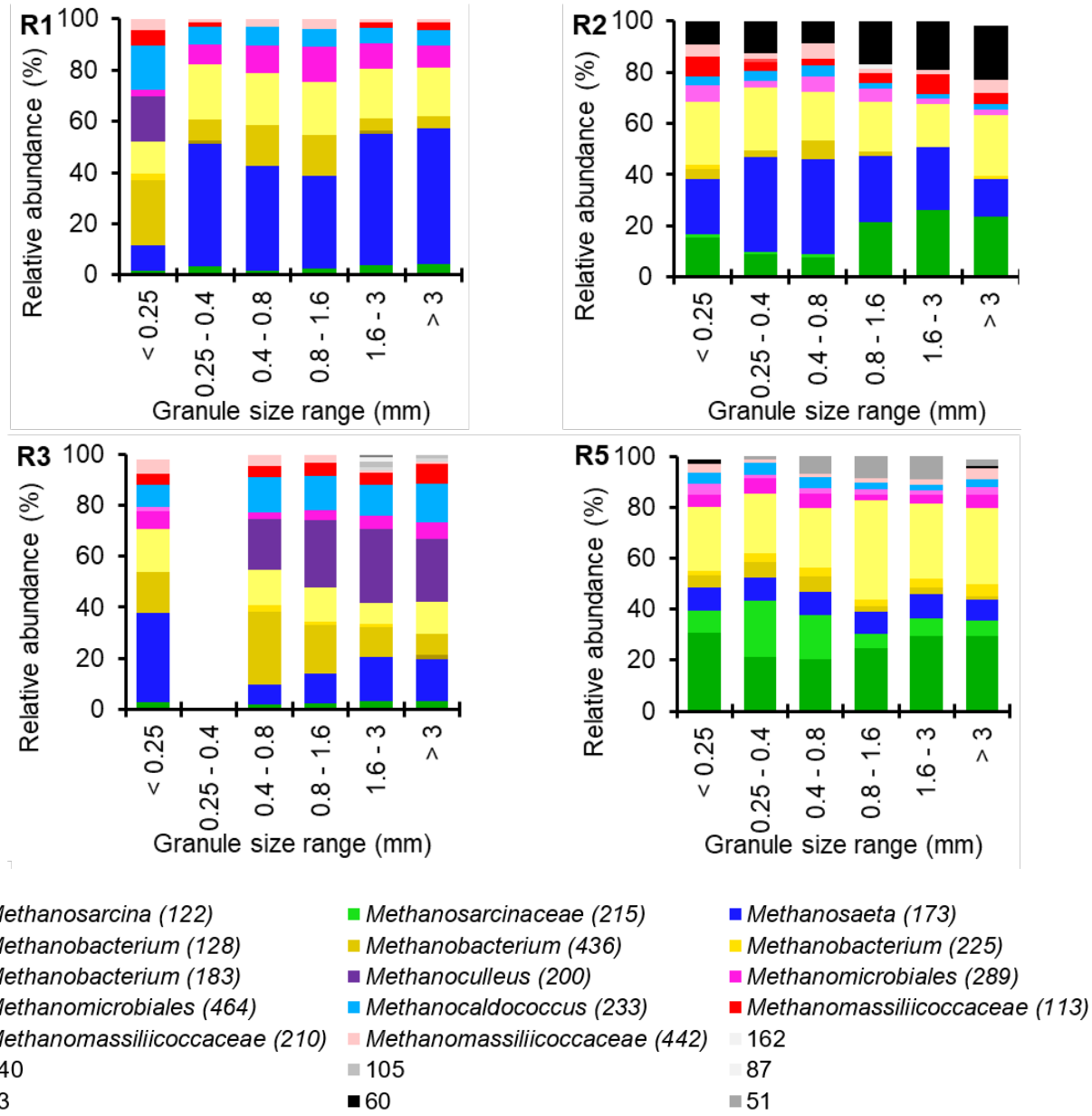

Figure 16. Relative T-RF abundance of mcrA *amplicons digested with enzyme MwoI of sieved granule fractions from R1, R2, R3 and R5, calculated from a single extraction. Number in brackets after assignments refers to the number of base pair (bp) of the specific T-RF. Grey and black coloured sections are T-RFs that were not assigned to a taxa*

T-RFLP profiles of sieved granules indicate variation of methanogenic community related to sizes of granule (figure 15 and 16). Although any trend in methanogenic structure with respect to granule size seems undiscernible.

Major characteristics of methanogenic structure and granule size are as follows:
(R1) Dominated by *Methanosaeta*, lower abundance in granules between 1.6-0.8 mm.
(R2) Both *Methanosarcinaceae* and *Methanosaeta* dominating, high similarity between granule sizes.
(R3) Dominated by *Methanosaeta*, lower abundance in granules between 1.6-0.25 mm.
(R5) High similarity in methanogenic composition between granule sizes.

Links between methanogenic community structure indicated by the T-RFLP profiles and the size of sieved granules were further visualized with NMDS (figure 17 and 18). Points close to each other have greater similarity in methanogenic community structure than samples far apart. The

points belonging to the same reactor clustered together, indicating that the methanogenic community was more influenced by the reactor than by the size of granules. R2 and R5 had the most similar methanogenic community structure, represented by both datasets, indicated by closely clustered points. In R1 and R3 the points are less clustered, especially in *Mwo*I were granules less than 0.25 mm have the largest deviation compared to the rest of the reactor granules. Distance from a point to a vector indicate association which increases with proximity. *Methanosaeta* is strongly associated with reactor R1 and *Methanosarcinaceae* with R5, independent on enzyme

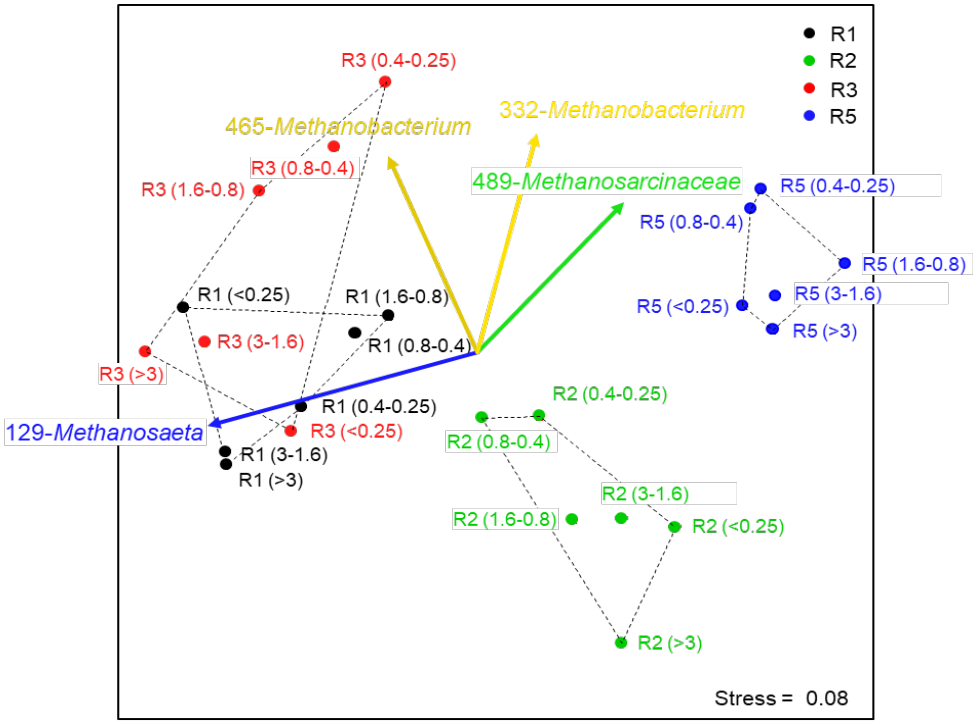

Figure 17. NMDS plot of T-RFLP profiles of mcrA *amplicons digested with enzyme BstNI. Calculated using Bray-Curtis Index n-MDS. Groups belong to the reactors R1, R2, R3 and R5. Points are granules of specific size range specified in parenthesis (mm). Arrows indicate vector of methanogen and number represent the T-RF in base pair*

used in the T-RFLP. *Methanobacterium* have ambiguous association, but were closest to R3 in *Bst*NI.

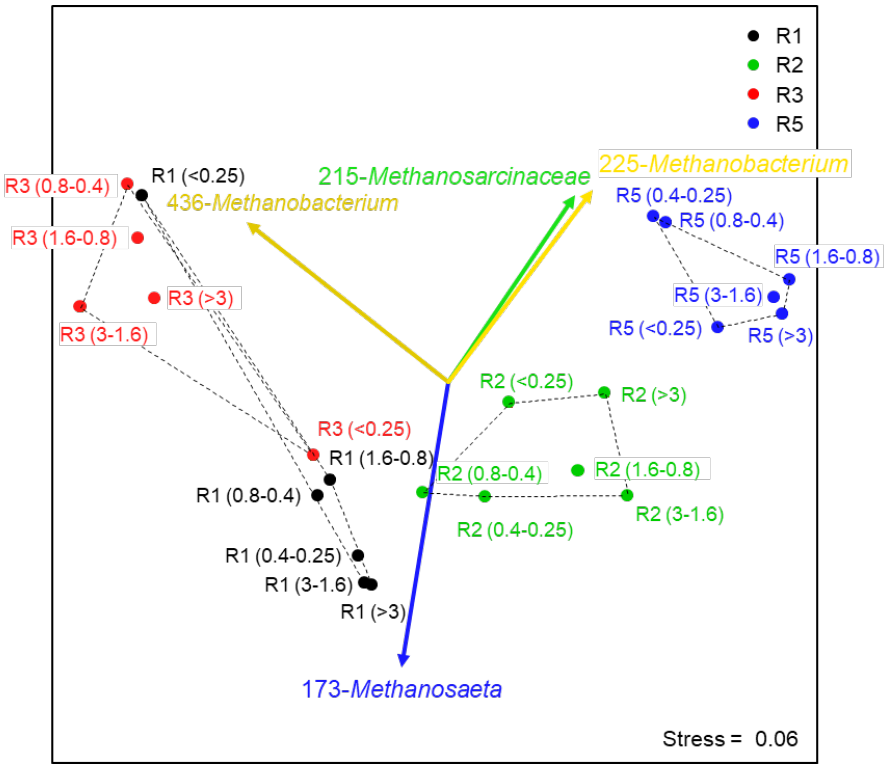

Figure 18. NMDS plot of T-RFLP profiles of mcrA *amplicons digested with enzyme MwoI. Calculated using Bray-Curtis Index n-MDS. Groups belong to the reactors R1, R2, R3 and R5. Dots are granules of specific size range specified in parenthesis (mm). Arrows indicate vector of methanogen and number represent the T-RF in base pair*

4 Discussion

4.1 Granule size distribution and volatile solids content

R1-R3 had dominant abundance of granules larger than 3 mm (figure 8), which are larger compared to several previous studies (Batstone & Keller, 2001; Nishio & Nakashimada, 2013). In Batstone & Keller (2001) the granules were counted instead of being measured by volume as in present study. Thus, the size distribution in that study may be skewed in favour of large granules, which contribute more volumetrically compared to small granules. In respect of size distribution R4 and R5 had higher similarity, both more evenly distributed and a larger fraction of granules < 1.6 mm than R1-R3.

By visual observation it was noted that some large granules (> 3 mm) from R1-R3 were floating in the media, suggesting a lower density in big granules compared to smaller ones, possibly explained by higher porosity. This would in part explain the upper boundary of the size of a granule i.e. if granules become more porous with size, they are more likely to me washed out of the reactor the larger they grow. A weak trend of increasing VS/TS ratio with granule size larger than 0.4 mm was observed in all reactors (figure 10), which is in contrast with Bhunia & Ghangrekar (2007), reporting of increasing trend only between 0.04-0.65 mm and decreasing trend in granules larger than 0.65 mm. The effect of the fraction inorganic inert matter in granules is not discussed much in literature. However, it may be argued that a high VS content reflects a dense microbial community, with would give R4-R5 a disadvantage given their high ash content (figure 9), possibly linked to high salinity levels.

4.2 Shape and size of granules and links to methanogenic community

From the T-RFLP fingerprinting of prevalent methanogens it is evident that *Methanosaeta* was the dominant acetoclastic methanogen in reactor R1 and R3, while in R2, R4 and R5 *Methanosarcinaceae* was more abundant (figure 14). According to De Vrieze et al. (2012) a reactor with dominating community of *Methanosarcina* will be less likely to fail, because of its tolerance towards shocks and stresses. In contrast, *Methanosaeta* is known to be very sensitive to perturbation. R4-R5 operated at elevated levels of salinity (conductivity of about 39 mS/cm), which can explain the dominating relative abundance of *Methanosarcinaceae* over *Methanosaeta*. These reactors also had the highest hydrogenotrophic activity (figure 13), which is not surprising since the greater part of methanogens present can use the hydrogenotrophic pathway, including *Methanosarcinaceae*.

The shape and structure of the granules varied between the reactors, R1-R3 had distinct oval shape while R4-R5 granules were fragmentated and with rough shape. According to Pol et al. (2004), the filamentous methanogen *Methanosaeta* plays a key role in the granulation process, which was high in abundance in R1-R3. *Methanosaeta* was only found in low abundance in R4-R5 (figure 14), which may explain the fragmented state of the granules. Pol et al. (2004) suggests that optimal growth conditions for *Methanosaeta* will enhance the granulation process, forming intact granules. Karakashev et al. (2005) reported that full-scale reactors with long HRT favour a dominant presence of *Methanosaeta*, because of their slower growth. The life length of a granule may be a key factor. If granules grow quickly and disintegrate due to poor structure, more fast-growing methanogens may have an advantage i.e. *Methanosarcina*. For granules to harbour a benefitting population of *Methanosaeta*, a long SRT with favourable conditions is therefore required. Further studies may include, how to improve conditions for *Methanosaeta*.

The result from the degradation assays (figure 11, 12) showed that acetate, propionate and *n*-butyrate was degraded at a significantly higher rate and more methane was produced in R1 and R3 (*Methanosaeta* dominated), compared to R2, R4 and R5 (*Methanosarcina* dominated). Suggesting significant difference in active VFA oxidizing microorganisms. This confirm the reports of Karakashev et al. (2005), which states that reactors containing low levels of VFA are dominated by *Methanosaeta*, while high levels of VFA are common in *Methanosarcina* dominated reactors. As such, in a broader sense, following the anaerobic degradation of complex substrate it is possible that *Methanosaeta* dominated reactors utilize VFA degradation pathways to a greater extent, compared to *Methanosarcina* dominated reactors, which may be more likely to degrade other compounds instead. Since this study did not include investigation of the degradation of reactor feedstock, it is difficult to draw any conclusion about methane yield of either systems. If reactor conditions are ideal without stresses, the growth of *Methanosaeta* is favoured with subsequent low concentration of acetate. Such a system is however vulnerable to shocks and dependent on sources of waste that will not be inhibitory for *Methanosaeta*. In such an event, a knockout of *Methanosaeta* would halter the VFA consumption and possibly result in adverse and acidic conditions. As discussed previously, a community dominated by *Methanosarcina* is likely more stable despite shock and stress as suggested by De Vrieze et al. (2012).

Despite of the acetogenic activity converting propionate and *n*-butyrate into acetate (figure 12), no accumulation of acetate was measured to any large extent in the degradation assays. Acetate consumption and methane production showed strong correlation, suggesting that acetate was in fact cleaved into methane. Although SAO may have contributed to the degradation of acetate, those reactions are favoured during high ammonia and/or thermophilic conditions (Westerholm, 2012; Westerholm et al., 2016) at which the degradation test did not operate in. It is plausible to expect that the stress induced by high salinity in R4-R5 favour SAO, although from this study it is not possible to say. At the end of the degradation assays when all substrates were consumed the cumulative methane production was expected to be roughly the same in all reactors, because of the same moles of initial substrate. This was found not to be the case. It was observed that at day 0 of the degradation assay the reactor bottles differed in organic acid composition to a small extent (mostly ethanol). These acids likely originate from the reactor water they were stored in prior to inoculation, giving each reactor assay slightly different starting substrates. Also at the end of the assay an accumulation of organic acids such as ethanol, valerate, *i*-butyrate, 1-propanol etc. was observed (data not presented), explaining to some extent the differences in SMP (table 5). This proposes that plenty of specialized microorganisms are active, which can make the degradation unpredictable. In R4 a temporary production of propionate and acetate was observed at day 7, but later consumed at day 10 (figure 12). The lag phase seen in R2 (figure 11) indicate a growth of

acetate degraders before rapid acetate consumption occurred. Also, when degrading *n*-butyrate by R2, R4, R5 a lag phase is seen until day 7, this is likely because of low abundance of *n*-butyrate degraders at day 0. Suggesting that *n*-butyrate is not a common substrate to be degraded in those reactors.

The clustering depicted by the NMDS plots (figure 17, 18) of granules belonging to the same reactor indicate that the methanogenic community of granules is more influenced by the reactor (such as reactor type, substrate, process conditions etc.) than by the size of the granules. It is reported by Bhunia & Ghangrekar (2007) that the methanogenic activity increases with size of granules, but after a "peak size" is reached the activity decreases due to diffusion limitations of substrates. It is likely that an "optimal or most active" granule size is not general but wastewater and/or reactor specific. A future study on degradation in different sizes of granules coupled with investigation of microbial community may be interesting to conduct.

Ultimately the methanogenic composition is characterized by numerous influencing parameters of the specific reactor. The performance and yield of the reactor is tightly connected to the methanogens present, which are influenced by type of waste, stresses etc. It is thereby important to recognize that rectors using waste waters containing high levels of inhibitory substances have a disadvantage compared to reactors without stresses. Because microorganisms that have evolved tolerance (e.g. *Methanosarcina*) to stresses most commonly suffer in other aspects, like reduced degradation efficiency, but may still be preferred because of operation stability. As such, the ideal methanogenic community structure may vary depending on waste treated. I propose that a reactor with high levels of stresses may benefit from having a diverse methanogenic community dominated by *Methanosarcina*, while a reactor with ideal condition and high control of influents may yield more methane if dominated with *Methanosaeta*.

4.3 Considerations of methods

One concern in this study was that the heterogenous nature of the granules would give a large variation in result of the degradation assays. Although in this study only duplicate sampling were used, this was found only to be the exception. With samples from reactor R3 significant variation was noted in the methane production in the degradation assays (figure 11, 13). It seems in general that the fatty acids measured by HPLC had a high degree of similarity, while production of methane deviated more. Possibly explained by inaccuracy in gas analysis procedure rather than differences between granules.

Of major importance when considering the setup of the degradation assays is the inoculum to substrate ratio. When using granules which contain a concentrated amount of active microorganism as in present study, a low relative volume of inoculum to substrate can be used compared to other sources which contain less dense microbial biomass. However, not low enough that an accumulation of VFAs occur due do incomplete degradation as stated by Angelidaki et al. (2009). Accumulation of VFA at the end of this study was only found to a small extent.

Using both the restriction enzymes BstNI and *Mwo*I proved to be a potent method for characterizing the methanogenic community. Especially since it was sometimes possible to exclude ambiguous assignments by searching corresponding T-RF with the other enzyme. T-RFs < 50 was excluded because they infer with the primer peak in the electrophoresis, although such fragments are present and may represent a taxa. By using two enzymes, although a methanogen may be excluded

by one enzyme (< 50 bp), it is still possible it is represented by the other. The major difference between the two enzymes was the assignment of *Methanobacterium*, this can be explained by the absence of a restriction site in some species when digested with *Bst*NI.

Although the database used for assignment of *mcrA* amplicons offers a fast and effective way to assess relative abundance of methanogenic community, it is not complete. There are still gaps and unassigned T-RFs, for the *Bst*NI data (average 23%) and for *Mwo*I (average 6%). This stresses the importance of extending the database, by isolation and sequencing of still unknown methanogens.

5 Concluding remarks

Granules from UASB and EGSB reactors investigated were predominantly 3 mm or larger from paper waste reactors and more evenly distributed from reactors treating methylcellulose. In general, larger granules had slightly higher VS content than average. The dominating methanogens found in anaerobic digesters studied, ranked in order of prevalence include: *Methanosaeta, Methanobacterium, Methanosarcinaceae, Methanocaldococcus, Methanomicrobiales, Methanomassiliicoccaceae, Methanoculleus.* The methanogenic community differed in granules of particular sizes, but no clear trend could be interpreted. Instead the community was found to be highly influenced by reactor conditions such as high salt concentration in methylcellulose waste, which favoured *Methanosarcinaceae*. While in other reactors, treating paper and sugar waste during low stress conditions, was dominated by *Methanosaeta*. Reactors dominated by *Methanosaeta* had superior VFA degradation rates, while *Methanosarcinaceae* dominated reactors had higher hydrogenotrophic activities.

Methanogens perform such a critical role in AD and their activity may indicate process failure, which make them a good target for monitoring. Further development of screening methods of methanogenic community will help to diagnose anaerobic reactors and increase the understanding of shifts in community when process parameters change.

In this study the focus has been on the methanogenic community, although in a full-scale AD reactor many more groups of microorganisms are present and equally important. A more comprehensive study of microbial composition, sequencing both the *mcrA* and 16S rRNA gene using MiSeq Illumina is planned in the future.

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Appendix 1

Sample height (m) from reactors R1-5

Reactor	Height (m)					
R1	1.2	3.2	5.2	7.2		
R2	1.6	3.7	5.6	10.7		
R3	0.5	2.0	3.5	5.0	6.5	8.0
R4	2.7	6.0	9.3			
R5	3.0	6.0	8.0			

Mineral media components

Component A				Component B	
Compound	(mg/L)	Compound	(mg/L)	Compound	(mg/L)
KCl	600	$ZnCl_2$	4.72	KH_2PO_4	1000
$MgCl_2 \times 6\ H_2O$	600	H_3BO_3	4.96	NH_4HCO_3	8190
$CaCl_2 \times 2\ H_2O$	200	biotin	0.04		
$Na_2S \times 9\ H_2O$*	500	folic acid	0.04		
$FeCl_2 \times 4\ H_2O$	42.36	pyridoxine	0.2		
$CuCl_2 \times 2\ H_2O$	0.86	thiamine	0.1		
$CoCl_2 \times 6\ H_2O$	1.94	riboflavin	0.1		
$MnCl_2 \times 4\ H_2O$	1.64	nicotinic acid	0.1		
$Na_2MoO_4 \times 2\ H_2O$	0.86	Ca-pantothenate	0.1		
$NiCl_2 \times 6\ H_2O$	3.28	B12	0.1		
$Na_2WO_4 \times 2\ H_2O$	0.36	p-aminobenzoate	0.1		
$Na_2SeO_3 \times 5\ H_2O$	0.8	lipoic acid	0.1		

*added right before assay preparation