Impact of management measures on the biogas microbiome on the example of feedstock changes

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“The more I learn, the more I realize how much I don't know.”

Albert Einstein

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Abstract

Regarding the current political and social-economic situation, the biogas industry in Germany is standing at a crossroad as a transition to a residue-based biogas production is imminent. In future biogas plants, the microbial communities will often be exposed to varying process conditions while an overall stable process has to be ensured. Hence an understanding of how biogas microbiomes response to management measures and how these responses affect to process efficiency is of great importance. Therefore the overall aim of this study was to investigate the impact of feedstock changes on the biogas microbiome structure and deduce potential impacts on the process performance to recommend process operation conditions.

The study was separated into three phase: (1) The development of the microbial community during a long-term anaerobic digestion of maize and sugar beet silage (2) The elucidation of a nexus between the microbial diversity level and the stress tolerance potential by supplying animal manure or ammonium carbonate. (3) The verification of the potential of biogas-producing microbiomes to handle a profound feedstock change.

This study revealed *i.a*. that members from the phylum *Bacteroidetes* and the order *Spirochaetales* have an affinity to easy degradable compounds such as sugars, ethanol and acetate, the main compounds of the sugar beet silage. Hence they are assumed to play a crucial role in the acido- and acetogenesis, the phase where organic acids are produced. Secondly, this study revealed that a bacterial community with a few dominant members led to a functional more flexible archaeal community (reactors fed with sugar beet silage) which was more stress resistant to elevated TAN concentrations compared to a bacterial community with higher amount of more evenly distributed community members combined with a more rigid archaeal community (reactors fed with maize silage). However, with a careful counteracting, the process could be stably operated with 75% chicken manure (based on VS). Interestingly it was observed that the disappearance of members of the phylum *Cloacimonetes* can potentially be used as an indicator for an upcoming process disturbance due to increasing TAN concentrations. Last but not least, a profound feedstock exchange from maize silage to sugar beet silage and vice versa resulted in a short-range decrease or increase in the biogas yields according to the chemical feedstock complexity without a longstanding negative impact on the overall biogas production. This indicates that the two feedstocks sugar beet and maize silage potentially do not contain chemical compounds that are difficult to handle during anaerobic digestion compared to the impact the nitrogen-rich chicken manure.

Zusammenfassung

Angesichts der aktuellen politischen, wirtschaftlichen und gesellschaftlichen Situation steht die Biogasbranche in Deutschland an einem Wendepunkt, da der Übergang zu einer reststoffbasierten Biogasproduktion bevorsteht. Zukünftig werden die mikrobiellen Gemeinschaften häufig variierenden Bedingungen ausgesetzt sein, während ein insgesamt stabiler Prozess gewährleistet sein muss. Das Verständnis, wie Biogas-Mikrobiome auf Managementmaßnahmen reagieren und wie sich diese Reaktionen auf die Prozesseffizienz auswirken sind daher von zentraler Bedeutung. Das übergeordnete Ziel dieser Studie war es, den Einfluss von Einsatzstoffvariation auf die Mikrobiomstruktur zu untersuchen und mögliche Auswirkungen auf die Prozesseffizienz und damit Handlungsempfehlungen für eine optimierte Prozessführung abzuleiten.

Die vorliegende Arbeit wurde in drei Phasen unterteilt: (1) Anpassungspotential einer mikrobiellen Gemeinschaft an die Monofermentation von Mais- bzw. Zuckerrübensilage, (2) Aufklärung eines Zusammenhangs zwischen dem mikrobiellen Diversitätsniveau und der Anpassung an erhöhte Ammoniumkonzentrationen und (3) Auswirkungen eines Wechsels der Einsatzstoffe auf die Mikrobiomstruktur und die Prozesseffizient.

Ein zentrales Ergebnis dieser Studie ist, dass Vertreter der Abteilung *Bacteroidetes* und der Ordnung *Spirochaetales* eine Affinität zu leicht abbaubaren Verbindungen wie Zuckern, Ethanol und Acetat haben, was darauf hindeutet, dass sie eine zentrale Rolle in der Acido- und Acetogenese spielen. Darüber hinaus konnte gezeigt werden, dass eine Bakteriengemeinschaft mit wenigen dominanten Mitgliedern zu einer funktionell flexibleren Archaeengemeinschaft führt, die gegenüber erhöhten TAN-Konzentrationen resistenter war als eine gleichmäßig verteilte Bakteriengemeinschaft. Trotz einer kurzzeitigen Prozessstörung konnte ein stabiler Prozessverlauf mit 75% Hühnertrockenkot erreicht werden. Eine Abnahme der relativen Häufigkeit von Mitgliedern der Abteilung *Cloacimonetes* könnte diese Gruppe als Indikator für eine bevorstehende Prozessstörung aufgrund erhöhter TAN-Konzentrationen kennzeichnen. Nicht zuletzt führte ein Wechsel von Mais- zu Zuckerrübensilage und umgekehrt zu einer kurzfristigen Verringerung oder Erhöhung der Biogaserträge, ohne dass der Gesamtprozess langfristig negativ beeinflusst wurde. Dies weist darauf hin, dass die beiden ausgetauschten Einsatzstoffe keine chemischen Verbindungen enthalten, die während des anaeroben Abbaus im Vergleich zu den Auswirkungen des stickstoffreichen Hühnertrockenkots für das Mikrobiom schwer zu handhaben sind.

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Abbreviations

°C	degree Celsius
µl	microliter
16S rRNA	small subunit of the ribosomal ribonucleic acid
A	ammonium
AD	anaerobic digestion
bp	base pair
C	control
CH_4	methane
CM	chicken manure
CO_2	carbon dioxide
CSTR	continuously stirred tank reactors
Cy5	Indodicarbocyanine at the 5′-end
d	days
DGGE	denaturation gradient gel electrophorese
DNA	deoxyribonucleic acid
dNTPs	deoxyribose nucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen/German Collection of Microorganisms and Cell Cultures
Eq.	Equivalent
FM	fresh mass
GC	gas chromatography
h	hour
H_2	hydrogen
HAc	acetic acid
HPr	Propionic acid
HRT	hydralic retetion time
l	liter
M	manure
mg	milliliter
MS	maize silage
ng	nanogramm
NGS	next generation sequencing
NH_3	ammonia
NH_4^+-N	Ammonium-nitrogen
NMDS	non metric dimentional scaling
OLR	organic loading rate
OTU	operational taxonomic unit
PCR	polymerase chain reaction

qPCR	quantitative polymerase chain reaction
R^2	Goodness of the vector fit
s	second
SBS	sugar beet silage
SM	swine manure
TAN	total ammonium nitrogen
TKN	total Kjeldahl nitrogen
TRF	terminal restriction fragment
TRFLP	terminal restriction fragment lenght polymorphism
TS	total solids
TVFA/TA	total volatile fatty acids/total alkalinity
UPGMA	unweighted pair group method with arithmetic mean algorithm
VDI	Verein Deutsche Ingengieure
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten / Association of German Agricultural Analytic and Research Institutes
VFA	volatile fatty acids
VS	volatile solids

1 Introduction

Methane (CH_4) is a colourless, nontoxic and odourless gas with a calorific value comparable with gasoline and diesel (Gerthsen, 2008). It is produced as the last step during degradation of organic matter under anaerobic conditions. The formation of methane takes place in numerous environments, such as sediments, rice fields, fens and in the rumen of ruminants. Because of the high calorific value of CH_4, this natural occurring process has been transferred into biotechnological applications, such as biogas plants, as it can be used as a substitute of fossil fuels, for heat and electricity production and as vehicle fuel (Angelidaki et al., 2011; Schink, 1997; Schnürer, 2016; Weiland, 2010).

In biogas plants, organic matter is degraded into biogas, a mixture of mainly CH_4 and carbon dioxide (CO_2). The first documented biogas plants were operated in Bombay, India in the 1850s and in the United Kingdom in the 1890s, whereby the produced biogas was used for lightning and heating (Bischofsberger *et al.*, 2005; Stadtmüller, 2004). Over the course of time, the interest in biogas as an energy source has been depending on for example the oil prices during the oil crisis in the 1970s and more recently due to the limited assets of fossil fuels as well as climate changes caused by the use of fossil fuels. Next to the use of wind, water and solar for the production of renewable and climate friendly energy also the use of biomass for biogas production has increased during the last years. In Germany, 13.1% of the energy consumption came from renewable sources in 2017, whereof the use of biomass accounts for the highest amount (FNR, 2018).

1.1 The process of anaerobic digestion

Many microorganisms use the chemical energy saved in organic matter to gain energy for the synthesis of cell-own compounds and for cell growth. This energy conversion is carried out through reduction-oxidation reactions, where one component is oxidised and one is reduced, i.e. one component give electrons away (electron donor) and one gain them (electron acceptor). If the organic matter is oxidised with an extern compound as electron acceptor it is

called respiration. If this extern electron acceptor is oxygen (O_2), a complete oxidation (degradation) of the organic matter into CO_2 and water (H_2O) takes place. If no O_2 is present other inorganic compounds, such as nitrite, sulphate or CO_2 can be used as electron acceptor (anaerobic respiration). A third possibility to gain energy is through fermentation, where the microorganism uses an internal electron acceptor produced during degradation (Angelidaki et al., 2011; Fuchs, 2007).

The willingness for a compound to take or give electrons is expressed through the redox potential. The higher the redox potential is for a redox couple (e.g. O_2/H_2O), the better it can serve as electron acceptor. The difference in the redox potential between two redox couples (ΔE) is directly proportional to the amount of free Gibbs energy (ΔG) - the maximum of energy that the organism can gain from the reaction and use for e.g. cell growth. O_2 is the best electron acceptor and the highest amount of energy can be gained through aerobic respiration. For example the ΔG from aerobic respiration of glucose to CO_2 and H_2O accounts for -2870 kJ mol^{-1} while the conversion of glucose into CO_2 and CH_4 yields only -390 kJ mol^{-1}. However energy cannot just disappear, hence is the remaining energy from glucose stored in CH_4, making this compound so energy rich (Madigan et al., 2009; Schink, 1997).

Because of the small amounts of energy gained during the anaerobic degradation of organic matter a complete degradation cannot be performed by one microorganism alone. Glucose for example can be oxidized into acetic acid by one microorganism, but the further degradation of acetic acid into CH_4 and CO_2 is performed by other microorganisms. Regarding the often highly complex chemical composition of organic matter (cellulose, hemicellulose, starch, sugars, proteins, nucleic acids, fats) the process of biogas production requires a great microbial diversity. Among the members of the biogas microbiome the two different domains *Bacteria* and *Archaea* are the most investigated, as it is well known that they are working together in order to degrade the chemical complex organic matter into energy-rich CH_4. Other, less investigated, organisms within the biogas microbiome are virues/phages as well as funghi and protists (Theuerl et al., 2019b). Members of the domain *Bacteria* degrade organic matter into mainly acetic acid, CO_2 and hydrogen (H_2), which serve as substrate for the methanogenic *Archaea* that produces CH_4 as an end product. In general, the biogas production chain can be divided into four steps: 1) hydrolysis, 2) acidogenesis, 3) acetogenesis and 4) methanogenesis, where the first three steps are performed by *Bacteria* and the last one by *Archaea* (Angelidaki et al., 2011; Schnürer, 2016).

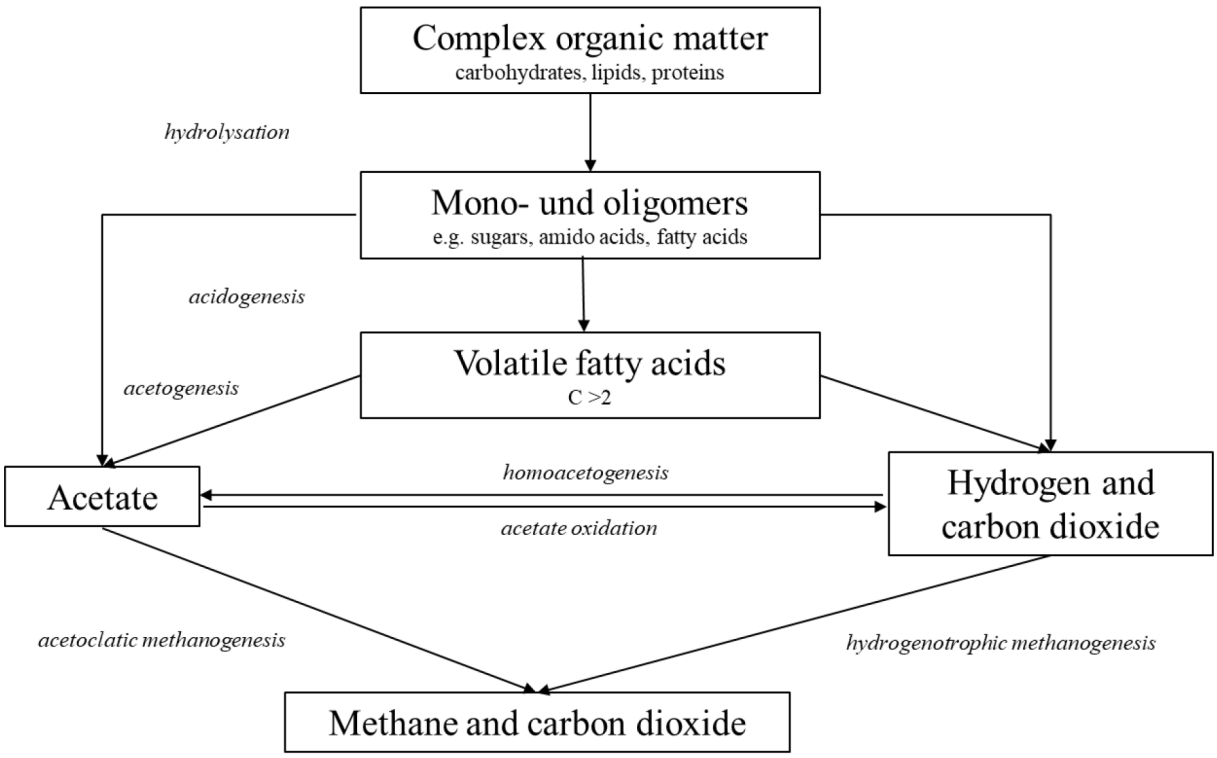

Figure 1-1: Flow chart of the biogas production chain

Complex organic matters consist of macromolecules such as carbohydrates, lipids and proteins that cannot be taken up through the cell wall, thus have to be degraded outside of the cell. This first step of the biogas production chain, the **hydrolysis**, is performed by bacteria able to produce exo-enyzmes such as cellulases, amylases and proteases (Vinet and Zhedanov, 2010). These exo-enzymes are released by the microorganisms through the cell wall and hydrolyses the macromolecules into soluble oligo-, di- and mono molecules such as sugars, fatty acids and amino acids (Angelidaki et al., 2011; Gujer and Zehnder, 1983). One bacterial species alone is not able to produce all required exo-enzymes in order to degrade the whole variety of macromolecules in the organic matter hence to degrade the high variety of chemical compounds within the biomass different microorganisms are necessary (Vinet and Zhedanov, 2010). This first step of the biogas production chain is often considered to be the rate limiting step, especially if fibre-rich biomasses are converted (Pavlostathis and Giraldo-Gomez, 1991, Azman et al., 2015, Shrestha et al 2017). After hydrolysis the oligo-, di- and monomers are taken up into the cell where they are further degraded into intermediates such as different volatile fatty acids (VFA) during **acidogenesis**. VFAs longer than two carbon atoms, such as propionic acids, butyric acids and valeric acids, have to be further degraded

into acetic acid, CO_2 and H_2 in the **acetogenesis** before they can be converted into CH_4. In the last step, the **methanogenesis**, the production of CH_4 is performed by methanogenic *Archaea*. The methanogenic *Archaea* can be divided into three groups due to their metabolism: acetoclastic, hydrogenotrophic and methylotrophic methanogens. The acetoclastic and hydrogenotrophic pathways are thought to be the most important in the biogas production community. The acetoclastic *Archaea* cleave acetate into CH_4 and CO_2 whereas the hydrogenotrophic *Archaea* produces CH_4 through oxidation of H_2 with CO_2 as electron acceptor (Ahring, 2003; Angelidaki et al., 2011).

The degradation of higher VFA, such as propionic acids, is an endergonic reaction, meaning that energy is needed in order to perform the reaction. However, the reaction can take place if the H_2 concentration is kept low, for example through H_2 scavenging microorganisms, such as hydrogenotrophic methanogens. Hence, the bacteria performing the acetogenesis need the hydrogenotrophic methanogens to remove the produced H_2 and the hydrogenotrophic methanogens need the H_2 produced by the acetogenic bacteria. This type of close relationship, when the organisms depend on each other to survive, is called syntrophy (Leng et al., 2018; Schink, 1997).

Besides the four main steps of the biogas production chain also other intermediate steps may occur. In the homoacetogenesis specific bacteria use CO_2 and H_2 for energy recovery while acetate is produced as end product. Also the other way around is possible, where bacteria oxidize acetate into CO_2 and H_2. The homoacetogenesis is an exergonic reaction and the acetate oxidation an endogenic reaction. Hence, the bacteria performing the acetate oxidation has to live in a syntrophic relationship with H_2 scavenging microorganisms. The conversion of acetate into biogas over this relationship is called syntrophic acetate oxidation (SAO) (Hattori, 2008; Westerholm et al., 2016).

The production of biogas is thus a biochemical process of different degradation steps, performed by different *Bacteria* and, at the last step, *Archaea* where the products of one step serve as substrate for the other one. Hence it is important, to ensure suitable environmental conditions for several different microbial groups in order to perform an efficient degradation of organic matters into biogas (Theuerl et al., 2019b).

1.2 The microorganisms present during anaerobic digestion

The degradation of organic matters into energy-rich biogas is performed through a close cooperation of different microorganism from the domains *Bacteria* and *Archaea*. Because of the higher substrate spectra for the bacterial community the amount and diversity of this domain is higher compared to the archaeal community, which only have a substrate spectra limited to acetate, CO_2, H_2 and other one carbon compounds (Liu and Whitman, 2008; Tang et al., 2015).

During the last two decades several studies have been performed to investigate the microbial community composition in anaerobic digestion systems. (e.g. Alsouleman et al., 2016; Liu et al., 2009; Rademacher, 2013; Theuerl et al., 2018). In 2011 Nelson et al conducted a meta-study, where they collected all available 16S rRNA-gene sequences obtained from methanogenic anaerobic digestion. Overall 19.388 sequences were analysed, from which around 85% belonged to *Bacteria* and 15% *Archaea*. The four largest phyla found within the Bacteria were *Firmicutes, Bacteroidetes, Proteobacteria* and *Chloroflexi*, from which the three first phyla were found in almost every study. From the domain *Archaea,* most sequences were assigned to the phylum *Euryarchaeota*.

Members of the bacterial phylum *Firmicutes*, mainly from the class *Clostridia,* are thought to play an important role in the first step of the biogas production chain as many of the known species are cellulolytic and amylolytic and hence are able to degrade complex polymers. The genera *Clostridium*, *Ruminococcaceae*, *Sedimentibacter* are some examples of Bacteria detected with hydrolytic capabilities. This phylum also contains some species known to live in syntrophic relationships, for example from the genus *Syntrophomonas* as well as members able to perform the already mentioned acetate oxidation, such as *Thermacetogenium phaeum*, *Tepidanaerobacter acetatoxydans*, *Clostridium ultunense* or *Syntrophaceticus schinkii* (Maria Westerholm et al., 2018).

In the phylum *Bacteroidetes* Nelson et al. (2011) found that only around 50% could be classified to any known class, from which the main part belonged to the order *Bacteroidetes* and further to the family *Porphyromonadacea* with genera like *Parabacteriodes*, *Petrimonas*, *Paludibacter* and *Proteiniphilum*. Described members from this family have shown capability to degrade sugars and protein with VFAs such as acetic acid and propionic acid as fermentation products. From the phylum *Proteobacteria* around 70% could be assigned to a

described genus. Many species in the phylum are able to grow on organic acids, for example are the species *Smithella* and *Syntrophobacter* able to degrade propionate in syntrophic relationship with hydrogen consumer. *Chloroflexi* was not found in all reactors and the knowledge of what they do is low. 60% of the found sequences were classified as unclassified *Anaerolineaceae.*

From the domain *Archaea* most of the found sequences were assigned to the phylum *Euryarchaeota* with the two, for the anaerobic digestion important, classes *Methanomicrobia* and *Methanobacteria.* Around 60% of the sequences were assigned to *Methanomicrobia* and the most sequences could further be assigned to the obligate acetoclastic genus *Methanothrix*. Other, for the anaerobic digestion important genuses belonging to the class is the mixotrophic Methanosarcina and the hydrogenotrophic *Methanoculleus*. Sequences belonging to the class *Methanobacterium* counted for around 8% whereas the most sequences were assigned to the hydrogenotrophic genus *Methanobacterium.*

1.3 Feedstocks used for anaerobic digestion in Germany

For the anaerobic digestion with the goal to produce energy-rich methane a wide range of biomasses, such as sewage sludge, municipal bio-waste, residues from livestock husbandry and landscape management, aquatic plants and algae as well as energy crops are used as feedstocks in biogas plants around the world

In Germany the use of energy crops and animal manure are of great importance since the Renewable Energy Sources Act (Erneuerbare-Energien-Gesetz, EEG) came into force in year 2000. The Renewable Energy Sources Act secured the plant operator remuneration based on the generated electrical power produced as well as on which feedstock used for digestion. The remuneration for feedstock was only given for selected feedstock, such as energy crops and animal manure, but not for bio-waste like food and industrial residues, which is the reason that most biogas plants in Germany are operated with energy crops and animal manure. The remuneration was secured for 20 years after start-up and the Renewable Energy Sources Act led to a high increase in the number of biogas plants in Germany, from around 1000 in the year 2000 to 8700 in the year 2016 (FNR, 2018).

In Germany around 16.7×10^6 ha are used for agricultur of which around 2.4 10^6 ha are used for production of energy crops. There has been an increase in the last years as the area used for crop production was 1×10^6 ha in 2004 and in 2.4×10^6 ha in 2007. Out of this 2.4 $\times10^6$ ha around 1.3×10^6 ha are used for biogas production, whereof 0.9×10^6 ha are maize cultivation (FNR, 2019). Maize has many advantages for biogas production as it has a high energy yield per hectare crop area, is easy to cultivate and has no specific demands on the growth site (FNR, 2018). However has this increase of energy crop cultivation, especially the mono-cultivation of maize, had some negative effects. The conversion of species-rich grasslands into less diverse arable land has led to loss of biodiversity, which further reduced soil quality. Furthermore it has caused discussions about land occupation for energy crop cultivation instead of food production (Herbes et al., 2014a; Lüker-Jans et al., 2017).

To counteract this trend an upper limit of 60% of the use of maize and cereal was introduced with the amendment of the Renewable Energy Source Act in 2012 and further reduced to 50% in 2016/2017. However, due to the secured remuneration for 20 years many biogas plants are still operated with high amounts of energy crops but with the amendments the use of other feedstocks, such as bio waste and industrial waste, is easier and changes in the feedstock composition have been noted during the last years. Compared to 2014, the amount of energy crop decreased from 53% to 49% and the use of maize silage from 73% to 69% whereas the use of bio waste as well as industrial and agricultural residues rose from 5% to 7% (FNR, 2018).

1.4 Operational Parameters and inhibitions of the process

The main macromolecules of all organic matter are carbohydrates, proteins and fats. Due to the chemical composition of these macromolecules, different amounts of biogas and CH_4 contents can be gained during digestion (Table 1-1).

Table 1-1: Theoretical biogas yields and methane contents the main macromolecules of organic matter (VDI-4630, 2006)

Substrate	**Biogas yield** [$L_N\ kg_{VS}^{-1}$]	**Methane** [%]
Carbohydrates	750	50
Protein	800	60
Fat	1390	72

The actually reached values for biogas yields and methane contents are however lower than these theoretical values. The actual amount of biogas depends for example on the structure and bioavailability of the chemical compounds contained in the used feedstocks, operational parameters like temperature and on the adaptation of the microbial community to these parameters (Theuerl et al., 2019b; VDI-4630, 2006).

Depending on the feedstock composition the required hydraulic retention time (HRT), the time the feedstock remains in the biogas plant, in order to gain the maximal degradation degree and hence the maximum amount of biogas, differs. Easy degradable compounds, such as sugars, amino acids and alcohols, can be degrade within hours, whereas proteins, lipids and hemicellulose need a couple of days while cellulose may need up to several weeks (Steffen et al., 1998). A sufficient HRT is also important considering the microorganisms present within the digester. As the energy recovery under anaerobic conditions is very low, the growth rates of the microorganism are slow, with generation times of several days or even weeks. Therefore slow growing microorganism cannot establish in required amounts to efficiently degrade the supplied biomass if the HRT is too short, leading to a wash-out of process relevant microorganisms (Drosg, 2013; Westerholm, 2012). However, a long HRT results in a low loading rate: The organic loading rate (OLR) is defined as the amount of feedstock given to the biogas plant per day and reactor volume. A too high OLR, in combination with a too short HRT, can lead to process disturbances or even process failure (Theuerl et al., 2019b).

The reactor temperature has high influence on the microbial structure and efficiency. If the optimal growth temperature of an organism is between 20 and 45°C they are called mesophilic and between 45 and 80°C thermophilic (Madigan et al., 2009). Most reactors are operated under mesophilic conditions, with temperatures between 37 and 42°C and some rectors are operated under thermophilic conditions, between 50 and 57°C (FNR, 2018, 2016). The operation under thermophilic conditions is more efficient due to higher microbial activity at higher temperatures, however is the process more sensitive to disturbances at higher

temperatures, most likely because of a lower microbial diversity and hence a lower potential of functional redundancy. Temperature fluctuations should not exceed 1°C under thermophilic conditions whereas reactors operated under mesophilic conditions might cope with fluctuations of up to 3°C (Drosg, 2013; Westerholm and Schnürer, 2019).

In addition, intermediates produced during degradation can lead to process disturbances. During degradation of nitrogen rich compounds, such as proteins and urea, ammonium is produced. Ammonium cannot be further degraded under anaerobic conditions but accumulates in the reactor. Ammonium, or more likely ammonia, has an inhibitory effect on mainly the acetoclastic methanogens of the genus *Methanothrix.* In aqueous solutions is ammonium in equilibrium with ammonia, which shifts towards ammonia with increasing temperature and pH. However it has been shown in several studies, that an adaptation of the microbial community can take place and that the degradation of acetate can be performed by syntrophic acetate oxidation, a cooperation between bacteria degrading acetate to carbon dioxide and hydrogen, which can be further converted to methane by hydrogenotrophic methanogens (Rajagopal et al., 2013; Westerholm et al., 2016)

As different microorganisms are able to degrade different compounds and have different tolerance ranges against environmental factors it is important that changes in a biogas plant are carried out with caution. Whether it is a feedstock change, increasing OLR or a temperature change the microbial community needs enough time to adapt to the new conditions. A process disturbance is often accompanied by an accumulation of volatile fatty acids (VFAs), followed by a declining biogas yield and/or methane content. An accumulation of acetic acid might indicate a disturbance of the acetoclastic methanogens as this is the wanted end product from the bacterial community. An accumulation of other acids most likely indicates a disturbance in the bacterial community or in the syntrophic relationship between hydrogen producing bacteria and hydrogen scavenging hydrogenotrophic archaea. If the acid production is not counteracted they will inhibit the microbial community, which might lead to complete process failure (Theuerl et al., 2019b)

1.5 Analysis of the microbial community

In order to investigate the microbial diversity of microorganisms and their physiological potential two different approaches are used: (1) culture-dependent or (2) culture-independent, molecular biological methods.

When using the first approach, the microorganisms from environmental samples are cultivated on specific growth media containing the required nutrients. The microorganisms best adapted to the chosen growth media or growth conditions will outcompete others. By performing several dilution steps and verification of the obtained cultures by e.g. sequencing the 16S rRNA gene ideally a pure culture which can be further physiological and genetically described can be found. However, it has been estimated, that only between 0.1-10% of all microorganisms have been cultivated (Lloyd et al., 2018; Zeyaullah et al., 2009). Although it is hard to obtain pure cultures this approach is still of high importance because of the amount of information gained from cultured strains. The characterization of a single organism provides information about its substrate utilization capacities as well as its produced by- and end-products. Moreover can the obtained pure cultures be genome sequenced, resulting in insight to the general genetic potential which can be further used for approaches on the up and down regulation of the microbial metabolism through altering physical, chemical and/or biological parameters (Curtis et al., 2012; Plugge, 2014).

Despite the high amount of information about organism present in the environmental sample gained by cultivation approaches the information is mostly not sufficient in order to evaluate the entire microbial diversity and dynamic variation over time. In order to perform community and diversity studies the use of molecular biological methods are more useful. With these methods can the community be investigated independent of specific growth conditions of the present organisms. The basis for many molecular biological methods is the deoxyribonucleic acid (DNA) that carries the genomic information for e.g. growth and function/activity. The first step of DNA isolation is the cell lysis, which can be either made by chemical or mechanical treatment or a combination of both, followed by purification of the obtained DNA from the environmental matrix and cell components. The next step for many molecular biological applications is the polymerase chain reaction (PCR) , where a specific fragment/region of the DNA, e.g. the 16S rRNA gene, is amplified (Cabezas et al., 2015; Talbot et al., 2008).

When investigating environmental samples, such as from a biogas plant, the sample contains several hundred to thousand different Bacteria and Archaea. One way to investigate the diversity of the community in the sample is through genetic fingerprint methods. With these methods, also the dynamic over the course of time as well as community differences between different habitats can be investigated. Frequently used fingerprints methods are denaturation gradient gel electrophorese (DGGE) and terminal restricted fragment length polymorphism (TRFLP) (Cabezas et al., 2015; Su et al., 2012), whereas DGGE is based on the separation of DNA sequences according to their GC-content (Muyzer et al., 1993) and TRFLPs on the fragment length after the use of restriction enzymes (Liu et al., 1997). In TRFLP the PCR is performed with fluorescence labelled and then digitated with one or more restriction enzymes (Osborn et al., 2000). The sample is then analysed in a capillary sequencer where only the fluorescence labelled terminal restriction fragments (TRFs) are detected, which can be compared between different environmental samples or with TRFs of known species resulting in a rough classification of unknown TRFs within the environmental sample.

To assign the detected TRFs to specific organisms, a parallel cloning-sequencing approach can be performed. For this a PCR approach is carried out using the same primer pairs (in this case unlabelled) and PCR conditions as for the TRFLP analysis. The obtained PCR products are then ligated into a plasmid vector and transformed into a cell host. The cells are grown on an agar medium containing antibiotics, enabling a selection between cells containing the insert and those who do not. The plasmids are then extracted from the cell and can be used for other applications, such as Sanger sequencing.

The Sanger-sequencing was the most common sequencing method for several years This method is also called the chain-termination sequencing and is set up as four parallel reactions, one for each nucleotide. The DNA-fragment, DNA polymerase, dNTPs as well as fluorescence dideoxinucleoitides (ddNTPs) are added to each reaction. The incorporation of one of the four different ddNTPs leads to termination of the DNA elongation, resulting in a mixture of different fragments ending with an A, C, T or G. Each mixture is separated through electrophoresis, revelling the position of each nucleotide. With this method fragments with up to 1000 base pairs (bp) can be sequenced (Corley, 2004; Shendure et al., 2017.

During the last years new sequencing technologies, the so called next generation sequencing (NGS) approaches, were developed that enables the parallel analysis of a large amount of DNA, whereof the Illumina sequencing technique is currently the most used one. With NGS a

large number of short reads (< 500bp) are sequenced and afterwards aligned to a reference applying complex data analysis. Using NGS either amplicon sequencing, hence sequencing a specific fragment of the DNA extracted from an environmental sample, or metagenomics sequencing, were the whole genome from the DNA of the sample is sequenced, can be performed. However, the short reads are one negative aspect of the NGS, which has led to the development of the third generation sequencing, the real-time single molecule sequencing, enabling read lengths of more than 20 000 bp.

The time-consuming and complex nature of the modern sequencing techniques, regarding sample preparation and especially data evaluation and interpretation, is a potential bottleneck for their application when monitoring community dynamics under varying environmental conditions. Therefore, established potential "old-school" techniques, such as the TRFLP, are still valuable for microbiome screening (De Vrieze et al., 2018).

1.6 System ecological theories

In order to describe the world around us different theories and hypotheses have been developed. The use of these is needed in order to plan and predict different scenarios. Considering the field of ecology most theories have been developed for macroorganisms, hence plants and animals The implementation of the theories in microbial ecology are less used due to for example the small size and similar morphology of microorganisms. However the rapid development of different molecular biological methods, such as different NGS-methods, have reduced these difficulties (Prosser et al., 2007).

There are several theories trying to explain and describe biodiversity. The two main theories are the niche theory and the neutral theory of diversity. According to the first one is selection and competition between species essential for community development and this theory is the oldest and most acknowledged one. Opposite to this, says the neutral theory the random process and ecological drift are determining for the development (Chave, 2004; Fisher and Mehta, 2014; Hubbell, 2001, 2005; Pocheville, 2015).

Some other ecological theories are for example the insurance hypothesis, the species-area-relationship and resource insurance hypothesis. The insurance hypothesis says that a community with a high amount of different species is more stable as species reacts different to

changes and one species might compensate for another (Lynch et al., 2004; Yachi and Loreau, 1999). The species-area-relationship says that the number of different species increases with the volume of the habitat (Bell et al., 2004; Prosser et al., 2007) and the resource insurance hypothesis says that in a habitat with increasing resources also the diversity increases (Hall et al., 2000; Lynch et al., 2004).

What distinguish a stable community from a disturbed one? A disturbance of the system can be defined as a "significant change in the functionality within the microbial community leading to severe and unaccepted decreases in biogas/methane generation and requiring counteraction to be overcome" (Theuerl et al., 2019b). Three different responses of the microbial community to disturbance have been described: either the community is resistant, resilient or functional redundant. In case of a resistant community a disturbance does not affect the microbial community composition, it remains unchanged. A resilient community changes during the disturbance, but returns back to the former composition, whereas organisms of a functional redundant community can be replaced by other organisms with equal or similar or equivalent functional capacities (Allison and Martiny, 2008).

In order to describe and compare different communities the calculation of diversity indices can be used. The richness is the most basic and direct one: the number of detected species in a population. In molecular biology this number can be defined as for example number of detected operational taxonomic units (OTUs) or number of detected TRFs. For the calculation of the evenness there are several different indices. The evenness also takes the abundance of the different species into account. If all species have the same abundance the community is perfectly even and the higher the abundance of single species are, the more uneven is the community. One way to calculate the evenness is with the Gini index, which is the area between perfect evenness and the Lorenz curve of the data. The higher the Gini index is, the more uneven is the community (Cabezas et al., 2015; Daly et al., 2018; Wittebolle et al., 2009). Marzorati et al. (2008) suggested that if 20% of the detected organisms (OTUs) account for 80% cumulative abundance (high Gini index) this is a highly specialized community that is fragile to changes and might be sensitive to disturbances. In contrast is a community where 20% of the organisms account for 25% of the cumulative abundance (low Gini index) thought to be too even and not able to react to stress exposure.

Also different multivariate statistical methods can be used in order to better understand the obtained data. Cluster analysis calculates the dissimilarity of the different samples and groups

them into categories and the data are often visualised as a dendrogram. Non metric dimensional scaling (NMDS) ranks the distances of each sample and reduces them to a single plot, which can be visualized in a two or three dimensional space. Environmental vectors can be calculated and the direction and length in the plot indicates its effect on the community (Cabezas et al., 2015; Paliy and Shankar, 2016; Ramette, 2007; Talbot et al., 2008).

The members of the microbial community can be divided into generalist and specialists. The generalists are thought to be able to live under various conditions and are often found in different habitats whereas the specialists are thought to perform metabolic functions specific for their habitat (Jousset et al., 2017; Theuerl et al., 2019b). According to the concept of microbial resource management (MRM), which was introduced by Verstraete et al. (2007), there are three main questions that should be answered: Who is there, who is doing what, and with whom are they doing it?

1.7 The aim of the study

The aim of this study was to investigate the impact of management measures on the biogas microbiome on the example of feedstock changes.

This study consisted of three consecutive phases:

(1) the development and adaptation of a microbial community to the anaerobic digestion of maize and sugar beet silage, two feedstocks that significantly differ in their chemical composition
(2) the potential nexus between the microbial diversity level and the stress tolerance potential of the microbial community to elevated total ammonium nitrogen concentrations induced by different ammonium sources
(3) the potential of biogas producing microbiomes to handle a profound feedstock exchange from sugar beet silage to maize silage and vice versa

This approach enabled (i) an system ecological view of existing biotic and abiotic relationships and interactions, (ii) the opportunity to reveal if and how members of the microbiome are affected by changing environmental condition and (iii) the identification if and how members of the microbiome can adapt to potentially unfavourable process conditions

which might result in the detection of potential microbial process indicators usable for process monitoring in order to be predicted and prevented process disturbances.

2 Dynamic variation of the microbial community structure during the long-time mono-fermentation of maize and sugar beet silage

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2.1 Summary

This study investigated the development of the microbial community during a long-term (337 days) anaerobic digestion of maize and sugar beet silage, two feedstocks that significantly differ in their chemical composition. For the characterization of the microbial dynamics, the community profiling method terminal restriction fragment length polymorphism (TRFLP) in combination with a cloning-sequencing approach was applied. Our results revealed a specific adaptation of the microbial community to the supplied feedstocks. Based on the high amount of complex compounds, the anaerobic conversion rate of maize silage was slightly lower compared with the sugar beet silage. It was demonstrated that members from the phylum *Bacteroidetes* are mainly involved in the degradation of low molecular weight substances such as sugar, ethanol and acetate, the main compounds of the sugar beet silage. It was further shown that species of the genus *Methanosaeta* are highly sensitive against sudden stress situations such as a strong decrease in the ammonium nitrogen (NH_4^+-N) concentration or a drop of the pH value. In both cases, a functional compensation by members of the genera

Methanoculleus and/or *Methanosarcina* was detected. However, the overall biomass conversion of both feedstocks proceeded efficiently as a steady state between acid production and consumption was recorded, which further resulted in an equal biogas yield.

2.2 Introduction

One important objective for the future energy supply worldwide is to disengage from the dependence on fossil fuels and nuclear energy and instead extend the use of renewable energy sources. In this context, the production of biogas, containing energy-rich methane, is one important technique for energy production. Biogas is produced through the anaerobic digestion (AD) of organic matter, e.g. energy crops and animal manure. The production of biogas is unique among renewable energies, because it is suitable for the simultaneous production of electricity and heat, as a fuel and as a substitute for natural gas (FNR, 2013). In addition, the production of biogas is independent of daily and seasonal as well as weather-related fluctuations. Therefore, this technology, or more precisely, this process can be used for securing the basic supply of electricity. For the last years, there has been an increased cultivation of energy crops in Germany, which are used as feedstock for the production of energy-rich biogas (Balussou et al., 2012) whereby maize accounts for the largest share (FNR, 2012). Maize offers several advances as feedstock for the AD, including a high amount of dry matter and a high potential biogas yield as well as low requirements of fertilization and plant protection products during cultivation. On the other hand, there is an increasing criticism concerning the cultivation of maize caused by negative influences on soil fertility and biodiversity. As a consequence, the research efforts for alternative feedstocks, for example sugar beet, which have a similar potential to maize in terms of the resulting biogas yield, are intensified. The conversion of biomass into biogas is an anaerobic process mediated by a complex microbial community. The process can roughly be divided into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. In the first phase, particular organic polymers, such as carbohydrates, lipids and proteins, are hydrolyzed into sugars, fatty acids and amino acids, which are further degraded into the intermediates volatile fatty acids (VFAs), acetate, alcohols, carbon dioxide (CO_2) and hydrogen (H_2) during the acidogenesis and the acetogenesis. In the last phase, methane (CH_4) is produced either from acetate (acetoclastic) or from hydrogen and carbon dioxide (hydrogenotrophic) (Gujer and Zehnder, 1983).

The first three phases are conducted by organisms from the domain *Bacteria*, whereby the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Chloriflexi* are the most abundant ones (Nelson et al., 2011). The last phase, the methanogenesis, is performed by representatives from the domain *Archaea* where the three main orders *Methanobacteriales*, *Methanomicrobiales* (both hydrogenotrophic) and *Methanosarcinales* are prevalently found (Nelson et al., 2011). The latter order can be divided into the obligate acetoclastic family *Methanosaetaceae* and the mixotrophic family *Methanosarcinaceae*, which are the physiological generalist among the *Archaea* as they can switch, depending on prevailing conditions, between the two main metabolic pathways (Liu and Whitman, 2008).

In order to investigate the high community complexity in regard of the composition and dynamic of the process-involved microorganisms, different molecular biological methods are frequently applied (Carballa et al., 2011; Fotidis et al., 2014; Guo et al., 2010; Klocke et al., 2007; Regueiro et al., 2012). The most commonly used methods, for example cloning/sequence, quantitative “real-time” polymerase chain reaction (qPCR) or community profiling techniques like the terminal restriction fragment length polymorphism (TRFLP) or the denaturing gradient gel electrophoresis (DGGE), are based on analyses of the 16S rRNA gene. This gene is well known for its high phylogenetic resolution power for detection of microbial relationships. A combination of different methods and a correlation with the process parameters enable a complementary and comprehensive investigation that allows to link community structure information to its phenotypic role in its respective habitat.

In this study a comparative investigation between the mono-fermentation of maize and sugar beet silage was performed. It was assumed that maize silage will be characterized by a diminished biodegradability due to a higher amount of organic polymers resulting in a higher structural and functional microbial diversity. In comparison with this, sugar beet silage has a higher amount of easy degradable compounds that probably favours the development of secondary degraders. Therefore, our research objective was to investigate the specific community adaptation due to differences in the chemical compositions of the energy crops as well as the community changes in a long-term experiment of 337 days during mono-fermentation in regard to possible nutrient deficits.

2.3 Results and discussion

2.3.1 Biogas production kinetics of maize and sugar beet mono-fermentation

The analysis of the investigated feedstocks maize silage and sugar beet silage showed great differences in their chemical composition (Table 2-1). The total solid (TS) was around two times higher in the maize silage compared with the sugar beet silage. However, the maize silage had higher amounts of complex polymeric compounds such as lignin, cellulose, hemicellulose, starch, crude fat and crude protein, whereas the sugar beet silage contained more easy degradable compounds such as sugar, ethanol and acetate.

Table 2-1: Chemical composition of the supplied feedstocks maize silage (MS) and sugar beet silage (SBS) as well as the ratio of each compound in comparison of MS and SBS. Values are given as single measurements of a composite sample.

Parameter	Unit	MS	SBS	MS : SBS
TS	[% FM]	27	14	2 : 1
VS	[% TS]	96	95	1 : 1
Lignin	[g kg_{FM}^{-1}]	6	4×10^{-2}	142 : 1
Cellulose	[g kg_{FM}^{-1}]	58	5	12 : 1
Hemicellulose	[g kg_{FM}^{-1}]	49	4	14 : 1
Starch	[g kg_{FM}^{-1}]	0.8	0.2	4 : 1
Sugar	[g kg_{FM}^{-1}]	0.01	0.17	1 : 19
Crude fat	[g kg_{FM}^{-1}]	1.0×10^{-4}	5.3×10^{-6}	20 : 1
Crude protein	[g kg_{FM}^{-1}]	20	7	3 : 1
TKN	[g kg_{FM}^{-1}]	3.2	1.3	2.5 : 1
NH_4	[g kg_{FM}^{-1}]	0.06	0.11	1 : 2
Ethanol	[g kg_{FM}^{-1}]	2	24	1 : 12
Acetate	[g kg_{FM}^{-1}]	5	10	1 : 2

TS=total solids, FM=fresh mass, VS=volatile solids, TKN = total Kjeldahl nitrogen, NH4+-N = ammonium nitrogen

The complex compounds have to undergo all four process phases before biogas is produced, whereby the hydrolysis is considered to be the rate-limiting step (Pavlostathis and Giraldo-Gomez, 1991). In contrast to that, the compounds of the sugar beet silage can more or less be directly converted into biogas. For each feedstock, three parallel continuously stirred tank reactors (CSTRs) with a working volume of 3 l were operated at mesophilic conditions (40°C). The mentioned differences in the chemical composition of the feedstocks are reflected in the kinetics of the biogas production (Figure 2-1).

Shortly after feeding, the easy degradable com- pounds were degraded resulting in the maximum biogas production in both reactor systems, whereby the sugar beet reactors yielded higher biogas production rates than the maize reactors.

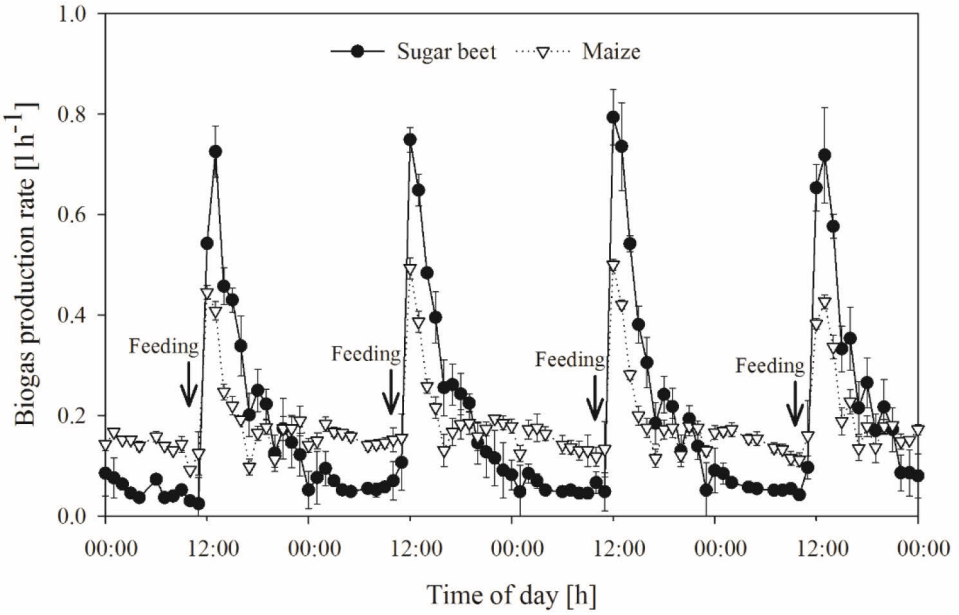

Figure 2-1: Highly temporal resolution of the kinetics of the biogas production rates over four days at OLR 2.0 g_{VS} L^{-1} d^{-1} for both the anaerobic digestion of maize silage and sugar beet silage. Shown are mean values including the standard deviation of the three parallel reactors per feedstock.

After this first consumption/conversion phase, only more complex compounds such as starch, hemicellulose and cellulose were available for microbial degradation. As the maize silage was characterized by a higher amount of these compounds, here the biogas production was higher over time, whereas only a low biogas production was found in the sugar beet reactors around 12 h after feedstock addition. Nevertheless, the mean biogas yield was equal for both feedstocks with 0.64 ± 0.02 l_N g_{VS}^{-1} day^{-1} (with 51% CH_4) for maize silage and 0.67 ± 0.01 $l_N g_{VS}^{-1}$ day^{-1} (with 54% CH_4) for sugar beet silage, which is in agreement with substrate-specific biogas yields previously published by (KTBL, 2009).

2.3.2 Reactor performance and process efficiency

All reactors were inoculated with digestate from an agricultural biogas plant feed with a mixture of different energy crops and animal manure. Compared with the inoculum, the results showed a decrease in the TS and volatile solids (VS) of the digestates for both systems after the changeover from the feedstock mixture to the sole substrates maize and sugar beet silage (Table S1). During the subsequent experimental phase, an enrichment of the TS and VS was recorded in the maize reactor systems, which was accompanied with a decrease in the degradation degree of VS from 90 ± 0.1% at day 33 to 78 ± 0.3% at day 337. In contrast, the degradation degree of VS in the sugar beet system was rather constant over the entire trail

period with 87 ± 0.6%. Also these differences may be explained by the differences in the chemical composition of the feedstocks as the lower degradation degree in the maize reactors is caused by the high amount of complex compounds of the supplied feed- stock. The hydrolyzable compounds hemicellulose and cellulose of the maize plant material are protected from biodegradation as the anaerobically non-degradable lignin is forming a matrix that surrounds the (hemi-) cellulose microfibrils (Kirk and Farrell, 1987; Ress et al., 1998) resulting in a diminished biodegradability and hence a lower degradation rate. However, the overall degradation degree was slightly higher, but in a general agreement with practical experiences where the mean VS degradation degree is reported with 76% (FNR, 2010). It can be assumed that the overall process proceeded efficiently and that there was a steady state between acid production and consumption as no VFA accumulation was recorded (Table S1).

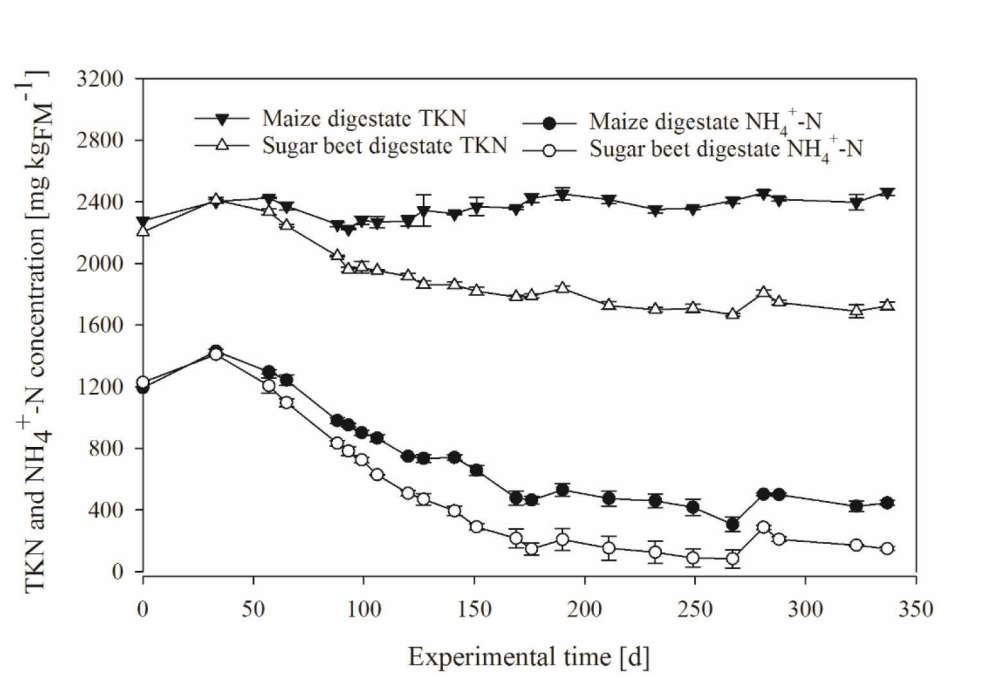

Figure 2-2: Total Kjeldahl nitrogen (TKN) and ammonium nitrogen (ammonium-N, NH_4^+-N) concentration of the maize reactor digestate (MD) as well as the sugar beet reactor digestate (SBD) over the entire experimental time as mean values including standard deviation of the three parallel reactors per feedstock.

The total Kjeldahl nitrogen (TKN) was rather constant in the maize reactors, but decreased in the sugar beet reactors until around day 141. These findings indicate that the sugar beet reactors required more time to reach stationary conditions caused by the generally lower TKN in the sugar beet silage (Figure 2-2). On the other hand, the NH_4^+-N concentration constantly decreased in both reactor systems (Figure 2-2). Considering that no TKN was accumulated, this indicates that only low protein degradation took place.

It can be assumed that the present microorganisms utilized the more easy accessible carbon compounds as primary energy source whereby the proteins were only degraded into amino acids, which were used for their cell growth but not mineralized to ammonium nitrogen. Several studies confirmed that the protein degradation capacity decreased when high amounts of sugar are present (Breure et al., 1986; Tommaso et al., 2003). This seems to be the case especially in the sugar beet reactors.

2.3.3 Long-time adaptation of the bacterial communities to different feedstocks

For each feedstock digestion experiment, three parallel CSTRs were operated either with maize silage or sugar beet silage. The TRFLP results are given as median values of the three parallel operated CSTRs per feedstock digestion experiment. To ensure a high functionality of the initial starter-community (Wittebolle et al., 2009), the inoculum used for the start-up consisted of a microbial community specifically adapted to the digestion of a diverse mixture of energy crops and animal manure.

The results of this study showed that the bacterial community structure is directly influenced by the supplied feedstocks, as an adaptation of the bacterial starter-community was found already at day 33, the end of the first organic loading rate (OLR) stage (Figure 2-3). The calculated pairwise distance between these two sampling points (“inoculum” and “day 33”), considering both the changes in the number as well as the relative abundance of each detected terminal restriction fragment (TRF), showed a change in the community structure of 49% in the maize reactors and 64% in the sugar beet reactors.

The community organization became more even (

Table S2), meaning that the bacterial community members became more equally distributed in their relative abundance (Marzorati et al., 2008; Read et al., 2011; Verstraete et al., 2007). The three most abundant TRFs from the starter-community (TRF-150bp, TRF-163bp and TRF- 180bp) decreased while for example TRF-224bp (family *Ruminococcaceae*) in the maize reactors or TRF-84bp and TRF-93bp (both related to the family *Porphyromonadaceaea*) in the sugar beet reactors gained importance (Figure 2-3, Table 2-2)

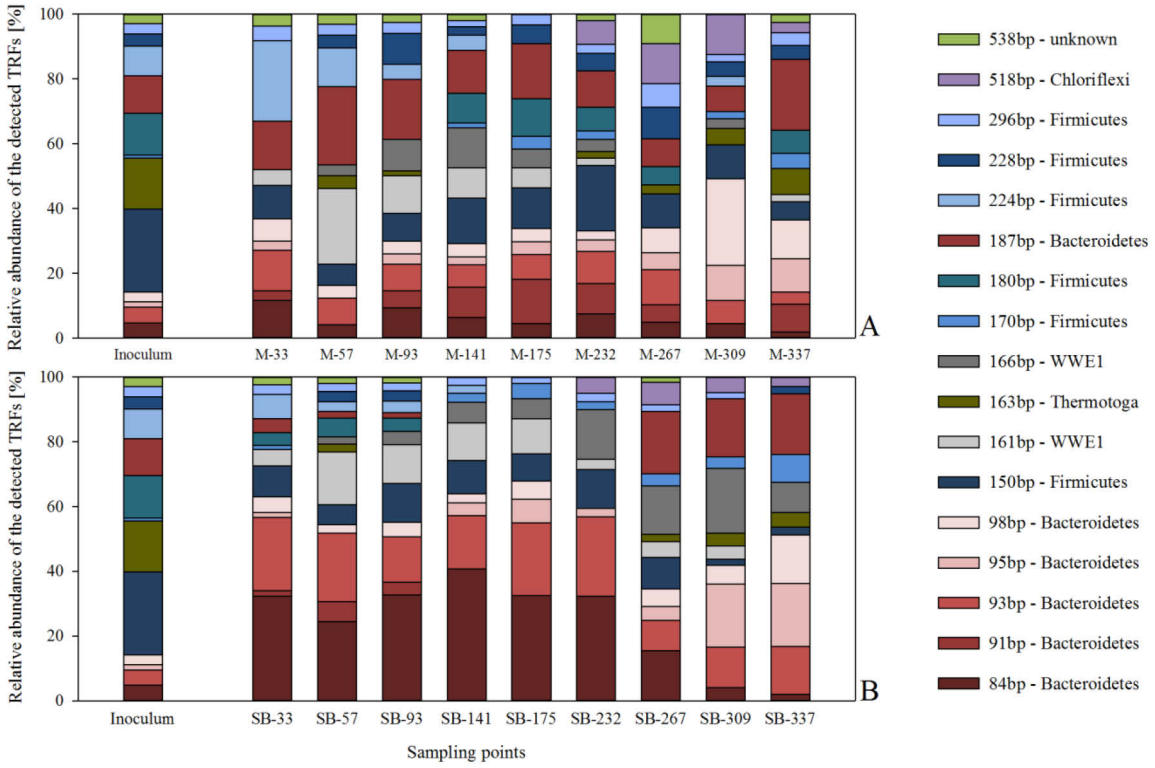

Figure 2-3: Structure of the bacterial community involved in the biomethanation process of the maize silage (A) and sugar beet silage (B). Shown is the relative abundance of the detected terminal restriction fragments (TRFs) as a function of the percental fluorescence intensity of each individual TRF in relation to the total fluorescence intensity. Colored bars symbolize TRFs in base pairs (bp) which were identified by 16S rRNA gene sequence libraries. Only TRFs with a relative abundance over 5 % in at least one sample are shown. Each sampling point is given as median value of three biological replicates (parallel reactors) and three technical replicates (three DNA extracts per reactor).

Table 2-2: Phylogenetic assignment of the detected bacterial TRFs by screening of 16S rRNA gene sequence libraries using the RDP Classifier as well as selected species examples from the identified families and their physiological potential to identify the functionality of the dominant groups within the maize and sugar beet reactors.

Phylogenetic assignment [phylum, class, order, family]	**Species examples and their potential function within the AD process chain**		**Reference**
Bacteroidetes, Bacteroidia, Bacteroidales, Porphymonadaceae (TRF-84bp, TRF-93bp, TRF-95bp)	*Palidibacter propionicigenes*	Sugar fermentation	Ueki et al. (2006)
	Petrimonas sulfuriphila	Mono- and disaccharid fermentation	Grabowski et al. (2005)
	Proteiniphilum acetatigenes	Protein degradation	Chen and Dong (2005)
Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae (TRF-98bp)	*Prevotella ruminicola*	Utilisation of starch, non-cellulosic polysaccharides, and simple sugars	Purushe et al. (2010)
Fibrobacteres, Fibrobacteria, Fibrobacterales, Fibrobacteraceae (TRF-150bp)	*Fibrobacter succinogenes*	Only cellulose is hydrolysed and metabolised; removes xylose-rich hemicelluloses to gain access to cellulose	Suen et al. (2011)
Firmicutes, Clostridia, Clostridiales, Ruminococcaceae (TRF-224bp)	*Saccharofermentans acetigenes*	Utilisation of starch, non-cellulosic polysaccharides, and simple sugars	Chen et al. (2010)
	Clostridium sufflavum	Degradation of mono- and disaccharides, xylane and cellulose, but no starch	Nishiyama et al. (2009)
	Ruminococcus albus	Highly cellulolytic, degrade cellulose and hemicellulose	Suen et al. (2011)
Firmicutes, Clostridia, Clostridiales, Lachnospiraceae (TRF-296bp)	*Cellulosilyticum ruminicola*	Degradation of cellulose, hemicellulose and pectin	Cai et al. (2010)
	Lachnospira pectinoschiza	Pectin degradation	Cornick et al. (1994)
	Clostridium xylanovorans	Mono- and disaccharid fermentation, non-cellulolytic, non-acido./acetogenic	Mechichi et al. (1999)
Chloriflexi, Anaerolineae, Anaerolineales, Anaerolineaceae (TRF-518bp)	*Levilinea saccharolytica*	Utilisation of monosaccharides, peptides and amminoacids and pyruvate	Yamada et al. (2006)
	Longilinea arvoryzae	Fermentation of divers carbohydrates (incl. hemicellulose) and proteins; enhanced growth in co-cultivation with hydrogenotrophic methanogens	Yamada et al. (2007)
WWE1 candidate division	*"*Candidatus *Cloacamonas acidaminovorans"*	Fermentation of amino acids, sugars, and carboxylic acids; synthrophic	Pelletier et al. (2008)
Unknown *Bacteroidetes* (TRF-91bp, TRF-187bp) Unknown *Firmicutes* (TRF-150bp, TRF-170bp, TRF-180bp) Unknown *Thermotogae* (TRF-163bp)	No functional relation possible		
Unknown *Bacteria* (TRF-161bp, TRF-228bp, TRF-538bp)	No phylogenetic and no functional relation possible		

In comparison with the more even (Gini coefficient of 0.42 ± 0.01) but highly diverse (richness of 48 ± 8) and dynamic bacterial community of the maize reactors (

Table S2, Figure 2-3A), the bacterial community in the sugar beet reactors remained rather stable as TRF-84bp and TRF-93bp were predominant with a relative abundance between 15–28% and 9–17% until day 232 (

Table S2, Figure 2-3B). As the NH_4^+-N concentration in the reactors reached the minimum values between day 232 and 267 a community change (dissimilarity) of around 30% was recorded by the calculated pairwise distance between these two sampling points, resulting in a community re-organization: in the maize system, TRF-98bp (family *Prevotellaceae*, phylum *Bacteroidetes*) was predominant with an abundance of 17% followed by TRF-518bp (family *Anaerolineaceae*, phylum *Chloriflexi*), TRF-95bp (family *Porphyromonadaceae*, phylum *Bacteroidetes*) and TRF-150bp (class Clostridia, phylum *Firmicutes*) with 7–8%. In contrast to that, the former most dominant TRF in the sugar beet reactors (TRF-84bp) decrease to less than 3%, while TRF-187bp (order *Bacteriodales*), TRF-166bp (WWE1 candidate division), TRF-98bp and TRF-95bp gained importance each with up to 12%.

To conclude, these findings showed that the present bacterial community in the maize reactors consisted of a more even distribution of different phyla, whereby the sugar beet reactors were dominated by members of the phylum *Bacteroidetes* with abundances of up to 47%. In contrast to the sugar beet silage, which showed a very narrow substrate spectrum, the digestion of maize silage provided a wide range of metabolites whereby each requires a specific conversion pathway. As the bacterial community in the maize reactors has to be able to perform a successive and complementary biomass conversion with a functional redundancy among diverse phylogenetic groups (Table 2-2), it is not surprising that the maize reactor systems showed a higher bacterial diversity and higher dynamic variations over time then the sugar beet reactor systems. The high abundance of *Bacteroidetes* within the sugar beet reactors indicates that these microorganisms hold an important part as secondary degrader in the second and third step of the biomass conversion chain, which is in accordance with previously published studies (Hanreich et al., 2013; Ito et al., 2012).

2.3.4 Long-time adaptation of the archaeal communities to different feedstocks

As expected, the archaeal population structure revealed a lower diversity than the bacterial one in both reactor systems with a richness of 6 ± 2 and 7 ± 2 TRFs in the maize and sugar beet system, compared with 48 ± 8 and 41 ± 9 TRFs in the bacterial community (

Table S2). These findings have previously been reported several times, e.g. by Carballa et al. (2011), Liu et al. (2009) and Regueiro et al. (2012). This is also in agreement with the fact that *Bacteria* are involved in the first three steps of biomass transformation with a high variety of substrates whereby *Archaea* are restricted to a very narrow nutrient spectrum in terms of acetate, methyl- group containing compounds as well as CO_2 and H_2.

Similar to the bacterial community, the results showed an adaptation of the archaeal starter-community to the supplied feedstocks already at day 33 (Figure 2-4): the inoculum was dominated by hydrogenotrophic *Archaea* (represented by TRF-338bp/*Methanobacterium* sp. and TRF-428bp/*Methanoculleus* sp.), caused by the rather high VFA and NH_4^+-N concentrations, which are known to inhibit the obligate acetoclastic genus *Methanosaeta* (Karakashev et al., 2005; Schnürer et al., 1999).

After 33 days of reactor performance, the hydrogeonotrophic methanogens were replaced by members of the most versatile (mixotrophic) and stress-tolerant methanogenic family *Methanosarcinaceae* (De Vrieze et al., 2012; Liu and Whitman, 2008), more precisely by the genus *Methanosarcina* (represented by TRF-625bp and TRF- 627bp), which were under the detection limit within the starter-community. This evident shift in the archaeal community indicates that the reactors, or better, the occurring communities, had to deal with a sudden stress, the feedstock change, and adapted to the new conditions (De Vrieze et al., 2012).

From day 33 to day 57, the calculated dissimilarity values still showed high structural changes between the communities. The changes decreased evidently over time until the community shift reached 1% in the maize system and 5% in the sugar beet system between day 93 and 141, meaning that the archeal community structure became more and more similar over time. During the start-up phase, which was accompanied by a decrease in the NH_4^+-N level (Figure 2-2), the genus *Methanosaeta*, represented by the TRF-108bp, became predominant with 86% of the total archaeal community in the maize reactors, whereas the abundance of the genus *Methanoculleus* decreased. This can be explained as the inhibitory effect of high NH_4^+-N concentration on the acetoclastic methanogenes pathway (e.g. Fotidis et al., 2014; Schnürer

and Nordberg, 2008) was successively reduced over time. Interestingly and first described in this study, a further decrease of the NH_4^+-N concentration promoted the re-occurrence of the genus *Methanoculleus* (between day 175 and 267, Figure 2-4A). After day 267 until the end of the experimental phase, the NH_4^+-N concentration in the maize reactors was kept rather constant at around 450 mg kg_{FM}^{-1}. Consequently, the *Methanoculleus*-related TRF-428bp completely disappeared, and finally, the archaeal community of the maize reactor was clearly dominated by the obligate acetoclastic genus *Methanosaeta* (TRF-108bp, 87%). Thus, the results revealed an antagonistic behaviour between the genera *Methanosaeta* (symbolizes by TRF-108bp) and *Methanoculleus* (TRF-428bp), in the maize reactor systems (Figure 2-5A).

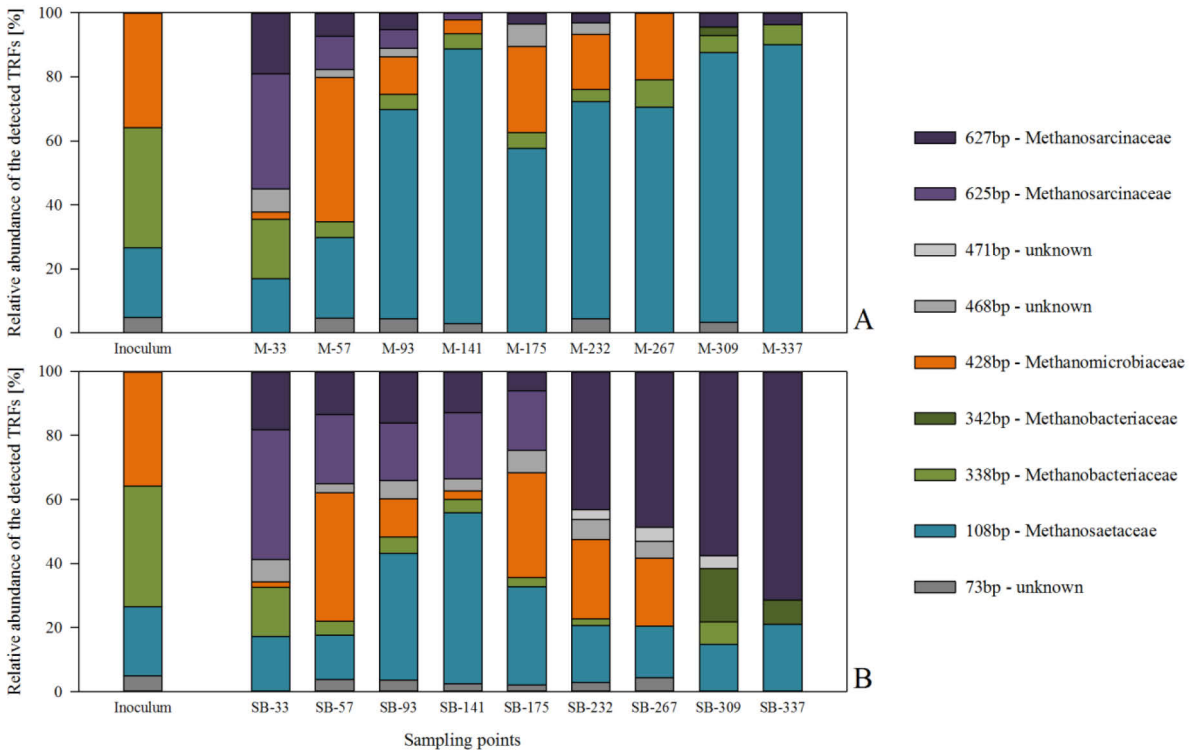

Figure 2-4: Structure of the archaeal community involved in the biomethanation process of the maize silage (A) and sugar beet silage (B). Shown is the relative abundance of the detected terminal restriction fragments (TRFs) as a function of the percental fluorescence intensity of each individual TRF in relation to the total fluorescence intensity. Colored bars symbolize TRFs in base pairs (bp) which were identified by 16S rRNA gene sequence libraries. Each sampling point is given as median value of three biological replicates (parallel reactors) and three technical replicates (three DNA extracts per reactor).

Apparently, species of the genus *Methanosaeta* are highly sensitive against sudden stress situations, whereby *Methanoculleus* seems to be more robust. Moreover, it can be supposed that the decreasing and especially the low NH_4^+-N concentration influence not only the archaeal community composition, but also the bacterial one. This in turn may lead to the

production of metabolites, which favour the presence of *Methanoculleus*. For example, between day 175 and 267, members from family *Anaerolineacea* (phylum *Cloriflexi*) became abundant (Figure 2-3A, Table 2-2). It is known that the growth for example of the genus *Longilinea* is enhanced in co-culture with hydrogenotrophic methanogens (Yamada et al., 2007). Nevertheless, after keeping the NH_4^+-N concentration rather constant to avoid a process failure, the genus *Methanosaeta* is dominating the archaeal community again.

Similar results were found for the sugar beet reactors, although the relative abundance of both genera *Methanosaeta* and *Methanoculleus* were lower compared with the maize reactors (Figure 2-4B). A significant antagonistic behaviour between *Methanosaeta* and *Methanoculleus*, which is already discussed above, was recorded until day 232 (Figure 2-5B). Afterwards, the high abundance of *Methanosarcina* overlapped or more precisely mitigated this correlation.

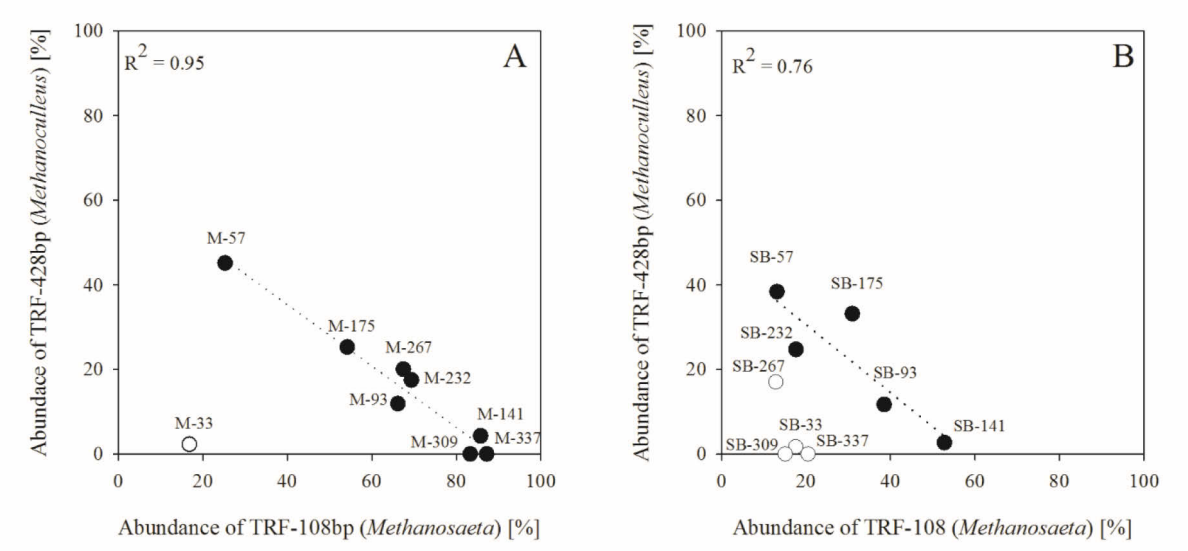

Figure 2-5: Correlation between the abundance of TRF-428bp (related to *Methanoculleus*) and TRF-108bp (related to *Methanosaeta*) in the maize reactors (A) and the sugar beet reactors (B). Only sample indicated by a full black dot were considered for correlation. R^2 is the correlation coefficient.

Finally and in contrast to the maize reactor systems where *Methanosaeta* became predominant after stabilizing the NH_4^+-N concentration, *Methanosarcina* (TRF-627bp) prevailed in the sugar beet reactor at a NH_4^+-N concentration around 200 mg kg_{FM}^{-1}. In regard to the general high abundance of *Methanosarcina* in the sugar beet reactors, it has to be mentioned that the VFA concentration and the pH value were measured shortly before feeding; hence, it can be assumed that the rapid utilization of the easy degradable compounds in the sugar beet silage led to an increased VFA concentration, followed by a drop in the pH values shortly

after feeding. Consequently, the higher occurrence of *Methanosarcina* compared with *Methanosaeta* might be explained by their lower sensitivity to a drop in pH due to their spherical form and thus higher volume-to-surface ratio as well as their growth in cell clusters, which altogether limit the intake of acetate in the archaeal cell (De Vrieze et al., 2012).

2.3.5 Conclusions

To conclude, this study provided interesting insights into the highly dynamic variation of the microbial communities, which converted maize and sugar beet silage into methane-containing biogas. According to the chemical differences of the supplied feedstocks, both the bacterial and the archaeal communities showed a clear substrate adaptation resulting in a feedstock-specific kinetic of the biogas production. It was demonstrated that members from the phylum *Bacteroidetes* are mainly involved in the degradation of low molecular weight substances such as sugar, ethanol and acetate, the main compounds of the sugar beet silage. In contrast to this, the structural and functional broader phylum *Firmicutes* were found in a higher abundance in the maize reactors due to their capacity of degrading complex polymers, especially cellulose. For both investigated systems, it was further shown that *Methanosaeta*, an indicator for a good-performing process, is highly sensitive against sudden stress situations such as a strong decrease in the NH_4^+-N concentration or a drop in the pH value. In both cases, the overall process did not failed, but rather was compensated by members from the genera *Methanoculleus* and/or *Methanosarcina*. All in all, the results of this study showed that maize and sugar beet silage are suitable feedstocks for AD as the biogas production process resulted in an equal amount of methane-containing biogas although the biogas was produced by different, highly feedstock-adapted microbial communities. Nevertheless, it has to be considered, especially by the biogas plant operators, that the AD of sugar beet silage seems to be more susceptible to stress. Therefore, it is advisable to ensure a high structural and functional microbial diversity, especially when a new biogas plant is started.

2.4 Experimental procedures

2.4.1 Reactor construction and operation

Laboratory-scale CSTRs were used to investigate the adaptation, the efficiency and the dynamics of process relevant microorganisms during the AD of maize silage and sugar beet silage. For each feedstock, three parallel CSTRs with a working volume of 3 l were operated at mesophilic conditions (40°C). To ensure a high diversity of the starter-community, all reactors were inoculated with digestate from a mesophilic agricultural biogas plant operated with mixed manure, grass and maize silage. This digestate was diluted 1:2 with tap water before inoculation. The reactors were started with an OLR of 0.5 $g_{VS} l^{-1}$ day^{-1} and slowly, after a minimum of 2 weeks, increased with 0.5 g_{VS} $l^{-1} day^{-1}$ until a final OLR of 2g_{VS} l^{-1} day^{-1} was reached (VDI-4630, 2006). A correction of the TS for the calculation of the OLR was performed according to Weissbach and Strubelt (2008a, 2008b). To avoid process inhibition through lack of micronutrients, 10 µl g_{VS}^{-1} of the trace elements solution DSMZ 144 were added to each feeding according to Elhussein and Weiland (2009). Differences in the volume flow were balanced with tap water to gain the same hydraulic retention time (HRT) in all reactors. After day 267, the NH_4^+-N concentration was kept at a stable level of around 450 mg l^{-1} in the maize reactors and 200 mg l^{-1} in the sugar beet reactors. To calculate the required amount of NH_4^+-N, the digestate was analysed twice a week, and the difference between the actual and the desired value was added as ammonium carbonate (powder) with the feeding the day after the analysis.

The amount of produced biogas was measured with a novel gas measurement system, which enables a high temporal resolution (Tölle and Huth, 2014). Additionally, the biogas was collected in gasbags to measure the gas composition twice a week using the portable analyser "Biogas Check".

Following chemical measurements and analyses of the feedstock and reactor digestate were performed according to VDLUFA (1997): pH, TS, VS, VFAs in terms of acetate, propionate, iso- and n-butyrate, iso- and n-valerate and capronate, alcohols, TKN and NH_4^+-N. Additionally, neutral detergent fibre, acid detergent fibre and acid detergent lignin were determined in order to calculate the amount of cellulose, hemicellulose and lignin of the feedstock as well as sugar and starch (Schönberg and Linke, 2012; VDLUFA, 1997)

2.4.2 Sampling and extraction of microbial DNA

During the start-up phase of the reactors, digestate samples were taken at the end of each OLR stage (day 33, 57 and 93). With achieving the final OLR, six further time points were selected for molecular biological analysis (day 141, 175, 232, 267, 309 and 337). The digestate samples were stored at −20°C until further analysis.

Total microbial genomic DNA was extracted using the PowerSoil®DNA Isolation Kit (MoBio Laboratories, USA). The DNA isolation was carried out according to the user's manual except for the mechanical lysis, which was performed using the FastPrep® instrument (MP Biomedical, USA) for 2 × 20 s at 5ms−1. For each sample, DNA from three subsamples was extracted. The extracted DNA was used as template for the TRFLP analyses as well as for the construction of 16S rRNA gene sequence libraries to characterize the diversity and dynamics of the bacterial and archaeal communities.

2.4.3 Analyses of the microbial community dynamics by TRFLP

To detect the microbial community dynamics of the reactors, the fingerprint method TRFLP was used as previously described by (Rademacher et al. (2012). For the amplification of the 16S rRNA gene, the bacterial primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 926MRr (5'- CCGTCAATTCMTTTRAGTTT-3') and the archaeal primer pair Ar109f (5′-ACKGCTCAGTAACACGT-3') and Ar912r (5'- CTCCCCCGCCAATTCC-TTTA-3') were used. For both the bacterial and the archaeal amplifications, the forward primer was labelled with Indodicarbocyanine (Cy5) at the 5′-end. All used primers were provided by Biomers (Germany). After verification of the PCR reaction in a 1.2% agarose gel, the PCR products were purified using Nucleospin®Gel and PCR Clean-up kit by Machery Nagel (Germany). The concentration of the purified PCR products was measured using a NanoPhotometer (Implen, Germany). A total amount of 200 ng PCR product was digested for 16 h at 37°C with MspI and Hin6I for the bacterial assay and with AluI for the archaeal assay. All enzymes were provided by Thermo Fisher Scientific Bioscience, Fermentas (Germany). The digested fragments (TRFs) were separated using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Germany) as described in (Rademacher et al. (2012) to obtain TRFLP profiles.

The further analysis and comparison of the TRFLP profiles was conducted using BioNumerics 7.1 (Applied Maths, Belgium). The TRFLP profiles of each sampling point

were evaluated separately in the fingerprint curve-processing window. Peaks lower than 185 relative fluorescent units were considered as “background noise”. False positive peaks were sorted out using the bleed throw detection. In the comparison window, a band matching was performed with a position tolerance of 0.1%. The band matching of the detected TRFs was exported to MS Excel and normalized to relative values of the total fluorescence. The median values of nine technical replicates for each feedstock digestion experiment were calculated, reloaded as character types into BioNumerics and subsequently used to calculate the pairwise distances between two sampling points applying the unweighted pair group method with arithmetic mean (UPGMA) algorithm with Pearson correlation. Pairwise distances were given as percentage of dissimilarity between microbial community profiles indicating the rate of community change. By this approach, the dissimilarity can be influenced by the number as well as the relative abundance of the detected TRFs.

2.4.4 Identification of detected TRFs by construction and screening of 16S rRNA gene sequence libraries

For the identification of the detected TRFs, a cloning/sequencing approach was applied. The PCR amplification of the 16S rRNA gene was conducted using the same primer pairs (in this case unlabelled) and PCR conditions as mentioned above. The PCR products were purified using the Nucleospin®Gel and PCR Clean-up kit by Machery Nagel (Germany). Cloning of 16S rRNA gene amplicons was per- formed according to (Rademacher et al. (2012). DNA sequencing was conducted by GATC Biotech AG (Germany).

The obtained sequences were processed using the software package BioNumerics 7.1 (Applied Maths, Belgium). After a quality check of the sequences, a multiple alignment was applied using default settings for the Needleman–Wunsch algorithm with a CLUSTW similarity calculation in combination with an UPGMA clustering using the Kimura-2-parameter correction. Based on this, alignment sequences were grouped into operational taxonomic units (OTUs) at 97% (*Bacteria*) and 99% (*Archaea*) sequence similarity required for the identification at the species level (Kim et al., 2011). All OTUs obtained in this study have been deposited to the European Molecular Biology Laboratory and are available under accession numbers LN624228-LN624323 (*Bacteria*) and LN624324-LN624341 (*Archaea*). Subsequently, OTUs were identified using the RDP Naïve Bayesian rRNA Classifier Version 2.6 (Wang et al., 2007). Reference sequences with a homology of at least 80%, which is required for the sequence correlation at the phylum level (Kim et al., 2011; Talbot et al.,

2008), were selected for a phylogenetic assignment of the defined OTUs from the microbial community. Additionally, the defined OTUs were cut virtually using the restriction digest tool of BioNumerics 7.1 to assign the detected TRFs of the reactor samples.

2.4.5 Ecological indices

To gain a better understanding of the ecological role of the investigated microbial community, various ecological indices were applied, which mainly based on the microbial resource management concept (Marzorati et al., 2008; Read et al., 2011; Verstraete et al., 2007). The richness (R) was deter- mined as the total number of detected TRFs. Additionally, we defined the Lorenz curve and the derived Gini coefficient for each sample, which is related to information about the community organization (Marzorati et al., 2008; Verstraete et al., 2007; Wittebolle et al., 2009). The higher the Gini coefficient, the more uneven is the community

3 Nexus between the microbial diversity level and the stress tolerance within the biogas process

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3.1 Abstract

To investigate whether there is a nexus between the microbial diversity level (taxonomic, functional and ecological) and the stress tolerance potential of the microbial community, the impact of different ammonium sources was evaluated. Therefore reactors adapted either to the anaerobic digestions of sugar beet silage or maize silage (SBS/MS) were supplemented with animal manure (M) or ammonium carbonate (A).

The results showed that increasing concentrations of total ammonium nitrogen (TAN) were not the only reason for community changes: the bacterial community in the reactors given animal manure became more similar over time compared to the reactors given ammonium carbonate. However, this study revealed that a bacterial community with a few dominant members led to a functional more flexible archaeal community (SBS reactors) which was more stress resistant under the experimental conditions. This indicates that a higher functional diversity within a certain part of the community, in the present study the archaeal community, is one important factor for process stability due to a higher tolerance to increasing amounts of

process-inhibiting metabolites such as TAN. Compared to this a bacterial community with higher amount of more evenly distributed community members combined with a more rigid archaeal community (MS reactors) showed a lower stress tolerance potential.

Moreover it was observed that the disappearance of members of the phylum *Cloacimonetes* can be used as an indicator for an upcoming process disturbance due to increasing TAN concentrations.

3.2 Introduction

The production of biogas by anaerobic digestion of biomass has become common practice because of its many advantages over other energy sources. Biogas production is independent of daily, seasonal and weather-related fluctuations and can therefore be used to secure basic energy supply. Biogas is suitable for the production of electricity and heat and it can be used as a substitute for fossil resources. A broad variety of biomasses can be used for anaerobic digestion, including energy crops, agricultural residues as well as municipal and industrial wastes. Therefore the production of biogas is important as it can be an essential part of sustainable concepts by integrating it into agricultural production systems.

Especially in Germany the use of energy crops and animal waste is common, whereby maize silage counts for the largest part because of its high yield (FNR, 2016). Lately the importance of residual materials, such as animal waste, has increased because of a changed legislation (German Renewable Energy Sources Act 2014 and 2016/2017). This legislation changed mostly due to the conflicts concerning the use of field area for energy crops in competition with food production. Moreover an increased use of residue material is intended to promote a sustainable circular economy. However, this change leads to a more heterogenic feedstock composition as well as a more dynamic feedstock supply. This is related to several challenges such as the need for new pre-treatment procedures, innovative reactor components and especially more resilient microbial communities.

For the production of biogas a close relationship between the process-involved microorganisms (*Bacteria* and *Archaea*) with different physiological capacities is required as they depend on each other's degradation products for survival. Hence the chemical

composition of the used feedstock is of great importance as it affects the development of different, in some cases feedstock/ substrate-specific, microbial communities (Alsouleman et al., 2016; Klang et al., 2015; Theuerl et al., 2018; Zhang et al., 2014).

Within a previous study by Klang et al. (2015) the microbial community development was investigated over time in order to follow the adaptation to the anaerobic digestion of sugar beet silage (SBS) or maize silage (MS) as sole feedstock. These two feedstocks differed in both their nutrient composition as well as in their chemical complexity. While SBS contains higher amounts of easy degradable compounds such as sugar and alcohols, MS with high amounts of cellulose has to be hydrolysed before the nutrients are accessible. The microbial communities in the SBS reactors were dominated by members of the phylum *Bacteroidetes*, whereas the bacterial community adapted to the digestion of MS showed a higher diversity and a more evenly distributed relative abundance of members from various phyla. Also the archaeal communities differed: the SBS reactors showed a combination of hydrogenotrophic and acetoclastic methanogens, whereas the reactors given MS were dominated by members of the genus *Methanothrix* (obligate acetoclastic).

Considering this, a feedstock change should be applied with caution due to the sensitivity of the microbial community to changing environmental conditions, which might lead to process failure and consequently to economic losses for the biogas plants operators (Alsouleman et al., 2016; Klang et al., 2015; Niu et al., 2015; Theuerl et al., 2015).

The change from an energy crop dominated feedstock regime to an operation based on residues, especially animal waste can be related to the risk of process inhibition due to increasing amounts of total ammonium nitrogen (TAN). TAN is a degradation product of nitrogen-rich compounds, such as proteins, nucleic acids and urea or uric acids, which are present in high amounts in manure, especially from chicken, but also for example in grass silage, slaughterhouse waste or kitchen waste (Kovács et al., 2015; Niu et al., 2015). Approximately 1.2 million tons of chicken manure are produced in Germany every year, of which about 97% are used as fertilizer on arable land (Statistisches Bundesamt, 2016). But considering that the theoretically methane potential of chicken manure in Germany is about 125 million m^3 and hence resulting in an electricity capacity for approximately 148.000 two-person households, chicken manure is a valuable feedstock for biogas production. However, the high amount of nitrogen in the chicken manure involves the risk of process inhibition as mentioned before. Several studies have dealt with the effect of increasing TAN

concentrations, as reviewed for example in Rajagopal et al. (2013) and Westerholm et al. (2016). According to the current state of knowledge free ammonia (NH_3) is assumed to be responsible for the inhibition since it diffuses passively into the cell causing proton imbalance (Chen et al., 2008; Rajagopal et al., 2013). The equilibrium between NH_3 and ammonium (NH_4^+) depends on the pH-value and temperature of the process; the higher these values are the larger is the amount of the putative toxic NH_3. It is well known that elevated TAN concentrations can negatively affect the production of biogas due to an inhibition of methanogenic archaea, whereby the obligate acetoclastic genus *Methanothrix* is the most sensitive one (De Vrieze et al., 2012). An inhibition of the obligate acetoclastic *Methanothrix* leads to an acetic acid accumulation as long as acetic acid is not converted into biogas by syntrophic acetic acid oxidation (Westerholm et al., 2016). An accumulation of other organic acids, such as propionic acids, indicates a disturbance in the inter species hydrogen transfer, hence in the syntrophic relationship between hydrogen producers and hydrogen scavengers (Goux et al., 2015; Hori et al., 2015; Leng et al., 2018). Hence, the accumulation of organic acids is an indication of an imbalance between acid producers and acid consumers (Goux et al., 2015; Hori et al., 2015; Li et al., 2015; Regueiro et al., 2015) which means that the formation of a metabolic intermediate, here the produced organic acids, exceeds the degrading capacity of the microorganisms involved in the subsequent process step.

However, it has also been shown, that the microbial community is able to adapt to higher TAN concentrations (Alsouleman et al., 2016; Niu et al., 2015). Nevertheless, the underlying process, especially the microbial response is still not completely understood and hence the handling of these feedstocks is still critical and requires further research.

In this study the genetic fingerprinting method Terminal Restricted Fragment Length Polymorphism (TRFLP) (De Vrieze et al., 2018; Liu et al., 1997) was used to investigate changes in lab-scale reactors adapted to either SBS or MS as these feedstocks were gradually exchanged with animal manure (M). A cloning/sequencing approach was used to taxonomically identify the obtained terminal restriction fragments (TRFs). The question to answer was whether the ammonium nitrogen compounds derived from the animal manure were the main reason for a changed microbial community structure. Therefore a second reactor was given ammonium carbonate (A) so that both reactors were operated at an equal, increasing TAN concentration in order to evaluate whether the communities developed similar or different over the course of time. Moreover, as the two highly adapted microbial communities (SBS and MS) differed distinctly in their structural composition and hence in

their functionality (Klang et al., 2015) a further question of this study was whether there is any nexus between the microbial diversity level and stress tolerance potential within the biogas process.

3.3 Material and Methods

3.3.1 Biogas fermenter operation and gas measurement

Six continuously stirred tank reactors (CSTR) with a working volume of 3 L were operated at mesophilic (40 °C) conditions. Prior to this study three of the fermenter had been operated either with SBS or MS as sole feedstock for approximately one year as described in Klang et al. (2015). Here all reactors were fed once a day and operated with an organic loading rate (OLR) of 2.0 $g_{vs}\ L^{-1}d^{-1}$ with a hydraulic retention time (HRT) of 86 days. In this study the HRT was shortened to 43 days due to the partly low total solids (TS) of the used animal manure. One fermenter of each fermenter system was operated equally to the first phase throughout the whole experiment as control (C), either with SBS or MS as a single feedstock. The TAN concentration in these two reactors was regulated to 0.2 resp. 0.4 g L^{-1} twice a week through addition of ammonium carbonate in order to avoid a nutrient limitation. One fermenter of each fermenter system was given increasing amounts of animal manure (SBS-M, MS-M). The manipulation was started through an exchange of 10% of the silages with swine manure followed by gradually exchange of silages with 10%, 30%, 50% and 75% chicken manure, based on the VS, over time. The length of each period was chosen based on the measured reactor parameters, especially on the stagnation of the increasing TAN concentrations. The third reactors were fed with SBS or MS and the TAN was increased to similar concentrations as in the M reactors through addition of ammonium carbonate (SBS-A, MS-A). This was made in order to elucidate whether the ammonium nitrogen compounds derived from the manure were the main reason for the expected microbial community change or if for example other compounds of manure, the microbial community present in the manure or intermediates produced during digestion influenced the community development. The reason for choosing ammonium carbonate was related to the fact that ammonium carbonate is slightly alkaline and acts as a buffer, so that a stabilizing or even an increase of the pH value could be expected in both reactors systems.

The produced biogas was collected in gasbags (Tesseraux Spezialverpackungen GmbH, Germany) and analysed at least twice a week. The biogas composition was analysed using the portable analyser "BiogasCheck" (Geotechnical Instruments Ltd., UK) and the volume was measured using a drum-type gas meter (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Germany).

3.3.2 Chemical analyses

The following chemical analyses of the digestates was performed at least once a week as previously published by Schönberg and Linke (2012): total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), volatile fatty acids (VFA) including the single acetic acid, propionic acid, iso- and n-butyratic acid, iso- and n-valeric acid and capronic acid as well as the ratio between the total volatile fatty acids and total alkalinity (TVFA/TA). Total ammonium nitrogen (TAN) was analysed at least two times every week and used to calculate the amount of ammonium carbonate required to operate the reactors under similar TAN concentration. Further the pH value was measured each day before feedstock supply. The amount of NH_3 was calculated according to Hansen et al. (1998). The used feedstocks were also analysed regarding their chemical characteristics, preliminary in order to calculate the OLR.

3.3.3 Microbial community analyses

Digestate samples for the molecular biological analyses were collected weekly and stored at 20°C until further analyses. Based on the applied feedstock change and the related changes within the prevalent chemical parameters 18 time points for each of the six reactors were chosen for microbial community analyses.

For each time point genomic DNA was isolated from three subsamples using the PowerSoil®DNA Isolation Kit (MoBio Laboratories, USA) following the protocol by Klang et al. (2015).

The microbial community composition was analysed using the genomic fingerprint method TRFLP targeting either the bacterial or the archaeal 16S rRNA gene. The polymerase chain reaction (PCR) and the restriction digest was performed according to Klang et al. (2015) using the primer pairs 27F/926MRr and the restriction enzyme MspI and Hin6I (*Bacteria*) and Ar109f/Ar912r and AluI (*Archaea*) whereby the forward primer were fluorescently labelled with Cy5. The separation of the digested fragments was performed using GenomeLab™ GeXP

Genetic Analysis System (AB SCIEX Germany GmbH, Darmstadt, Germany). A bioinformatical evaluation of the obtained data was performed as described by Klang et al. (2015) using the software package BioNumerics 7.6 (Applied Maths, Belgium).

The identification and the taxonomic assignment of the detected TRFs was carried out by the construction and screening of 16S rRNA gene sequence libraries. The PCR amplification of the 16S rRNA gene was conducted using the same primer pairs (in this case unlabeled) and PCR conditions as mentioned above. The PCR products were purified using the Nucleospin® Gel and PCR Cleanup kit by Machery Nagel (Germany). Cloning of 16S rRNA gene amplicons was performed according to Rademacher et al. (2012). DNA sequencing was conducted by Eurofins Genomics (Germany).

The obtained sequences were processed, grouped into operational taxonomic units (OTU) and virtually digested using BioNumerics 7.6 (Applied Maths, Belgium) as previously described by Klang et al. (2015). The OTUs were phylogenetically identified using the RDP Naïve Bayesian rRNA Classifier Version 2.6 (Wang et al., 2007). All OTUs obtained in this study have been deposited to the National Center for Biotechnology Information (NCBI) and are available under accession numbers MF769050-MF769233 (*Bacteria*) and MF774191-MF774209 (*Archaea*). All obtained sequences from this study were included in an in-house database, currently containing more than 3000 16S rRNA gene sequences from various biogas projects carried out at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) during the last ten years. Several plasmids (or better the 16S rRNA gene within the plasmids) has been analysed by a real TRFLP analyses in order to verify the virtual TRF with the real TRF of the obtained sequence.

3.3.4 Statistical analyses

Non-metric multidimensional scaling (NMDS) (Clarke, 1993) was performed with the R Project for Statistical Computing (R Team, 2018) using the packages “vegan” (Oksanen, 2018). The distance matrix was calculated using the Bray-Curtis algorithm (Bray and Curtis, 1957).

3.4 Results and discussion

3.4.1 Operation and reactors performance

Prior to this experiment, the CSTRs had been operated as triplicate with either sugar beet silage or maize silage for 337 days. In the here presented experiment the reactors SBS-C and MS-C were operated with only silages (equal to the first phase), in the reactors SBS-M and MS-M the silages were stepwise exchanged with increasing amounts of animal manure and the reactors SBS-A and MS-A were operated with silages and ammonium carbonate.

The average biogas yields for SBS-C and MS-C were 0.62 ± 0.06 $L_N\ g_{VS}^{-1}$ and 0.61 ± 0.08 $L_N\ g_{VS}^{-1}$, respectively (Figure 3-1A and C), values comparable with the first phase (Klang et al., 2015). With the introduction of animal manure in the feedstock supply the biogas yields in the reactors SBS-M and MS-M decreased gradually to approximately 0.40 $L_N\ g_{VS}^{-1}$. This decrease was expected as swine and chicken manure have lower biogas yields (0.42 $L_N\ g_{VS}^{-1}$ resp. 0.50 $L_N\ g_{VS}^{-1}$) due to a lower VS content in the feedstock (Cu et al., 2015; KTBL, 2009).

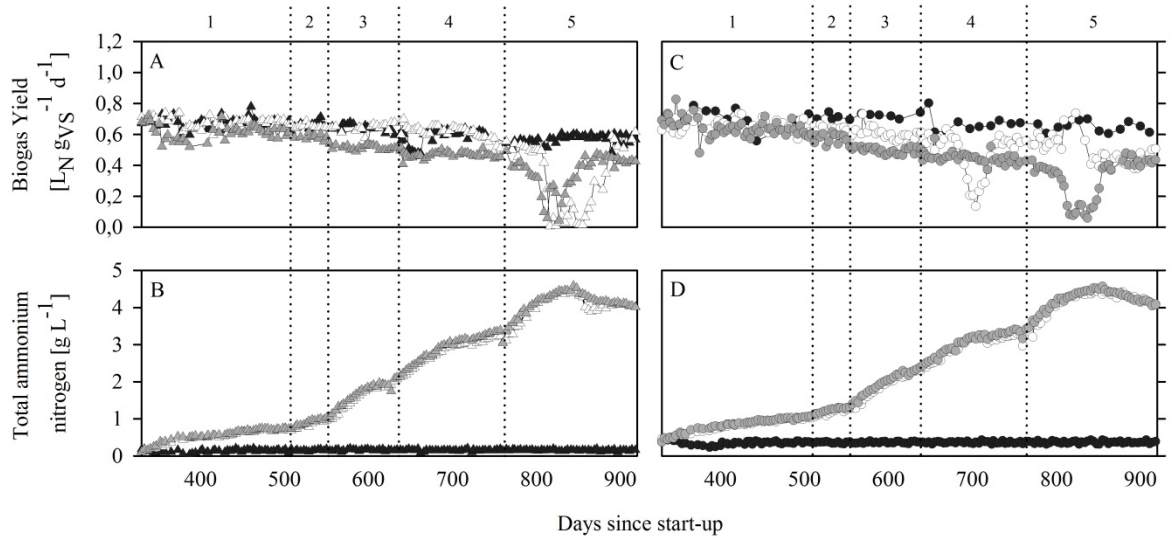

Figure 3-1: Operational parameters during the fermentation of sugar beet silage (A-B) and maize silage (C-D) with increasing amounts of animal manure respectively ammonium nitrogen. Shown are the biogas yield and the total ammonium nitrogen. Symbol identification: triangles = sugar beet silage reactor, dots = maize silage reactor, black = control reactor, white = reactor with addition of ammonium carbonate, grey = reactor with increasing amounts of animal manure: 1 = 90% silage + 10% swine manure, 2 = 80% silage + 10% swine manure + 10% chicken manure, 3 = 60% silage + 10% swine manure + 30% chicken manure, 4 = 40% silage + 10% swine manure + 50% chicken manure, 5 = 15% silage + 10% swine manure + 75% chicken manure.

Around day 800 the biogas yields in SBS-M and MS-M decreased, the TVFA/TA and thus the total VFA concentration (Figure 3-2), which consisted mostly of acetic acids (SBS-M: 3.6 $g_{HAc\text{-}Eq}\ L^{-1}$, MS-M: 3.1 $g_{HAc\text{-}Eq}\ L^{-1}$), increased. At this time point the TAN concentration in both

reactors had exceeded 4.0 g L^{-1}, an often reported inhibition threshold (Drosg, 2013; Rajagopal et al., 2013; Schnürer and Nordberg, 2008) indicating, that this is the main reason for the recorded disturbance (Figure 3-1B and D). Due to the buffer capacity of animal manure the pH value increased to 7.6 in all experimental reactors. The calculated amount of NH_3 was 0.3 g L^{-1}, a value that has also been reported to have inhibitory effects (Westerholm et al., 2016). In order to prevent further inhibition, or even a complete process failure, the feedstock supply in both reactors was stopped for a couple of days resulting in a subsequent decrease in the VFA concentration (monitored over the TVFA/TA ratio) and a slight increase in the biogas yield. The VFA concentration decreased faster in SBSM than in MS-M but the feedstock supply in both reactors had to be started and stopped several times before it stabilised. SBS-M was given the same amount of feedstock as before the disturbance after 28 days while the MS-M needed 40 days to stabilize.

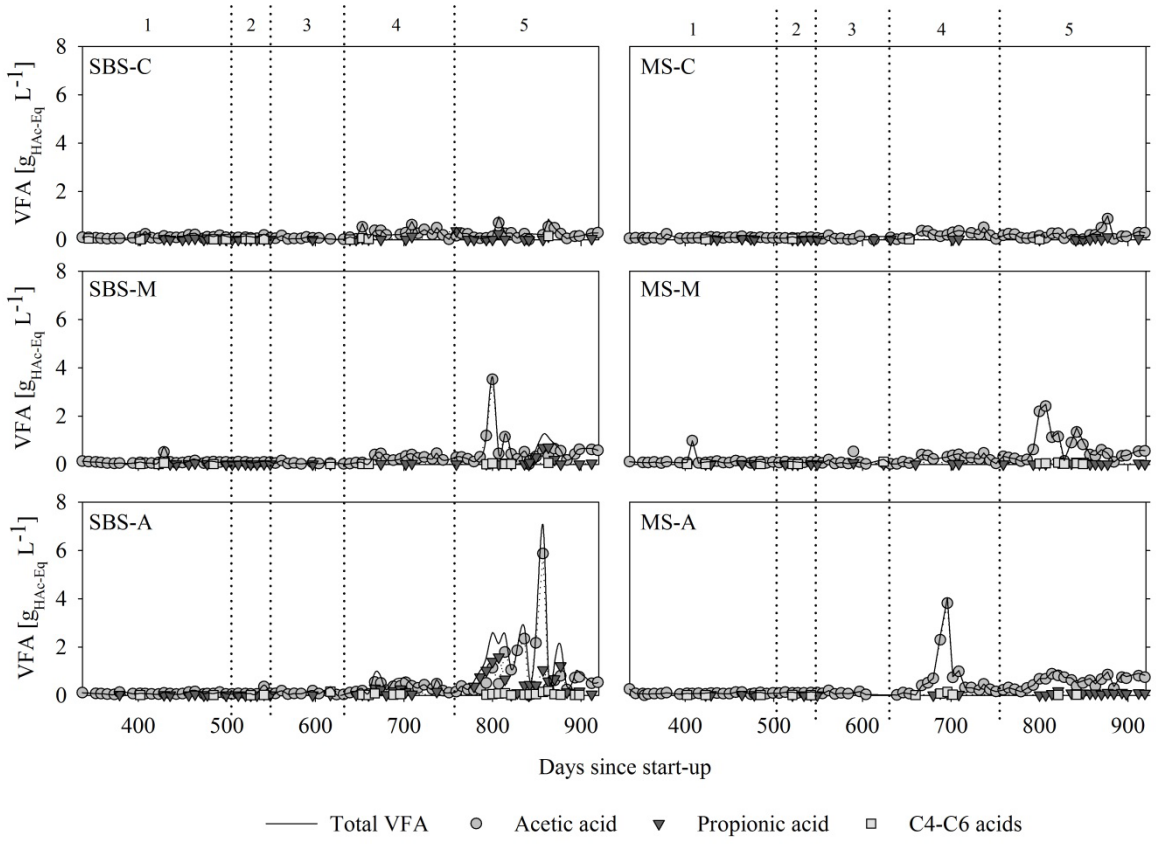

Figure 3-2: VFA concentration during the fermentation of sugar beet silage and maize silage (SBS-C, MS-C) with increasing amounts of animal manure (SBS-M, MS-M) and ammonium nitrogen (SBS-A, MS-A). 1 = 90% silage + 10% swine manure, 2 = 80% silage + 10% swine manure + 10% chicken manure, 3 = 60% silage + 10% swine manure + 30% chicken manure, 4 = 40% silage + 10% swine manure + 50% chicken manure, 5 = 15% silage + 10% swine manure + 75% chicken manure.

Interestingly, the amount of propionic acids in SBS-M increased during the disturbance phase (Figure 3-2). It has been reported by Felchner-Zwirello et al. (2013) that higher amounts of

acetic acids, which were observed in the beginning of the disturbance phase, not only indicate that the acetoclastic methanogens are inhibited but also might inhibit the propionic acid oxidizer. The degradation of propionic acid is thermodynamically unfavourable and only possible in a syntrophic relationship with hydrogen consuming organisms such as hydrogenotrophic methanogens (Liu and Whitman, 2008; Schink, 1997). Therefore a reorganization of the microbial community can be assumed over the disturbance phase as an adaption to the prevalent environmental conditions resulting in a community structure containing a syntrophic relationship of propionic acid oxidizers and hydrogenotrophic methanogens.

The biogas yield in the reactors with additional ammonium carbonate (SBS-A, MS-A) was comparable to reactors SBS-C and MS-C during the first part of the experimental phase (Figure 3-1A and C). However the microbial communities in the reactors SBS-A and MS-A seemed to react more sensitive to the increasing ammonium nitrogen than the microbial communities in the reactors digesting animal manure reactors SBS-M and MS-M. In the reactor MS-A a VFA accumulation was recorded already around day 690. Here the TAN concentration was around 3.0 g L^{-1} with a pH-value of 7.5 and a calculated NH_3 amount of 0.2 g L^{-1} (Figure 3-1B and D). The VFA concentration increased to 3.8 $g_{HAc\text{-}Eq}$ L^{-1} and, similar to the SBS-M and MS-M reactors, consisted mostly of acetic acid. Hence, it can be assumed, that acetoclastic methanogens were inhibited by increasing TAN concentrations also in the MS-A reactor. The feedstock supply was stopped for 11 days followed by 13 days with halve of the feedstock supply before an OLR of 2 g_{VS} $L^{-1}d^{-1}$ could be applied again. The biogas yield was comparable to the yield before the disturbance. In contrast, the disturbance of the reactor SBS-A occurred at the same time as the corresponding manure reactor. Here the feedstock supply had to be stopped and slowly started several times. An OLR of 2 g_{VS} $L^{-1}d^{-1}$ could be applied first after 64 days. In contrast to the other reactors, the acid spectra consisted mainly of propionic acids instead of acetic acid already at the beginning of the disturbance, suggesting a different microbial community composition in the SBS-A reactor. Propionic acid is produced during the primary fermentation and consumed during the secondary fermentation, hence an accumulation indicates an imbalance between these two processes. Due to the lack of electron acceptors the degradation of VFAs, such as propionic acid, during the secondary fermentation is only possible via the formation of H_2 (Leng et al., 2018; McInerney et al., 2010; Morris et al., 2013). Without subsequent utilization of the generated H_2 by hydrogen scavenging organism, such as hydrogenotrophic archaea, these syntrophic

bacteria would be inhibited by their own end product since excessive H_2 concentrations make the fatty acid oxidation thermodynamically impossible (Schink, 1997). Whereas an accumulation of acetic acid might indicate a disturbance of the acetoclastic archaea, an accumulation of propionic acid probably indicates a disturbance of the bacterial community. Regardless of where in the process chain the disturbance occurred, the detected propionic acid might indicate that the archaeal community in reactors SBS-A contained higher abundances of hydrogenotrophic methanogens than the other reactors since they can act as a syntrophic partner during degradation.

Concluding, the analysed chemical parameters differed as response to the feedstock change in the four experimental reactors (SBS-M, SBS-A, MS-M and MS-A). The earliest disturbance, defined through an acid accumulation, occurred in reactor MS-A at a TAN concentration of 3.0 g L^{-1} and lasted approximately 24 days. In the reactors SBS-M, MS-M and SBS-A the disturbance occurred first when the TAN concentration exceeded 4.0 g L^{-1} and lasted 28, 40 and 64 days respectively. However, regarding the predicted environmental stress based on elevated TAN concentrations it can be assumed (1) that the microbial community of the SBS reactors are more stress resistant than the community of the MS reactors and (2) that the manure treated reactors (SBS-M, MS-M) were more resistant than the ammonium carbonate treated reactors (SBS-A, MS-A). The first effect must be related to the occurring microbial community. The question at this point is whether there is any nexus between the microbial diversity level and stress tolerance potential within the biogas process. The second effect is probably related to the fact, that animal manure provided a complex matrix of nutrients, especially micronutrients which are essential for microbial growth. Furthermore, it is possible that the microbial community present in the swine and chicken manure influenced the latter community development.

3.4.2 Microbial community composition of the control reactors SBS-C and MS-C

In reactors SBS-C the most notable change during the beginning of phase two was the increased relative abundance of TRF 93 (Figure 3-3A, Figure 3-4B), which clearly dominated the bacterial community after day 449. Most of the recorded sequences with an in silico restriction site of 93 bp were assigned to the phylum *Bacteroidetes* and further to the family *Porphyromonadaceae*. Additionally, the NMDS (Figure 3-4B) revealed that the TRF 146, which could also be assigned to the family *Porphyromonadaceae,* was significant (R^2= 0.59, p-value = 0.001) for the community composition with a similar relative abundance over time.

Several described members of this family are known to convert easily degradable sugars and alcohols into acids (Chen and Dong, 2005; Grabowski et al., 2005; Hahnke et al., 2016; Jabari et al., 2012; Ueki et al., 2006). These finding confirm the results of the first phase (Klang et al., 2015) and show a further adaptation of the microbial community to the digestions of SBS.

The reactor MS-C was, similar as during phase one, characterized by a diverse and dynamic bacterial community with members of the phyla *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Spirochaetes*, *Synergistetes* and *Thermotogae*. Notable is the first increasing and then decreasing relative abundance of TRF 67 between day 505 and 638 (Figure 3-3D). This TRF was assigned to a sequence showing a similarity of 99% to *Defluviitoga tunisiensis* (NCBI reference sequence no: NR_122085), belonging to the phylum *Thermotogea*. *Defluviitoga tunisiensis* has been genome sequenced and described by Maus et al. (2016) as a hydrolytic bacterium, degrading a broad range of polysaccharides. Also the TRF 163 could most likely be assigned to the phylum *Thermotogea*, which was present with a similar abundance throughout the experimental time period. These results confirm the study by Klang et al. (2015) showing once again that chemical more complex feedstocks require a structural more diverse community with members of various phyla that ensure a high functional and ecological diversity to efficiently convert the provided biomass into biogas.

Despite the differences in the bacterial community composition between the two basic feedstocks one similarity was noted: TRFs assigned to the yet uncultivated phylum *Cloacimonetes* were found with similar and constant relative abundances throughout the experimental phase in both reactors (Figure 3-3A and D). In reactor SBS-C two different TRFs assigned to the phylum occurred: During the first three sampling points TRF 166 was the most abundant which was afterwards replaced by TRF 161. In MS-C TRF 161 was the most abundant one throughout the whole experimental phase. The phylum *Cloacimonetes* was first detected in a municipal wastewater plant and the cluster was called candidate division WWE1 (Chouari et al., 2005). The genome of a member was reconstructed from a metagenome dataset by Pelletier et al. (2008) and named "*Candidatus* Cloacamonas acidaminovorans". This most likely syntrophic bacterium ferments amino acids, sugars and carboxylic acids (Pelletier et al., 2008). Moreover, there are indications that other members of this phylum play an important role during cellulose degradation and/or in the uptake of fermentation products (Limam et al., 2014).

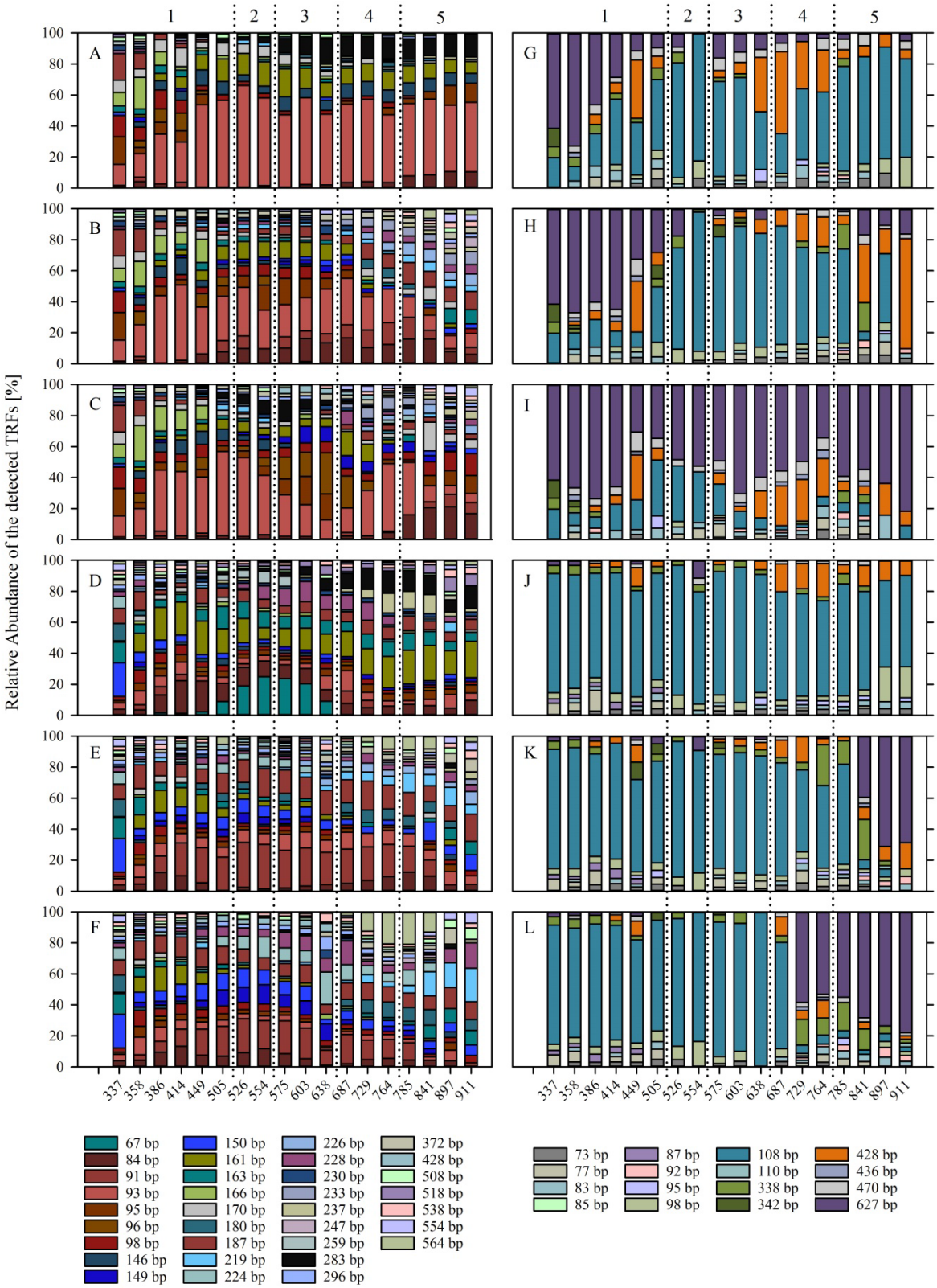

Figure 3-3: Structure of the bacterial (A-F) and archaeal (G-L) community of either the sugar beet (A-C and G-I) or maize silage (D-F and J-L) reactor systems with increasing amounts of animal manure (B, E, H, K) respectively ammonium nitrogen (C, F, I, L). Colored bars symbolize the detected terminal restriction fragments (TRFs) in base pairs (bp) and their relative abundance. For the bacterial community, only TRFs with a relative abundance over 5% in at least one sample are shown. Each sampling point is given as median value of three technical replicates. Numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

The archaeal community of the SBS-C reactor showed higher structural fluctuations over time than the archaeal community of the MS-C reactor (Figure 3-3G and J). In the end of phase one (Klang et al., 2015) and in the beginning of this experimental phase the SBS-C reactor was characterized by a high relative abundance of TRF 627, which was assigned to the genus *Methanosarcina*. Over the course of time TRF 627 was replaced by TRF 108, which was assigned to the genus *Methanothrix* (former *Methanosaeta* (Tindall, 2014)). However, the high relative abundance of TRF 108 was not stable throughout the whole experimental phase. At some time points (day 449 as well as 638 to 764) the TRF 428, assigned to the genus *Methanoculleus*, increased its relative abundance accompanied with a decrease of TRF 108 (Figure 3-3G). This functional complementary co-occurrence of the acetoclastic *Methanothrix* and the hydrogenotrophic *Methanoculleus* securing an overall stable biogas production process was already observed during phase one (Klang et al., 2015). In contrast, the reactor operated with MS showed a distinct dominance of the acetoclastic genus *Methanothrix* (TRF 108) throughout the whole experimental phase (Figure 3-3J).

The main difference in the operation of the reactors SBS-C and MS-C compared to the investigations described in Klang et al. (2015) was the shorter HRT as well as the regular addition of ammonium carbonate. Nevertheless, the results from the first phase could be verified here: A stable bacterial community composition with a comparable low number of detected TRFs and a few predominant community members seemed to lead to a more fluctual archaeal community. The easy degradable compounds in the SBS do not require the same diverse and complex bacterial community composition as the community degrading the chemically more complex MS. However, it seems that the bacterial community within the SBS-C reactor converted the given feedstock compounds into acids, with exception for acetic acids, and hence requires syntrophic relationships for further degradation and hence a more functional diverse archaeal community containing both acetoclastic and hydrogenotrophic methanogens. The results further indicate that the more diverse bacterial community within the MSC reactor might be capable of providing a wider range of metabolic pathways. During the primary fermentation different VFAs are produced, whereof acetic acid can immediately be used as substrate by acetoclastic methanogens. Other VFAs (e.g. propionic acid and butyric acids) have to be further degraded into usable substrates for the methanogens, which is only possible in syntrophic relationships with hydrogen scavenger. Possible hydrogen scavenger are hydrogenotrophic methanogens, but also for example homoacetogens, which channel hydrogen and carbon dioxide towards acetic acid.

If the only hydrogen scavenger were the detected hydrogenotrophic methanogens or if also other groups occurred cannot be answered, but nevertheless enabled the occurring microbial community the development of a predominant acetoclastic archaeal community dominated by the genus *Methanothrix*.

The question at this point is whether a stable and efficient process performance is related to a more diverse bacterial community (MS reactors) or to a more divers archaeal community (SBS reactors) even if environmental stress occurs (Allison and Martiny, 2008; Carballa et al., 2015).

3.4.3 Microbial community response to increasing amounts of animal manure (SBS-M, MS-M) or ammonium nitrogen (SBS-A, MS-A)

In the experimental reactors SBS-M and MS-M as well as in the reactors SBS-A and MS-A the TAN concentration was stepwise increased either through addition of animal manure or ammonium carbonate (Figure 3-1B and D). During the first part of this study the reactors with the same silage but with different TAN sources (SBS-M/SBS-A and MS-M/MS-A) showed a similar development of the microbial community as the corresponding control reactors (Figure 3-3 and Figure 3-4A). Hence the relative abundance of TRF 93 increased in the two SBS reactors (SBS-M, SBS-A) during the beginning of this experimental phase, but with increasing amounts of animal manure (starting at day 449, Figure 3-3B) or ammonium carbonate (starting at day 554, Figure 3-3C) its relative abundance decreased. However it seems that the ecosystem function of species assigned to TRF 93 was compensated by other members of the phylum *Bacteroidetes* as other TRFs, also assigned to this phylum, increased their relative abundances. The changes in the structural composition had no negative effect on the overall process performance (Figure 3-1 andFigure 3-2). These findings are confirmed by Goux et al. (2015) who showed that different members of the phylum *Bacteroidetes* are able to perform the same or similar metabolic pathways, resulting in the ability to quickly adapt to changing environmental conditions. Noteworthy is, that the detected *Bacteroidetes* members differed in the two reactors (e.g. TRF 84 and TRF 91 in SBS-M as well as TRF 95 and TRF 96 in SBS-A). It can be assumed that, although the organism had a similar metabolic pathway, the different TAN sources (animal manure or ammonium carbonate) influence the development of the community differently. Through the addition of animal manure the feedstock composition changed (compared to the mono-fermentation in the ammonium carbonate reactors) and it is also possible that the microbial community present in the animal

manure influenced the anaerobic digestion process. Nevertheless, according to the current state of knowledge it is not possible to explain the functional and hence the systems ecological specifications which lead to the establishment of different *Bacteroidetes* species under certain environmental conditions as comparative studies on the genetic potential in combination with the physiological capacities of single species are still missing.

Interestingly, members of the phylum *Cloacimonetes* (TRF 161 and TRF 166) disappeared in both reactors after day 687 (SBS-M) and day 729 (SBS-A) respectively, as the TAN concentration reached 3.0 g L^{-1}. With the disappearance of these bacterial community members a change in the bacterial diversity was recorded: The number of detected TRFs increased and the community members got more evenly distributed in both reactors. Between the sampling points 785 and 841 a process disturbance occurred, indicated by an acid accumulation in combination with a decreased biogas yield (Figure 3-1 andFigure 3-2), followed by a re-organization of the bacterial community. While the relative abundance of members from the phylum *Bacteroidetes* decreased several new TRFs became apparent (Figure 3-3B and C). None of these TRFs could be assigned to any sequence of the sequence libraries constructed in this study and hence no phylogenetic assignment was possible.

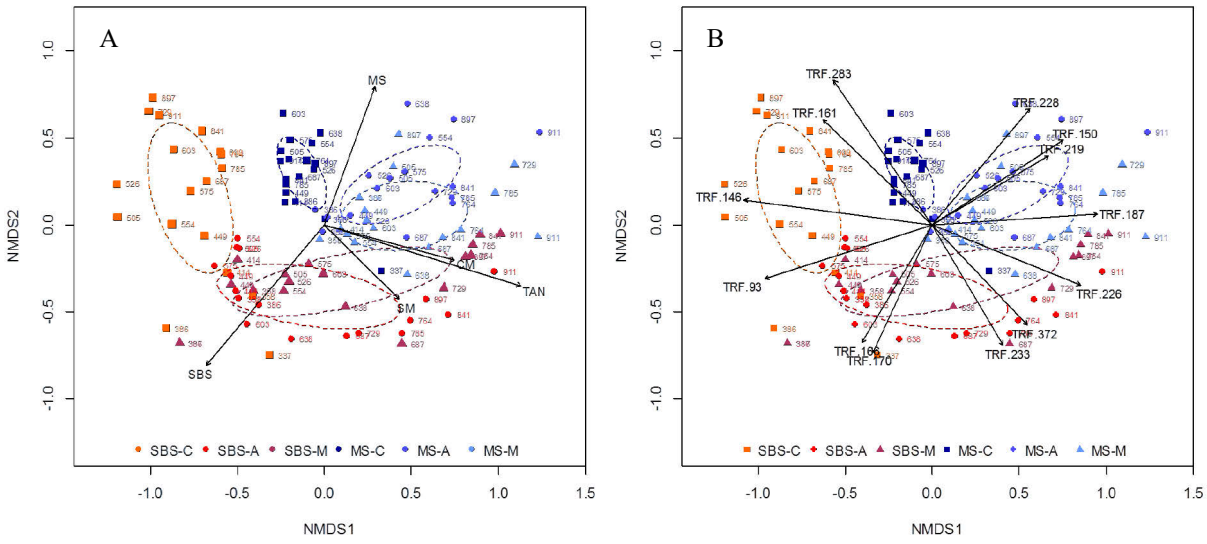

Figure 3-4: Non-metric multidimensional scaling (NMDS) analysis to elucidate the development of the bacterial community as response of increasing amounts of ammonium nitrogen either supplied by animal manure or ammonium carbonate. The final stress was 0.20. The p-value for all shown vectors is 0.001. Goodness of the vector fit R^2: TAN= 0.74, CM = 0.32, PM = 0.19, SBS = 0.58, MS = 0.38 (A). The vectors shown in (B) are the 10 TRFs with the highest abundance and with p-value = 0.001, $R^2 > 0.3$. TAN = total ammonium nitrogen, SM = swine manure, CM = chicken manure, SBS = sugar beet silage, MS = maize silage, C = control, M= animal manure, A = ammonium carbonate TRF = terminal restriction fragment.

In the MS-M and the MS-A reactors a change in the bacterial community compared to the control reactor (MS-C) occurred earlier than in the SBS reactors. In both reactors MS-M and MS-A changes were recorded already around day 414. The relative abundance of TRFs assigned to the phylum *Bacteroidetes* increased whereby TRF 91 showed the highest relative abundance. Also the TRFs 149 and 150 increased their relative abundances (Figure 3-3E and F, Figure 3-4B). Sequences assigned to TRF 149 showed a similarity of 93% to the species *Sedimentibacter hydroxybenzoicus* and *Sedimentibacter saalensis* (NCBI Reference Sequence: NR_029146.1 and NR_025498.1). Members of the genus *Sedimentibacter* are able to ferment amino acids and pyruvate but no carbohydrates. Their main fermentation products are acetate and butyrate while no hydrogen is produced (Breitenstein et al., 2002), indicating that these microorganisms are involve in the acido- and acetogenesis. Most sequences with the restriction site 150 were assigned to unknown *Firmicutes*, hence no deep phylogenetic assignment in combination with a potential functional or ecological prediction was possible.

Similar to the sugar beet reactors was the disappearance of members of the phylum *Cloacimonetes* (TRF 161) prior to process disturbance. The tolerance threshold regarding the TAN concentration was approximately 1.0 g L^{-1}, which was reached in MS-A at day 449 and in MS-M at day 505 (Figure 3-1, Figure 3-3E and F). With exception of this phylum the reactors MS-M and MS-A showed a more similar community development than the two SBS reactors until day 687; the time point as the process disturbance in MS-A occurred. After the disturbance the TRFs assigned to the phylum *Bacteroidetes* decreased and TRF 564 (assigned as unknown Bacteria) and later TRF 219 (assigned as unknown *Clostridia*) increased, whereby TRF 219 is likely significant for the bacterial community (R^2 = 0.3, p-value = 0.001) under the prevailing environmental conditions in MS-A (Figure 3-3and Figure 3-4B). The disturbance in reactor MS-M occurred later than in reactor MS-A, around the same time as in the SBS reactors (between day 785 and day 841), and the reorganization of the bacterial community during the disturbance was comparable to MS-A. Hence the community reorganization was similar in the SBS reactors and in the reactors MS-A and MS-M towards the end of the experimental phase (Figure 3-3 and Figure 3-4).

The reactors given manure became more equal over the course of time and at day 911 the community similarity was 73%. This result was not surprising as the feedstock mixture of both reactors contained 85% animal manure (based on VS). Compared to that, the SBS-A and MS-A reactors showed a community similarity of only 24%. This indicates that not only the

increasing TAN concentration but rather the different basics feedstocks (SBS or MS) still was an important factor which influenced the bacterial community structure.

The bacterial community similarities between SBS-M and SBS-A was 49% and between MS-M and MS-A 48% at day 911. This cannot be rated as that high, but compared to the community similarities between the control and experimental reactors (SBS-M and SBS-C = 17%, SMS-A and SBS-C = 24%, MS-A and MS-C = 15% as well as MS-M and MS-C = 23%) in combination with the above mentioned similar community reorganization, it can be assumed, that the increasing TAN concentration actually is an important driver factor for the community development. The NMDS analysis (Figure 3-4A) confirmed this assumption as the TAN concentration showed the highest correlation with the developed microbial communities (R^2=0.74). Consequently, the manure microbiome or members of this microbiome, entering the system by each feeding, had no significant impact on the community development. The finding that the microbial communities present in the swine and chicken manure do not established within the anaerobic digestion community confirms results of previous conducted batch experiments (data not published).

As mentioned above, members of the yet not cultivated bacterial phylum *Cloacimonetes* (represented by TRF 161 and TRF 166) disappeared prior to the recorded process disturbances in all four experimental reactors independent from the supplied TAN source or the supplied basic feedstock. Similar findings were observed by Alsouleman et al. (2016) who reported a strong decrease in the abundance of member of the phylum *Cloacimonetes* from 10% to a complete disappearance in direction to an upcoming process disturbance, where 50% poultry manure was supplied with the feeding. Hence, the disappearance of these organisms might indicate the possibility of an upcoming process disturbance due to increasing TAN concentrations. To confirm this, further investigations are needed.

In contrast to the bacterial community, the archaeal community behaved the other way around: The SBS reactors developed similar only until day 386, with a high relative abundance of TRF 627 (assigned to the genus *Methanosarcina*) whereas the MS reactors where dominated by TRF 108 (assigned to the genus *Methanothrix*) until day 687, as the process disturbance occurred in MS-A (Figure 3-3G and L). Therefore the results reported by Klang et al. (2015) as well as of the control reactors (SBS-C, MS-C) can be confirmed also in the reactors with increasing TAN concentrations: The structural more stable bacterial community of in the SBS reactors had a more dynamic archaeal community and the more

dynamic bacterial community in the MS reactors is combined with a more stable archaeal community composition (chapter 3.2).

In the SBS-M reactor the genus *Methanosarcina,* which is able to produce methane through both the acetoclastic and hydrogenotrophic pathway (De Vrieze et al., 2012), was replaced by the genus *Methanothrix* (TRF 108), an obligate acetoclastic methanogen, after day 505. Around day 638 also the hydrogenotrophic genus *Methanoculleus* increased its relative abundance slightly. Around, and especially after, the process disturbance the number of archaeal community members increased, including the most abundant genera *Methanobacterium* (TRF 338) and *Methanoculleus* (TRF 428) as well as *Methanosarcina* (TRF 627) and *Methanothrix* (TRF 108). At the end of the experiment *Methanoculleus* dominated the community of reactor SBS-M (Figure 3-3H). The change from the acetoclastic pathway of methane formation to a hydrogenotrophic methanogenesis at elevated TAN concentrations has been described in several studies (Alsouleman et al., 2016; Lv et al., 2014; Niu et al., 2015). Especially the hydrogenotrophic *Methanoculleus* have been reported as syntrophic partners of acetate oxidizing bacteria (Anderson et al., 2009; Manzoor et al., 2016; Schnürer and Nordberg, 2008; Westerholm et al., 2016). In the SBS-A reactor *Methanosarcina* remained the most dominant methanogen throughout the whole experimental phase, although the relative abundance varies over the course of time. Between day 505 and 575 *Methanothrix* is the second most abundant archaeal genus (Figure 3-3I) but as the TAN concentration was around 2.1 g L^{-1} (day 638) the TRF 428, assigned to *Methanoculleus*, increases its relative abundance. As the process disturbance occurred some TRFs that could not be assigned to any of the obtained sequences within the corresponding archaeal sequence library were recorded but they disappeared again. At the end of the experimental phase *Methanosarcina* clearly dominated the community in reactor SBS-A. The results suggest that not only the TAN concentration itself is responsible for the community formation as e.g. the two SBS reactors with different TAN sources (either degradation of animal manure or ammonium carbonate) showed different microbial community developments. Especially the archaeal community developed differently as the versatile archaeal genus *Methanosarcina* remained the dominant in SBS-A reactor, while the hydrogenotrophic genus *Methanoculleus* dominated the SBS-M reactor. Consequently, additional, currently unspecified factors such as e.g. trace elements derived from the animal manure seem to influence the development of the community in SBS-M.

Compared to the SBS reactors, the reactors MS-M and MS-A showed a similar change in the archaeal community composition with increasing TAN concentration, although the community changes occurred earlier in MS-A than in MS-M (Figure 3-3K and L). As already mentioned, the process disturbance in the MS reactor given ammonium carbonate occurred already around day 690 (Figure 3-2). This disturbance was combined with a complete reorganization of the archaeal community: Until day 687 *Methanothrix* dominated the community but after the disturbance *Methanosarcina* was the most dominant methanogen, followed by *Methanobacterium* and *Methanoculleus*. The same reorganization was observed after the process disturbance (between day 764 and day 841) in reactor MS-M (Figure 3-3K). After the reactor/process recovery, *Methanosarcina*, the most versatile methanogenic genus, was predominant. Regarding the current state of knowledge, these results are not surprising as the obligate acetoclastic methanogens (representatives of *Methanothrix*) have the least tolerance thresholds against TAN concentrations. Generally accepted and frequently described limit values for a process inhibition through increasing TAN for a not adapted community is between 3.0 and 5.0 g L^{-1} corresponding to 0.1-0.4 g L^{-1} NH_3 (Rajagopal et al., 2013; Schnürer and Nordberg, 2008).

The main question of this study was whether there is any nexus between the microbial diversity level and the stress tolerance within the biogas process. Generally it is assumed that a functional more diverse community indicates a higher stress tolerance potential due to the existence of functional redundant species, which reflects a higher community resilience (Carballa et al., 2015; De Vrieze and Verstraete, 2016). The results showed that a bacterial community with a few dominant members led, over the course of time, to a more flexible and hence functional more diverse archaeal community (SBS reactors) and that a bacterial community with higher amount of more evenly distributed community members (MS reactors) led to a more rigid archaeal community. Taken into account that the reactor disturbance was mainly caused by an inhibition at the archaeal level, the community in the SBS reactors seemed to be more stress tolerant against the induced inhibition by ammonium/ammonia nitrogen as the disturbance occurred first around day 800 in both reactors. The more diverse bacterial community of the MS reactors seems to be able to provide several metabolic pathways required for a complete conversion of chemical complex biomasses into acetic acids. However, the dominant occurrence of the acetoclastic genus Methanothrix made this reactors more susceptible as this genus is most sensitive to increasing TAN concentrations (De Vrieze et al., 2012), which was most obvious in reactors

MS-A as the disturbance occurred already around day 690. Moreover, the archaeal community within the SBS-M reactor was able to faster compensate the function of the inhibited organisms during a process disturbance (stabilization after 28 days) than the highly specialized Methanothrix-dominated community of the MSM reactor (stabilization after 40 days). Further investigations are needed in order to evaluate how the bacterial and archaeal community reacts to other stressors, for example changing temperatures, application-orientated feeding regimes or other process inhibitory compounds such as hydrogen or hydrogen sulfide.

3.5 Conclusion

A nexus between the microbial diversity level and the stress tolerance to elevated TAN concentration was evaluated. A bacterial community with a few dominant members led to a functional more flexible archaeal community (SBS reactors) which was more stress resistant under the certain experimental conditions than a bacterial community with higher amount of more evenly distribted community members combined with a more rigid archaeal community (MS reactors). Members of the bacterial phylum *Cloacimonetes* disappeared prior to the recorded process disturbances, this fact might be used as potential indicator for upcoming process disturbance caused by increasing TAN concentrations.

4 Effect of a profound feedstock change on the structure and performance of biogas microbiomes

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4.1 Abstract

In this study the response of biogas-producing microbiomes to a profound feedstock change was investigated. The microbiomes were adapted to the digestion of either 100% sugar beet or maize silage or of the silages with elevated amounts of total ammonium nitrogen (TAN) by adding ammonium carbonate or animal manure. The feedstock exchange resulted in a short-range decrease or increase in the biogas yields according to the level of chemical feedstock complexity. Twelve terminal restriction fragments (TRFs) were found in all reactors and can be considered as generalists. 13 TRFs were detected in the reactors operated with low TAN and seven in the reactors with high TAN concentration. TRFs assigned to the phylum *Bacteroidetes* and to the order *Spirochaetales* increased with the exchange to sugar beet silage, indicating an affinity to easily degradable compounds. The TAN-sensitive TRF 161 (phylum *Cloacimonetes*) showed no specific affinity to maize or sugar beet silage. The archaeal community remained unchanged. The reported findings showed a smooth adaptation of the microbial communities, without a profound negative impact on the overall biogas production indicating that the two feedstocks sugar beet and maize silage potentially do not contain chemical compounds that are difficult to handle during anaerobic digestion.

4.2 Introduction

The demand and use of renewable energy such as biogas have risen during the last decades because anaerobic digestion is attributed with several advantages such as a sustainable waste management or the integration into multi-faceted production systems for food, feed, bioenergy and biomaterials (Theuerl et al., 2019a) and because of the negative climate impact of fossil fuels.

Biogas is the end product of the anaerobic digestion of organic matter. It consists mostly of methane (CH_4) and carbon dioxide (CO_2), whereas the energy-rich CH_4 is the desired compound. For the production of biogas different kinds of organic matter can be used, such as wastewater sludge, municipal solid waste, residues from livestock husbandry and energy crops. According to the Renewable Energy Act in 2000, especially with the amendments in 2004 and 2009, most agricultural biogas plants in Germany are operated with energy crops and livestock residues (Daniel-Gromke et al., 2018; Theuerl et al., 2019a). The most used energy crop is maize, as it is easy to cultivate and has high biomass and biogas yields. Consequently the cultivation of maize was expanded (Statistisches Bundesamt, 2014, 2003). Although it is not fully quantified, negative effects of the Renewable Energies Act became obvious and led e.g. to the food versus fuel debate (e.g. Herbes et al. 2014b). Moreover the expanded cultivation of energy crops such as maize has several negative impacts, such as the loss of biodiversity and reduced soil fertility as well as soil degradation. In the last couple of years several amendments of the Renewable Energy Source Act have been made. In 2012 an upper maize limit of 60% was introduced which was further reduced to 50% in 2017 (Balussou et al., 2018; Herbes et al., 2014b). However, the transition to a circular bioeconomy is required, in which anaerobic digestion (biogas production) is a keystone for providing base load power which can be supplied on demand and serves as a sustainable residue management strategy (Theuerl et al., 2019a). Manifold residues from agricultural crop production, livestock husbandry and landscape management as well as from organic wastes from food processing, food consumption and from the organic fraction of municipal solid waste might be appropriate feedstocks for anaerobic digestion (Theuerl et al., 2019a). Consequently, plant operators will be forced to use a broad range of different feedstocks which are chemically very heterogeneous, variable over time and often available only in small quantities. This will lead to a more inhomogeneous feedstock supply and sometimes too rapid feedstock changes, which might cause problems for the microbial community in the biogas reactors as the available nutrients and the bioaccesibility might differ (Theuerl et al., 2019a, 2019b).

The production of biogas is a complex process where several different microorganisms using different metabolic pathways work together to degrade organic matter into biogas. The process can be divided into four main steps: the hydrolysis, the acidogenesis, the acetogenesis and the methanogenesis. The first three steps are conducted by anaerobic bacteria which gradually digest organic matter into mainly acetic acid, carbon dioxide and hydrogen. The methanogenesis is performed by methanogenic archaea, which convert the end products of the first three steps into biogas. Because of the low energy amount gained under anaerobic conditions one organism cannot perform a complete digestion so that several bacteria work together to digest the feedstock into the substrate used by the methanogens (Angelidaki et al., 2011; Schnürer, 2016; Weiland, 2010). This leads to a high diversity on the bacterial level, de Vrieze et al. (De Vrieze et al., 2018) for example detected up to 1000 different operational taxonomic units (OTUs) in one biogas plant. The development of the microbial community depends on the available substrates. Different feedstocks with different chemical composition and bioavailability lead to different microbial communities (Alsouleman et al., 2016; De Vrieze et al., 2015a; Han et al., 2016; Hanreich et al., 2013; Klang et al., 2019, 2015; Pap et al., 2016). Therefore knowledge of the microbial reaction to different feedstock compositions is highly important in order to avoid process disturbances, and changes in the feedstock composition have to be performed with caution. One well known factor leading to process disturbances are elevated concentrations of ammonium in equilibrium with ammonia (Theuerl et al., 2019b; Westerholm et al., 2016). Ammonium/ammonia is produced during degradation of nitrogenous compounds and cannot be further degraded under anaerobic conditions and accumulates in the reactors. The amount of ammonia depends on pH-value and temperature; the higher these values, the more ammonia occurs in the system. It has been shown that ammonia is the cause of process inhibition (Rajagopal et al., 2013; Theuerl et al., 2019b; Westerholm et al., 2016) as it can diffuse through the cell wall causing proton imbalance or potassium deficiency (Rajagopal et al., 2013). Furthermore it has been shown that the acetoclastic archaeal genus *Methanothrix* is the most sensitive one against elevated ammonia concentrations and that for example *Methanoculleus* or *Methanosarcina* tolerate higher concentrations (De Vrieze et al., 2012).

The objective of this study was to analyze the reaction of biogas microbiomes to a profound feedstock change from a chemically more complex to an easily degradable feedstock and vice versa. Prior to this study the microbiomes were adapted to the digestion of either 100% sugar beet or maize silage, of 100% sugar beet or maize silage with increasing amounts of ammonium carbonate or to the co-digestion of 25% silage and 75% animal manure. Based on

these preconditions we suspected (i) that the exchange of chemically different feedstocks will cause a significant change (increase or decrease) in the biogas yields, (ii) that the microbial communities will change in their taxonomic composition whereby it is of interest how long they will need to adapt to the new conditions, (iii) that the effects will be lowest for the reactor system where only 25% of the feedstock mixture was exchanged and (iv) that microorganisms or groups of microorganisms can be identified which have an affinity to either sugar beet or maize silage or which do tolerate elevated TAN concentrations. In order to elucidate these hypotheses the fingerprint method terminal restriction fragment length polymorphism (TRFLP) in combination with a cloning/sequencing approach was used to monitor the dynamics of the bacterial and archaeal communities which was further related to the occurring/changing environmental conditions using multivariate statistical analyses.

4.3 Materials and Methods

4.3.1 Reactor operation

For the eight weeks lasting study six continuously stirred tank reactors (CSTRs) with a working volume of three liters were used. Prior to this study, the reactors had been operated under different conditions for approximately three years. The reactors were adapted to the digestion of either sugar beet (SB) or maize (M) silage as single feedstock, to the digestion of sugar beet or maize silage with increasing amounts of ammonium carbonate or to co-digestion of either sugar beet or maize silage and animal manure (Klang et al., 2019). In this study the feedstock supply was changed, meaning that the reactors operated with sugar beet silage were given maize silage and vice versa. This means the reactor formerly fed with sugar beet silage was now given maize silage as single feedstock (SB-M1), the reactor formerly fed with maize silage was now given sugar beet silage as single feedstock (M-SB1), the reactor formerly fed with sugar beet silage and ammonium carbonate was now given maize silage and ammonium carbonate (SB-M2), the reactor formerly fed with maize silage with ammonium carbonate was now given sugar beet silage and ammonium carbonate (M-SB2), the reactor formerly fed with 25% sugar beet silage and 75% animal manure was now given 25% maize silage with 75% animal manure (SB-M3) and the reactor formerly with 25% maize silage reactor and 75% animal manure was now given to 25% sugar beet silage with 75% animal manure (M-SB3). The TAN concentration in the reactors SB-M1 and M-SB1 were kept at 0.3 g L^{-1} through addition of ammonium whereas the TAN concentration in the reactors SB-M2 and M.-SB2

were adapted to the concentrations in the reactors given manure (SB-M3 and M-SB3) twice a week. The trace element solution DMSZ 144 was added to every feeding in order to avoid process inhibition through lack of micronutrients according to Elhussein and Weiland (Elhussein and Weiland, 2009). The reactors were operated under mesophilic conditions and fed once a day. The organic loading rate (OLR) was 2.0 g_{VS} $L^{-1}d^{-1}$ with a hydraulic retention time (HRT) of 43 days. The biogas was collected in gasbags (Tesseraux Spezialverpackungen GmbH, Germany). The produced biogas amount was measured at least twice a week using a drum-type gas meter (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Germany) while the gas composition was analyzed using the portable analyzer "BiogasCheck" (Geotechnical Instruments Ltd. UK). Samples for chemical and microbiological analyses were taken at least once a week.

4.3.2 Chemical analyses

The pH-value was measured prior to feeding. The contents of total solids (TS), volatile solids (VS), volatile fatty acids (VFA, acetic-, propionic-, (i)-butyric-, (i)-valeric- and capronic acids), total Kjeldahl nitrogen (TKN) and total ammonium nitrogen (TAN) were analyzed according to Schönberg and Linke (Schönberg and Linke, 2012).

4.3.3 Molecular biological analyses

The DNA was extracted as triplicate using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The DNA was amplified with PCR according to Klang et al. (Klang et al., 2015) using the primer pair 27f/926Mr for the bacterial community and 109f/912r for the archaeal community. As the PCR product should be analyzed using TRFLP the forward primer of each assay was labeled with Cy5. The PCR products were purified using the Nucleospin Gel and PCR Clean-up (Macherey und Nagel, Düren, Germany). A restriction digestion was performed with the endonucleases *Msp*I and *Hin*6I for the bacterial community and *Alu*I for the archaeal community. The samples were analyzed using the capillary sequencer GenomeLab™ GeXP Genetic Analysis System (AB SCIEX Germany GmbH, Germany) according to Rademacher et al. (Rademacher et al., 2012). The phylogenetic assignment of the recorded terminal restriction fragments (TRFs) was conducted with the sequence library produced in Klang et al. (Klang et al., 2019, 2015). Additionally we used an in-house database, currently containing about 3000 16S rRNA gene sequences from various projects carried out at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) during the last ten years (Theuerl et al., 2018).

4.3.4 Bioinformatics and statistical analysis

The obtained electropherograms were analyzed using the software package BioNumerics (Applied Maths, Belgium) according to Klang et al. (Klang et al., 2015).

Only curves with a relative abundance over 1% were used for the statistical analysis. Non-metric multidimensional scaling (NMDS) (Clarke, 1993) and calculation of the Richness was performed with the R Project for Statistical Computing (R Core Team, 2018) using the packages "vegan" (Oksanen et al., 2018). The distance matrix was calculated using the Bray-Curtis algorithm (Bray and Curtis, 1957). The environmental vectors were calculated using envfit, the result was sorted after the highest R^2-values while the ten first vectors are shown. The calculations of the Gini index was performed using the R package ineq (Zeileis, 2014).

4.4 Results and Discussion

4.4.1 Chemical and operational parameters

In this study, the response of a profound feedstock change on the reactor performance and the microbial community was investigated. Prior to this change the reactors had been operated for 911 days, first with sugar beet silage or maize silage as a single feedstock for about a year (until day 337) (Klang et al., 2015), followed by an gradually exchange/addition of either animal manure or ammonium carbonate (until day 911) (Klang et al., 2019).

At the beginning of this study which was started at day 918 (after sampling), the two reactors, which had only been operated with silages since the experimental start-up, had an ammonium concentration below 0.3 g L^{-1}. As both sugar beet silage and maize silage are rather nitrogen-poor feedstocks, ammonium carbonate had to be added to avoid process disturbances due to a potential nutrient limitation (Klang et al., 2015). In the reactors given ammonium carbonate or animal manure the TAN concentrations were significantly higher with values of around 4 g L^{-1}, whereof around 340 mg L^{-1} was ammonia. This ammonium concentration is considered to be critically high, as values around 3.0 g L^{-1} often lead to reactor instabilities (Rajagopal et al., 2013; Westerholm et al., 2016). However, the microbial communities in the investigated reactor systems were adapted to this TAN concentration (Klang et al., 2019). The TAN concentrations were kept constant in all reactors throughout the whole experimental phase (data not shown).

The biogas yields, methane contents and volatile fatty acid concentrations over the course of time are shown in Figure 4-1. Directly after the feedstock change from sugar beet silage to maize silage in the reactors SB-M1 and SB-M2 the biogas yields decreased rapidly but increased again after only some days (Figure 4-1a and c). In contrast, increasing biogas yields were noted in the reactors M-SB1 and M-SB2 directly after the change from maize silage to sugar beet silage Figure 4-1b and d). In the reactors SB-M3 and M-SB3, the reactors fed with 75% chicken manure and only 25% silages, no notable changes in the biogas yields were observed (Figure 4-1e and f).

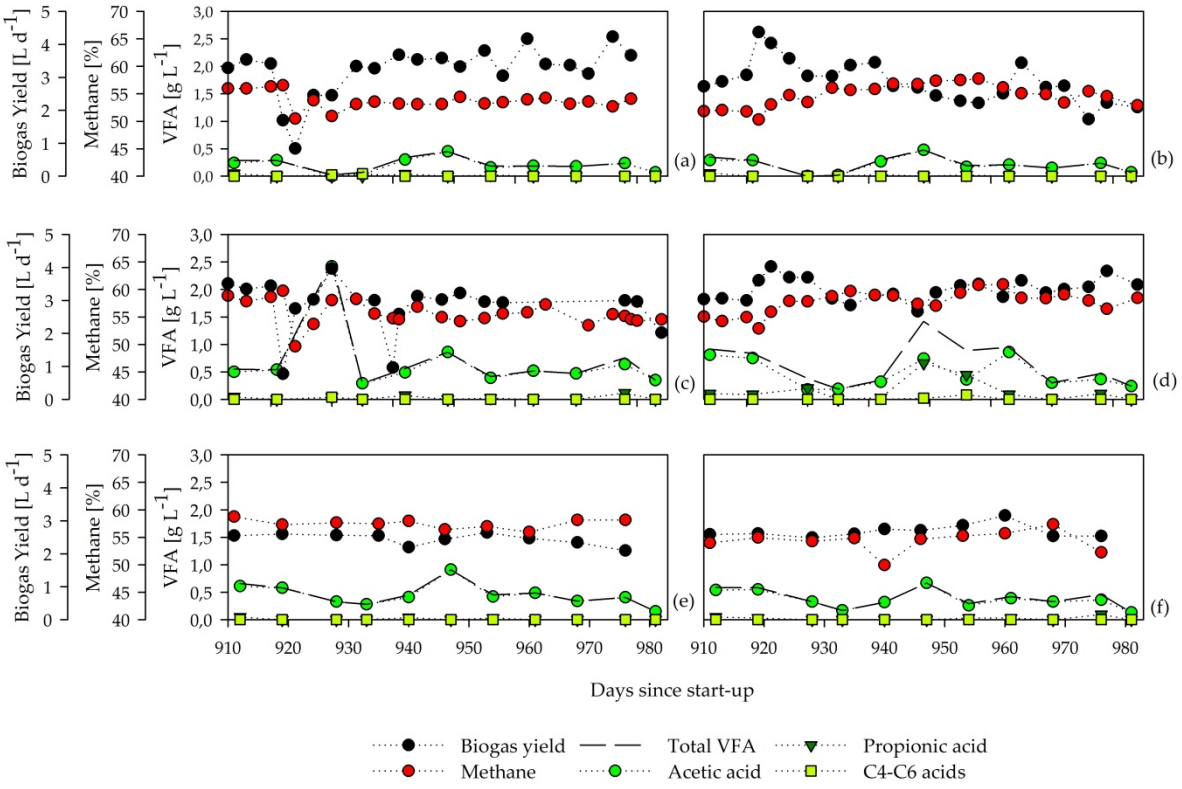

Figure 4-1: Biogas yields, methane contents and volatile fatty acids (VFA) of the investigated reactor systems SB-M1 (a), M-SB1 (b), SB-M2 (c), M-SB2 (d), SB-M3 (e) and M-SB3 (f) during a period of eight weeks.

The most probable explanation for the decreasing or increasing biogas yields is the different chemical composition of sugar beet silage and maize silage. The sugar beet silage contains higher amounts of easily degradable compounds, such as sugars, ethanol and acetid acid while maize silage has larger amounts of more complex compounds, such as cellulose, starch and hemicellulose which related kinetics of the biogas production rate in high temporal resolution (hours) for both feedstocks (Klang et al., 2015). Hence, the microbial community adapted to the conversion of sugar beet silage needed some time to hydrolyze the more complex

compounds in the maize silage into the substrates for the acido-/acetogenesis and hence the methanogenesis while the community given the easily degradable sugar beet silage instead of the maize silage could convert the given compound more or less directly into biogas shortly after addition.

Considering the accumulated VFAs, a notable change took place only in the reactors given ammonium carbonate (Figure 4-1c and d). In the reactor SB-M2 the concentration of acetic acids increased to 2.5 g L^{-1} some days after the feedstock change, indicating that the bacterial community was able to hydrolyze and ferment the supplied feedstock compounds but the methanogenic community could not utilize the produced acid at the same speed. However, the concentration decreased within one week, indicating a putative quick adaptation of the archaeal community. Compared to that, in the reactor M-SB2 an accumulation of propionic acid was noted approximately 30 days after the feedstock exchange, whilst the acetic acid concentration decreased. Apparantly, compounds in the sugar beet silage were degraded into propionic acid but the community was not capable of further conversion into biogas. This can be explained by the fact that methanogenic archaea only metabolize acetic acid, hydrogen (H_2) and one-carbon (C1) compounds, hence fatty acids such as propionic acid first have to be converted into these utilizable compounds until biogas can be produced in syntrophic relationships (Leng et al., 2018; McInerney et al., 2009; Morris et al., 2013). However, the VFA concentration in the silage reactors (SB-M1, M-SB1) was below 0.5 g L^{-1} (Figure 4-1a and b) and the in the manure reactors below 1.0 g L^{-1} (Figure 4-1e and f) throughout the whole eight weeks.

4.4.2 Bacterial community - general overview

The microbial community was investigated using TRFLP in combination with a cloning/sequencing approach. A total number of 150 different bacterial TRFs were found in all investigated reactors. Out of them, twelve TRFs (ca. 8%) were found in all six reactors at least at more than one time point with an overall median abundance of 31% (

Figure S1). Among them TRF 84, 92, 98, and 187 could be assigned to the phylum *Bacteroidetes*, TRF 167 and 218 to the phylum *Firmicutes*, and TRF 163 to the phylum *Thermotogae*; The TRFs 156, 228, 236, 373 and 538 remained unassigned. Calusinska et al. (Calusinska et al., 2018) reported that the core microbiome contained several members from the phylum *Bacteroidetes* and *Firmicutes* which is confirmed by several previous studies reporting high abundances of these two phyla in reactors producing biogas (De Vrieze et al.,

2016; Lee et al., 2017; Sun et al., 2013; Ziganshin et al., 2013). In accordance with previous studies, the TRF-related microorganisms seem to be able to exist under various environmental conditions as the six reactor systems differed in their TAN concentrations as well as in the degradability of the supplied feedstocks and might hence be putative generalists belonging to the biogas core microbiome (Calusinska et al., 2018; Theuerl et al., 2018).

Opposite to these generalists, several TRFs were found only in certain reactors and hence seem to be specialists that occupy particular ecological niches or fulfil specific ecosystem functions as they are e.g. important for the degradation of specific substrates (Theuerl et al., 2018). 13 TRFs were almost exclusively detected in the reactors operated with low TAN concentrations while seven TRFs mainly occurred in the reactors with high TAN concentrations (Figure S2 and Figure S3). The TRFLP profiles (Figure 4-2) as well as the NMDS ordination (Figure 4-3) clearly distinguish the reactor systems with low (SB-M1 and M-SB1) and high TAN concentration (SB-M2, M-SB2, SB-M3, M-SB3). Out of the ten most significant TRFs, seven TRFs are putatively important for the communities with low TAN concentration and three for the communities with high TAN concentrations.

Also the community evenness and richness of in the reactors differed: The mean values of the Gini indices were 0.44 ± 0.09 and 0.45 ± 0.08 for SB-M1 and M-SB1 respectively over the course of time, meaning that the communities in the reactors with low TAN concentrations were more unevenly distributed then the communities in the reactors with high TAN concentrations (SB-M2: 0.37 ± 0.05, SB-M3: 0.33 ± 0.06, M-SB2: 0.41 ± 0.06, M-SB3: 0.38 ± 0.04). Both a too high and a too low Gini index is assumed to be negative considering the ability of the occurring community to be resistant/resilient to sudden stress exposure as both community organisations might need time to adapt to changes: A too unevenly organized community might need time to recover after a change whereas a too evenly organized community might need time to react to changes caused by a lack of selective pressure; a "good" value is considered to be around 0.45 (Marzorati et al., 2008; Read et al., 2011; Wittebolle et al., 2009). The both reactors given ammonium carbonate (SB-M2, M-SB2) had lower Gini indices compared to the reactors with low TAN concentrations (SB-M1, M-SB1), hence the recorded VFA accumulation is supposed to be related to unfavorable community organization. The reactors given animal manure showed the lowest values, but no indication for a process instability/disturbance was observed. This might be related to the fact that in both reactors only 25% of the feedstocks were changed, which most likely was less important for a community adapted to the degradation of 75% animal/chicken manure.

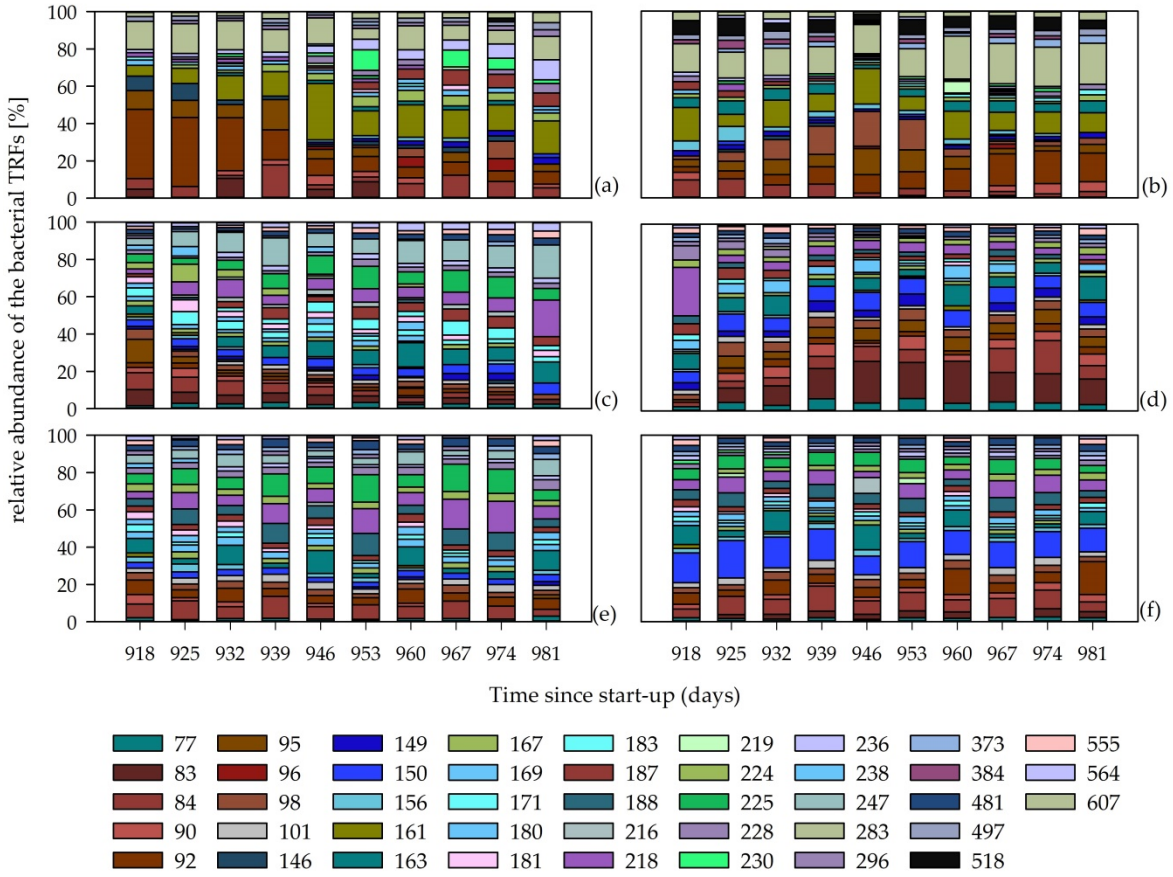

Figure 4-2: Structure of the bacterial community in reactors SB-M1 (a), M-SB1 (b), SB-M2 (c), M-SB2 (d), SB-M3 (e) and M-SB3 (f). Colored bars symbolize the detected terminal restriction fragments (TRFs) in base pairs (bp) and their relative abundance. For the bacterial community, only TRFs with a relative abundance over 3% in at least one sample are shown. Each sampling point is given as median value of three technical replicates. Numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

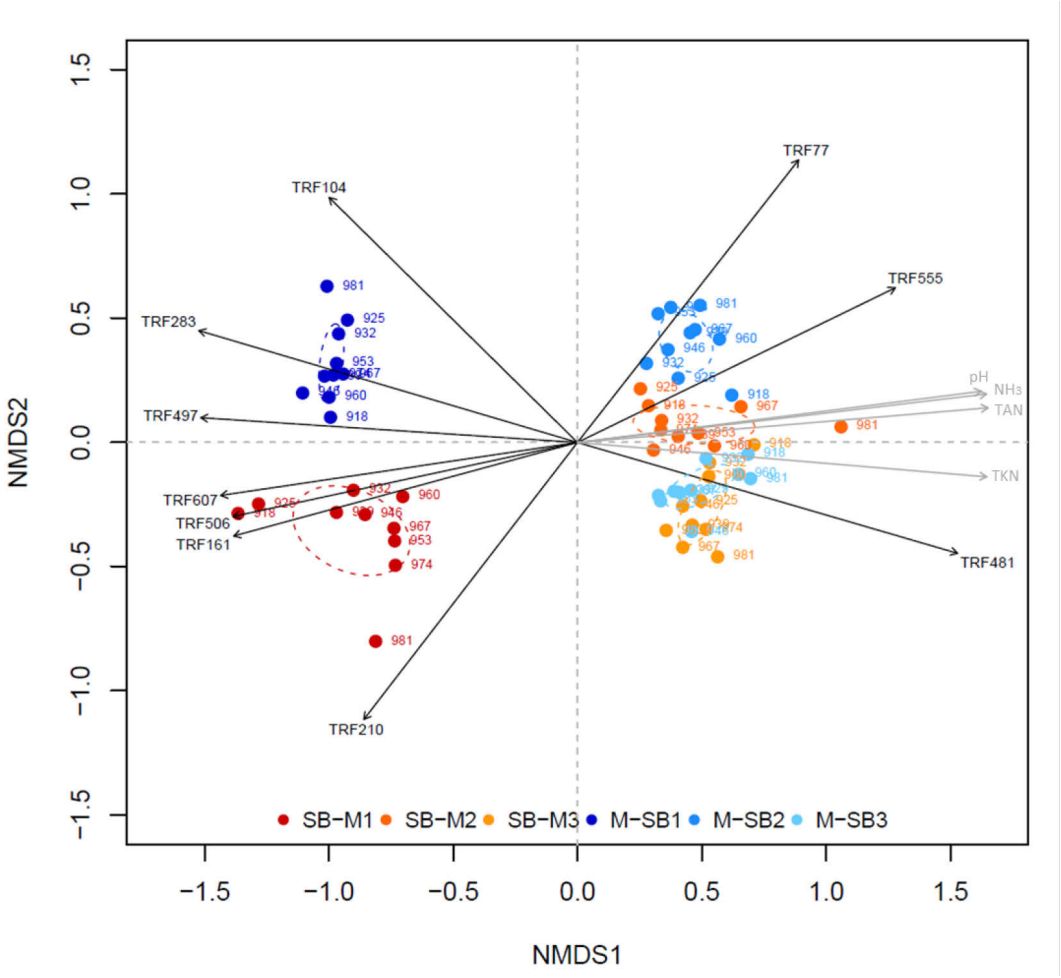

Figure 4-3: Non metric dimensional scaling (NMDS) of all six reactors (stress =0.14). The environmental vectors symbolize the ten most significant TRFs, sorted after R^2, p=0.001.

4.4.3 *Bacterial* community *at low TAN concentration*

The bacterial community in SB-M1 (Figure 4-2a) was clearly dominated by TRF 92, assigned to the phylum *Bacteroidetes*, in the beginning of the experimental phase but its relative abundance decreased over the course of time. The reactor M-SB1 (Figure 4-2b) showed an opposite behavior: the abundance of TRF 92 increased over the course of time. Hence this TRF disappeared as the sugar beet silage was changed into maize silage and increased as sugar beet silage was introduced. These findings further confirm the assumption, that certain members of the phylum *Bacteroidetes* have a high affinity to easily degradable compounds (sugars, alcohols and organic acids) and hence play a crucial role during the acido- and acetogenesis (De Vrieze et al., 2015b; Goux et al., 2015; Hanreich et al., 2013; Klang et al., 2015; Theuerl et al., 2018; Werner et al., 2011).

Out of the seven most significant TRFs, six belonged to the TRFs almost exclusively detected in the reactors with low TAN concentrations (TRF 161, 210, 283, 497, 506 and 607). Only two of these TRFs could be phylogenetically assigned: TRF 161 to the not yet cultivatable phylum *Cloacimonetes* and TRF 283 to the phylum *Spirochaetes*. Both TRF 161 and TRF 283 showed high relative abundances with values between 5 and 22% as well as 3 and 18% respectively whereas the abundances of the other characteristic TRFs were below 5% at all time points.

Members of the phylum *Cloacimonetes* can be assumed to be sensitive to TAN as it has been reported that their abundances decrease or even disappear at elevated TAN concentrations (Klang et al., 2019; Kovács et al., 2015; M. Westerholm et al., 2018). Considering this, the occurrence of members from the phylum *Cloacimonetes* in these reactor systems was expected. Additionally, there was no significant trend towards an increasing or decreasing abundance of TRF 161 over the course of time indicating that the TRF-related microorganisms had no specific affinity to maize silage or sugar beet silage.

The nearest neighbor assignable to TRF 283 had a similarity of only 93% to the next known species and can therefore only be assigned to the order *Spirochaetales.* Regarding their functional/ecological role within the biogas microbiome, less information is available. Gupte et al. [44] summarized that members of this order can be anaerobic, facultative anaerobic, or microaerophilic while they perform a chemoorganotrophic metabolism using carbohydrates or amino acids as carbon and energy sources. In the reactors with low TAN concentrations, the abundance of TRF 283 showed a tendency to increase in the reactor where the feedstock was changed from maize silage to sugar beet silage and to decrease when the change was the other way around. Hence the TRF-related microorganisms seem to have a higher affinity to more easily degradable compounds. However, the abundance of TRF 283 in reactor SB-M1 increased again at the last sampling point, so that one possible assumption might be, that the newly adapted community was able to degrade the maize silage into compounds suitable for the *Spirochaetales*.

A comparison of the bacterial communities only in the reactors SB-M1 and M-SB1 revealed that the bacterial community in reactor SB-M1 showed more dynamic variations/changes over time than the bacterial community in reactor M-SB1 (Figure 4-4a). The similarity between the first and the last sampling days (918 and 981) was 40% in reactors SB-M1 and 60% in M-SB1 (data not shown). The bacterial community in reactor SB-M1 became more diverse towards the end of the experimental phase (Figure 4-2a).

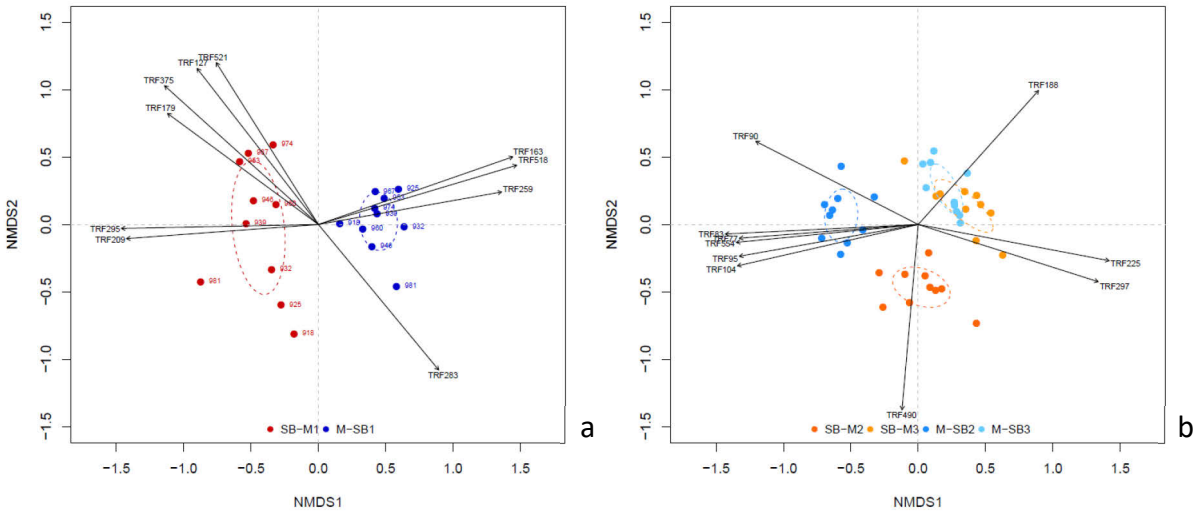

Figure 4-4: Non metric dimensional scaling (NMDS) of the reactors with low TAN (a), stress=0.15 and with high TAN (b), stress=0.20. The environmental vectors symbolize the ten most significant TRFs, sorted after r^2, p=0.001.

This higher diversity in the reactor where sugar beet silage was exchanged with maize silage is most likely caused by the higher amount of chemically more complex compounds within the maize silage which requires a higher functional and ecological diversity (De Vrieze et al., 2015b; Klang et al., 2015; Theuerl et al., 2019b, 2018; Zhu et al., 2018) This assumption is further confirmed as three of the SB-M1 characteristic TRFs (Figure 4-4a) could be assigned to the phylum *Firmicutes* (TRF 180, 210 and 296) which members are known to be mainly involved during the hydrolyses stage were chemically complex feedstock compounds such as cellulose are primarily degraded (De Vrieze et al., 2015b; Schnürer, 2016; Shrestha et al., 2017; Theuerl et al., 2019b; Zeeman et al., 2015). Compared to this, the four characteristic TRFs for the M-SB1 community were TRF 163, 259, 283 and 518. As mentioned above, the increasing significance of TRF 283 (order *Spirochaetales*) indicates that the TRF-related microorganisms have a higher affinity to easily degradable substrates which are provided by the sugar beet silage. In addition, TRF 163 could be assigned to the genus *Mesotoga*, the only known mesophilic genus of the phylum *Thermotogea* which is either assumed to be able to degrade different sugars with acetate, carbon dioxide and sulfide as endproducts (Ben Hania et al., 2013) or which are supposed to be involved in syntrophic acetate degradation (Nesbo et al., 2018). TRF 518 was assigned to the phylum *Chloroflexi* and further to the class *Anaerolineae*. An omic-based genome interpretation showed that members of this class are able to ferment carbohydrates with acetate as end-product, possibly live in syntrophic

relationships, are capable of cell adhesion and that some members are capable to hydrolyze cellulose (Xia et al., 2016).

Although a profound change in the feedstock regime was performed in this study, which generally should be carried out with caution (Theuerl et al., 2019b), the so far reported findings showed a smooth adaptation of the bacterial communities, without a long lasting negative impact on the overall biogas production. This indicates that the two feedstocks sugar beet and maize silage potentially do not contain chemical compounds that are difficult to handle during anaerobic digestion (Theuerl et al., 2019a).

4.4.4 Bacterial community at high TAN concentration

Although there was a high dissimilarity between the reactors with low and high TAN concentrations some similarities were observed considering the bacterial phyla. As already mentioned the microbial community of reactor SB-M1 was dominated by the TRF 92, assigned to the phylum *Bactreoidetes*, at the beginning of the experimental phase. Compared to that also the reactor SB-M2 showed a high dominance of members from the phylum *Bacteroidetes*, whereby the community was not dominated by one TRF but several different TRFs could be assigned to this phylum (e.g. TRF 84, 92, 95, 96, 98). Similar as in reactor SB-M1 the abundance of these TRFs decreased over the course of time as the community adapted to the degradation of maize silage (Figure 4-2a and c). In those reactors where the feedstock was changed from maize to sugar beet silage (M-SB1 and M-SB2) it was the other way around as the abundances of TRFs assigned to the phylum *Bacteroidetes* increased over the course of time. The NMDS of all six reactors showed that the TRFs 77, 481 and 555 were most significant for the community with high TAN concentration (Figure 4-3) while none of these TRFs showed an abundance higher than 5% (Figure S2 and Figure S3). Only TRF 77 could be properly assigned to the order *Syntrophobacterales* (phylum *Proteobacteria)* and showed a 96% sequence similarity to the species *Syntrophus aciditrophicus.* This species is able to degrade fatty acids and aromatic acids in a syntrophic relationship with hydrogenotrophic methanogens (McInerney et al., 2007). This finding is in accordance with previous studies which showed a predominance of the hydrogenotrophic pathway of methane formation under elevated TAN concentrations [e.g. 13,14,31,55,56]. Hydrogenotrophic archaea are also known as hydrogen scavengers, meaning they depend on hydrogen which is produced by their neighboring bacteria (such as members of the genera *Syntophomonas*, *Syntrophobacter*, *Syntrophus*, *Propionibacter*, *Pelotomaculum*, *Smithella* or *Clostridium* (Leng et al., 2018; Morris et al., 2013)) who in turn can only grow if the hydrogen is

consumed. Consequently, an inhibition of the function of hydrogenotrophic archaea by NH_4^+/NH_3 can lead to a process disturbance which is reflected not only in reduced methane yields, but also in acid accumulation as it was recorded in reactors SB-M2 and M-SB2.

The NMDS analysis of the four different reactors with high TAN concentrations showed three different clusters: (I) reactor SB-M2, (II) reactor M-SB2 and (III) the two manure reactors (SB-M3 and M-SB3) (Figure 4-4b). The most significant TRF for cluster I, the SB-M2 community, was TRF 490 that could be assigned to the phylum *Firmicutes*. This TRF was in the beginning of the experimental phase detected in both reactors given ammonium carbonate (SB-M2 and M-SB2) but its relative abundance decreased to below the detection limit in M-SB2 over the course of time, whereas it increased in the reactor SB-M2. This indicates that the TRF-related microorganisms have an affinity to the substrates available in the supplied maize silage. Compared to this, the NMDS pointed six out of the ten most significant TRFs for the reactor systems with high TAN concentrations towards cluster II (TRF 77, 83, 90, 95, 104, 554), reactor M-SB2 (Fig. 5B). Among them, the unassignable TRF 104 showed a significance towards the community with low TAN concentration when comparing all six reactor systems (Figure 4-3) but when only considering the reactors with high TAN concentrations it was characteristic for the community in reactor M-SB2 (Figure 4-4b). Hence the presence of this TRF seems to depend rather on the feedstock composition than on the TAN concentration with a preference for the degradation of compounds provided by the supplied sugar beet silage.

The two reactors operated with 75% chicken manure showed no significant community change over the course of time indicating that a change of 25% of a “process-uncritical” feedstock did not lead to profound effects on the microbial diversity and hence the overall reactor performance.

4.4.5 Archaeal community

The archaeal communities remained more or less unchanged in the six investigated reactor systems throughout the whole experimental phase (Figure 4-5).

The archaeal communities in the two reactors with low TAN concentrations were dominated by TRF 106 (Figure 4-5a and b), which was assigned to the genus *Methanothrix* (order *Methanosarcinales*). This genus is obligate acetoclastic, hence the members of this genus only produce methane from acetic acid (De Vrieze et al., 2012; Liu and Whitman, 2008). According to the assumption that this genus is abundant in well performing reactor systems

(Alsouleman et al., 2016; Klang et al., 2019; Regueiro et al., 2012), the VFA concentrations in the reactors SB-M1 and M-SB1 were low while the biogas yields and methane contents were constant indicating an undisturbed process. Therefore it can be concluded that the bacterial communities which adapted to the exchanged feedstocks over the course of time were able to degrade the provided chemical compounds into acetic acid while the archaeal community was not inhibited but could further degrade the intermediates into methane.

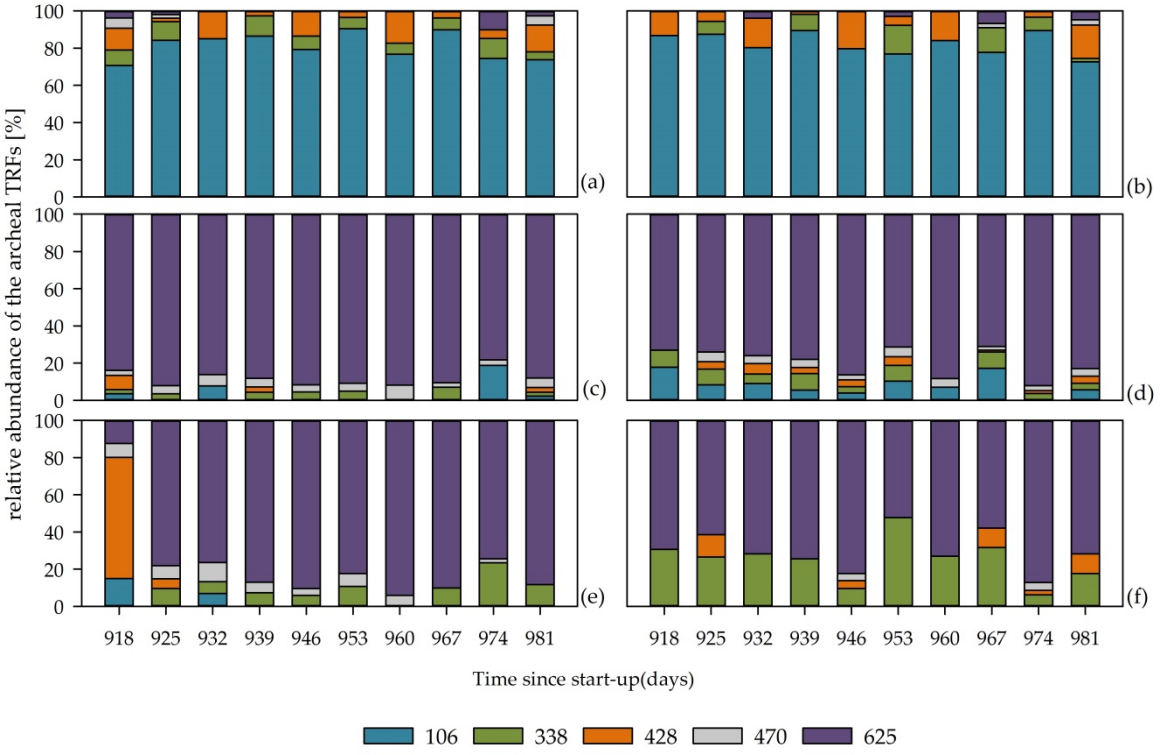

Figure 4-5: Structure of the archaeal community in reactors SB-M1 (a), M-SB1 (b), SB-M2 (c), M-SB2 (d), SB-M3 (e) and M-SB3 (f). Colored bars symbolize the detected terminal restriction fragments (TRFs) in base pairs (bp) and their relative abundance. Each sampling point is given as median value of three technical replicates. Numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

The reactors with high TAN concentrations were dominated by TRF 625 throughout the whole experimental phase (Figure 4-5c-f). This TRF was assigned to the genus *Methanosarcina* which members are able to convert both acetic acid as well as hydrogen (H_2) and CO_2 into biogas. Although this genus dominated the communities in reactor SB-M2 and M-SB2, the VFA accumulation (Figure 4-1c and d) indicated a disturbance in the reactors operated with only silages and ammonium carbonate. One possible explanation for this disturbance could be that changes in the bacterial communities forced the occuring members of the genus *Methanosarcina* to change their pathways of methane formation (De Vrieze et al., 2012). As an acetic acid accumulation occurred in reactor SB-M2 it can be assumed that the *Methanosarcina* community changed from the hydrogenotrophic to the acetoclastic

pathway. In the reactor M-SB2 an accumulation of propionic acid was observed which might indicate a change from the acetoclastic to the hydrogenotrophic pathway. Hence, it can be assumed that the bacterial community in reactor M-SB2 produced metabolites, in this case propionic acid, which can only be further converted in syntrophic relationships, whereas the bacterial community in reactor SB-M2 degraded the supplied feedstock into high amounts of acetic acid that is further degraded into biogas by acetoclastic methanogens. This assumption was already discussed by Klang et al. (Klang et al., 2019) and is strengthened by the observations in this study.

Beside the predominance of the genus *Methanosarcina*, in the reactor SB-M3, in which 25% of the feedstock composition was changed from sugar beet silage to maize silage, TRF 428, assigned to *Methanoculleus*, showed a high abundance at the first sampling day. Since *Methanoculleus* produces methane via the hydrogenotrophic pathway it was assumed that also *Methanosarcina* used this pathway, at least in the beginning of the experimental phase. Since no acid accumulation occurred it is not possible to conclude whether the acetoclastic or the hydrogenotrophic pathway was used throughout the experimental phase.

4.4.6 Conclusions

Different microorganisms have different nutrient requirements and hence different degradation capacities to certain feedstock compounds. This study showed that the chemical complexity of the supplied feedstocks (either various compounds or complex molecule structures) directly affects the diversity of the biogas microbiome and hence the process performance. As expected the exchange of maize and sugar beet silage against each other resulted in a short-term decrease or increase of the biogas yields according to the chemical feedstock complexity which could be related to a change in the taxonomic composition of the occurring microbial communities. Regarding the hypotheses, this study revealed that certain members of the phylum *Bacteriodetes* as well as members of the order *Spirochaetales* have an affinity to the conversion of easily degradable compounds, indicating their involvement in the acido-/acetogenesis. Moreover this study confirmed previous findings that members of the yet not cultivated phylum *Cloacimonetes* (symbolized by TRF 161) are sensitive to elevated TAN concentration as the respective TRF was only recorded within the reactor systems with low TAN concentrations, while no significant trend towards an increasing or decreasing abundance in relation to the feedstock exchange was detected.

Although established methods, such as TRFLP which was used in this study, are still of great value to study the microbial community dynamics over time in relation to the impact of changing environmental conditions, the disadvantage is the putatively low phylogenetic resolution which hamper a distinct identification of specific microbial process indicators. However, the results give further valuable information in order to better understand the biogas microbiome response to changing environmental conditions which are putatively risky for the process performance. With respect to the current methodological development and an urgent need to identify and verify microbial process indicators which are usable for monitoring the dynamic process of biogas production, new sequencing technologies, such as Nanopore sequencing, are expected to offer the possibility to capture the microbial diversity with high phylogenetic resolution down to the species level.

5 Overall analysis of the three experimental phases

In the previous three chapters the different experimental phases of the performed study are described separate from each other. The experiment was started using digestate from a mesophilic agricultural biogas plant fed with a mixture of energy crops (maize and grass) as well as cattle and chicken manure as inoculum. The digestate had a TAN concentration of around 4.0 g L^{-1} and was diluted with tap-water 1:2 prior to start-up. During the first 337 days of the experiment the reactors were operated as triplicate with sugar beet silage or maize silage as sole feedstock and the community development during the adaptation to the supplied feedstocks was investigated (phase 1). Between day 338 and 911 the microbial community response and the effects on the process performance to an increasing TAN concentration induced by a stepwise increase of the amount of animal manure or ammonium carbonate was studied (phase 2). During the last part, until day 981, the feedstocks sugar beet silage or maize silage were completely exchanged against each other (phase 3) and also here the microbial response and the effects on the process performance was investigated. With exception of the start-up phase the OLR was 2.0 g_{VS} L^{-1} d^{-1} throughout the whole experiment. The diversity and dynamic of the microbial communities were monitored using TRFLP combined with a cloning/sequencing approach.

During the first experimental phase a specific adaptation of the microbial community to the supplied feedstocks sugar beet silage and maize silage was recorded. The community development was related to differences in the chemical composition of the feedstocks. Sugar beet silage has higher amounts of easily degradable compounds, such as sugar, ethanol and acetic acid, whereas maize silage contains higher amounts of more complex compounds, such as cellulose, hemicellulose and lignin. The anaerobic degradation of the more easily degradable compounds led to a bacterial community with high abundances of members of the phylum *Bacteroidetes* whereas the reactors operated with maize silage showed a more even distribution with members of various bacterial phyla such as *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Spirochaetes*, *Synergistetes* and *Thermotogae*. The archaeal community changed from a predominance of hydrogenothropic methanogens (within the inoculum) to a *Methanosarcina*- or *Methanotrix*-dominated community in the reactors fed

with sugar beet silage or maize silage respectively. Regardless of the different community compositions the biogas yields and the methane amounts were similar in all reactors.

As the TAN concentration was increased, either through a changed feedstock composition or through addition of ammonium carbonate during the second experimental phase, a further community change took place (Fig. 4-1). Moreover, at a TAN concentration of 3 to 4 g L^{-1} a process disturbance occurred in all reactors, notable in an accumulation of VFAs. To counteract the process disturbance, the feedstock-supply was stopped and slowly restarted as the microbial community needed time to adapt to the higher TAN concentration. In this phase the former predominant acetoclatic genus *Methanothrix* in the maize reactors disappeared. Also the bacterial community changed. However this change was not as distinct as in the archaeal community, but occurred over the course of time. The two reactors given animal manure developed a more equal bacterial community structure then the reactors treated with ammonium carbonate. Compared to the reactors where the TAN concentration was not increased, a decrease of member to the yet not cultivable phylum *Cloacimonetes* (WWE1-cluster) to under the detection limit was recorded. Hence it is possible, that the disappearance of members of the phylum *Cloacimonetes* could be used as indicator for an upcoming process disturbance caused by TAN inhibition.

At day 918 the basic feedstocks sugar beet silage and maize silage were exchanged against each other. The addition of maize silage in the former sugar beet silage degrading reactors (SB1 and SB2) resulted in a decrease in the biogas yield due to the more complex chemical composition of maize silage. In the reactors M1 and M2, where maize silage was exchange with the more easy degradable feedstock sugar beet silage the biogas yield increased as the microbial community did not had any more complex compounds to degrade. In the reactors given sugar beet silage the relative abundance of members of the phylum *Bacteroidetes* increased, whereas its abundance decreased in the reactors given maize silage over the course of time. In the reactors where the feedstock mixture contained 75% animal manure (SB3 and M3) no changes, neither in the microbial community composition nor in the process parameters were observed.

In this chapter the results from the entire experiment are shown and discussed. For this overview a renewed band detection and export from BioNumerics were performed with all samples, so that small changes compared to the previous chapters may occur.

5.1 Chemical and operational parameters

The used feedstocks were sugar beet silage, maize silage, swine manure and chicken manure. Some of the main chemical characteristics of these feedstocks are shown in Table 5-1.

Table 5-1: Chemical composition of the used feedstocks. TS = total solids, FM = fresh mass, VS = volatile solids, TKN = total Kjehldahl nitrogen, TAN = total ammonium nitrogen, g = gram, L = liter, mg = milligram.

Parameter	Unit	Sugar beet silage	Maize silage	Swine manure	Chicken manure
TS	%FM	14	27	3	50
VS	%TS	95	96	66	76
TKN	g L^{-1}	1,3	3,2	2,5	27,2
TAN	mg L^{-1}	113	61	1675	3311
Lignin	mg L^{-1}	0,42	59,4	21	250
Hemicellulose	mg L^{-1}	35	486	69	800
Cellulose	mg L^{-1}	49	567	39	700

The TS of the swine manure is much lower than the TS of the other feedstocks and the VS is lower in the used livestock manures than in the silages, indicating a lower amount of organic compounds. Because of this, the HRT was shortened from phase one to two, from 86 days to 43 days. The nitrogen compounds are much higher in the chicken manure than in the other feedstocks and also the amount of lignin, hemicellulose and cellulose is higher in the chicken manure.

A NMDS of the chemical and operational parameters showed a difference between the reactors SB1 and M1 to the reactors SB2, SB3, M2 and M3 (Figure 5-1). The parameters TKN, TAN, NH_3-N and conductivity (r^2 >0.8) were highly relevant for the clustering of the reactors. In the reactors SB1 and M1 these parameters decreased during phase one and remained rather similar and constant throughout the rest of the experiment, with TKN values of around 1.5 g L^{-1}, TAN value of around 0.3 g L^{-1}, NH_3-N values of around 4 mg L^{-1} and conductivity values of around 4 mS cm^{-1} (Figure 5-2 A and D).

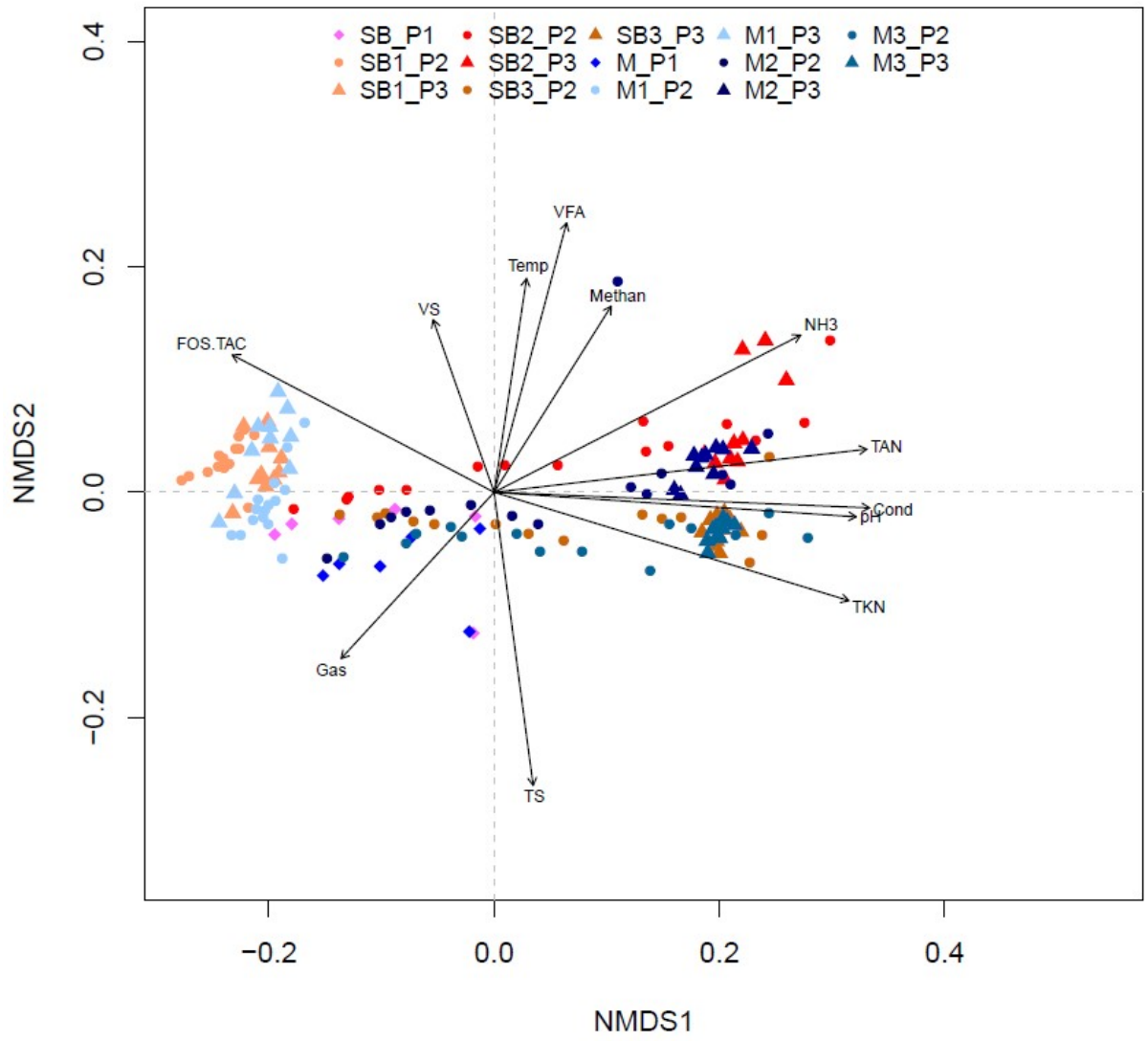

Figure 5-1: Nonmetric dimensional scaling (NMDS) of the chemical and operational parameters of the reactors SB1, SB2, SB3, M1, M2 and M3. The length and directions of the vectors indicates the influence of the parameter on the clustering (p=0.001). Only the time points used for the microbial analysis are shown. SB = sugar beet silage, M = maize silage.

In the reactors given ammonium carbonate or animal manure the values of these parameters increased over the course of time, the highest TKN value was around 6 g L^{-1}, TAN 4.4 g L^{-1}, NH_3-N 800 mg L^{-1}, pH 8 and conductivity 26 mS cm^{-1} (Figure 5-2 B, C, E and F) With exception of the TKN, the increase proceeded similar in the four reactors. The TKN value was higher in the reactors given animal manure then in the reactor given ammonium carbonate due to the nitrogen compounds in the manure.

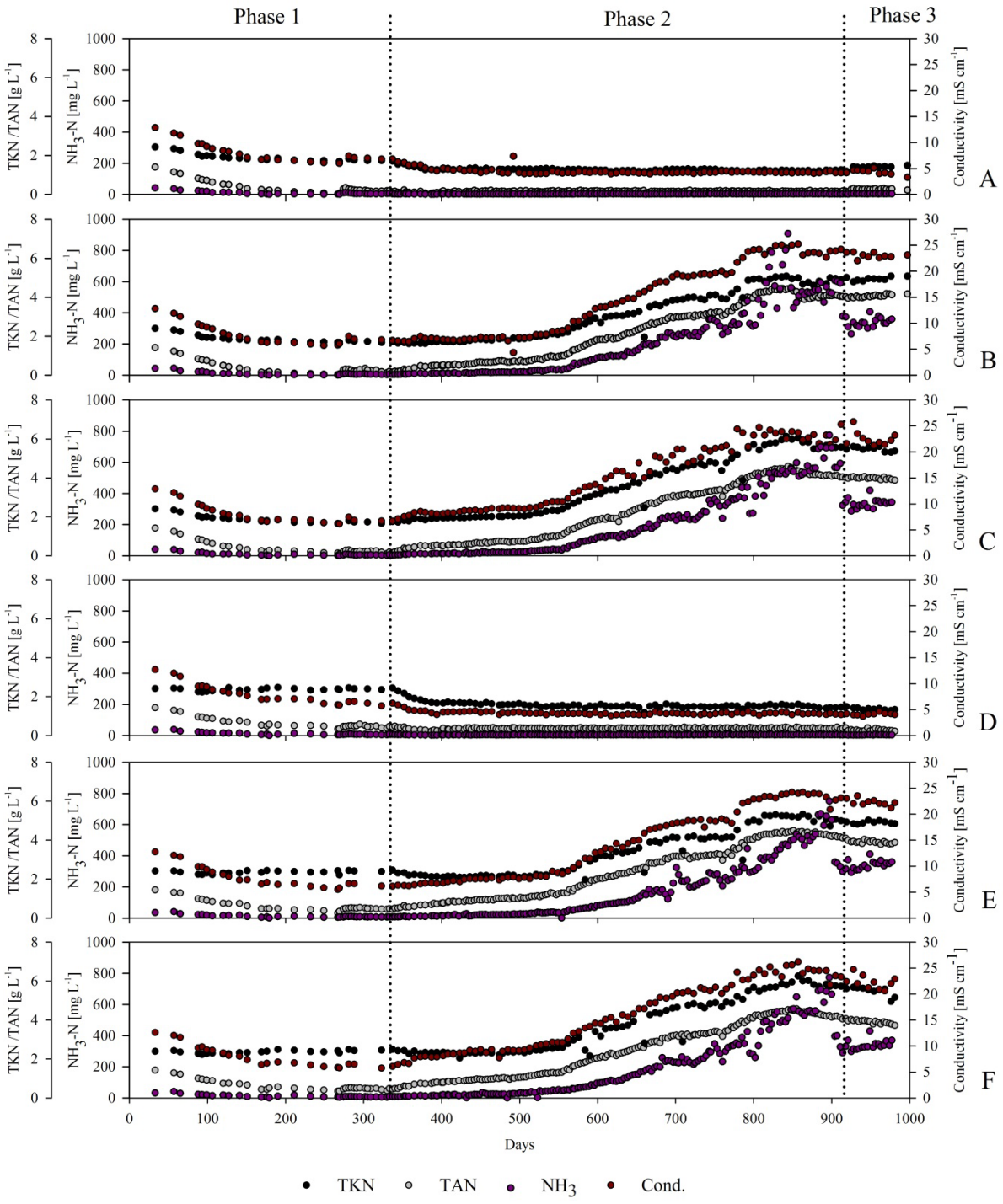

Figure 5-2: Development of the Total Kjeldahl nitrogen (TKN), total ammonium nitrogen (TAN), free ammonia nitrogen (NH_3-N), pH-value and conductivity in the reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E), M3 (F) over the course of time.

Conductivity is a measure of the salt content in the reactors and should not exceed 30 mS cm^{-1} due to possible osmotic pressure (Chen et al., 2008; De Vrieze et al., 2017, 2012). The increased conductivity in this study can mainly be attributed to an increasing release of NH_4^+.

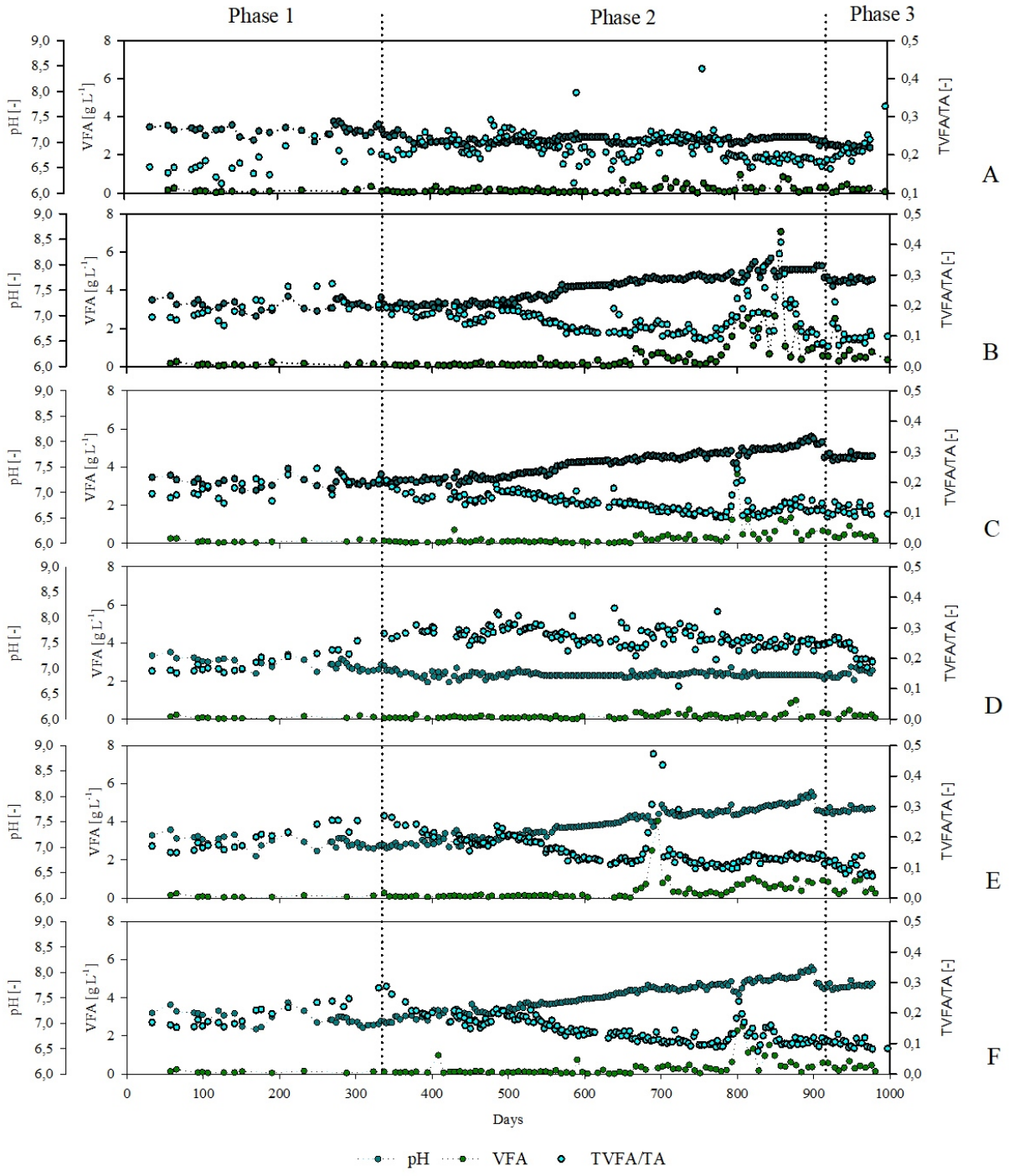

Figure 5-3: Development of the pH, volatile fatty acids (VFA) and TVFA/TA in the reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E), M3 (F) over the course of time.

Also the pH was important for the clustering: in the reactors SB1 and M1 it was around 7 throughout the whole experiment but due to the alkalinity of ammonium carbonate and animal manure it increased up to 8 in the reactors SB2, SB3, M2 and M3 (Figure 5-3).

The direction of the VFA vector in the NMDS plot showed that the concentration was not decisive for the clustering. The concentration was below 1 g L^{-1} most of the time, a value that indicates a stable process (Drosg, 2013). The exception was the disturbance in phase two were the VFA concentration increased to up to 7 g L^{-1}. During the disturbance, with high amounts of acids, the pH value only decreased with around 0.2. Through the addition of ammonium carbonate and animal manure the buffering capacity of the system is so high that the disturbance was not noted in a sinking pH value. This makes it clear that the pH value is not a good parameter to monitor the process stability. But neither the VFA concentration is a suitable parameter to detect a rapid change in the process as the analysis is rather time consuming and do not belong to standard equipment of biogas plant operators. The TVFA/TA is a faster way to get an impression of the situation in the reactor as the analysis is not that complicated. However, also this value has to be handled with caution. A value below 0.3 is generally considered to display a well-running process (Drosg, 2013), although the value should not be compared between reactors but only be used to monitor one reactor over the course of time. The TVFA/TA was higher in the reactors SB1 and M1 then in the reactors SB2, SB3, M2 and M3 (Figure 5-1 and Figure 5-3), although these reactors were operated under stable conditions with low VFA concentrations. As TVFA/TA is the ration between the amounts of volatile fatty acids and the buffer capacity, the addition of ammonium carbonate and animal manure was the reason for the lower TVFA/TA values in the reactors SB2, SB3, M2 and M3. Hence the TVFA/TA values were around 0.1 prior to the disturbance and in, for example, reactor M3 never exceeded 0.3 although the VFA concentration increased. Nevertheless, as the TVFA/TA first change when the disturbance is already happening, also this value is no indicator of an upcoming disturbance with the possibility to timely counteract before the process is profound negatively affected.

The biogas yield, the methane content, the amount of TS and VS as well as the temperature shows the lowest relevance for the distinction between the different reactors (Figure 5-1). The produced biogas per day increased in the beginning of phase one, during the start-up phase as the OLR stepwise increased with 0.5 g_{VS} $L^{-1}d^{-1}$ until the final OLR of 2.0 g_{VS} $L^{-1}d^{-1}$ was reached. After that the biogas yield remains rather stable with an average value of 3.7 L_N d^{-1} throughout the whole experiment, with exception of the disturbance at the end of phase two. In the reactors given manure the biogas yield decreased slightly due to the lower amount of volatile solids in the chicken manure (Figure 5-4). The methane content was also similar over the course of time, with around 55%.

The TS was higher in the reactors given maize silage as the TS of the maize silage was about twice as high as the TS of the sugar beet silage (Figure 5-4, Table 5-1: Chemical composition of the used feedstocks). Between phase one and two the TS decreased as the HRT was shortened, from 86 to 43 days and the reactors 1 and 2 were given tap water and the reactors 3 swine manure. With the introduction of chicken manure at day 505 the TS in the reactors SB3 and M3 increased again as chicken manure had a TS of 50% of the fresh mass (Table 5-1). The maize silage contains a higher amount of complex compounds, such as lignin that only can hardly be degraded by anaerobic bacteria (Li et al., 2018; Mulat, 2018). In the reactors given chicken manure the amount of VS decreases as the chicken manure only had a VS of 76%$_{TS}$ compared with around 95% for the silages (Table 5-1). The average temperature in the reactors was 41°C.

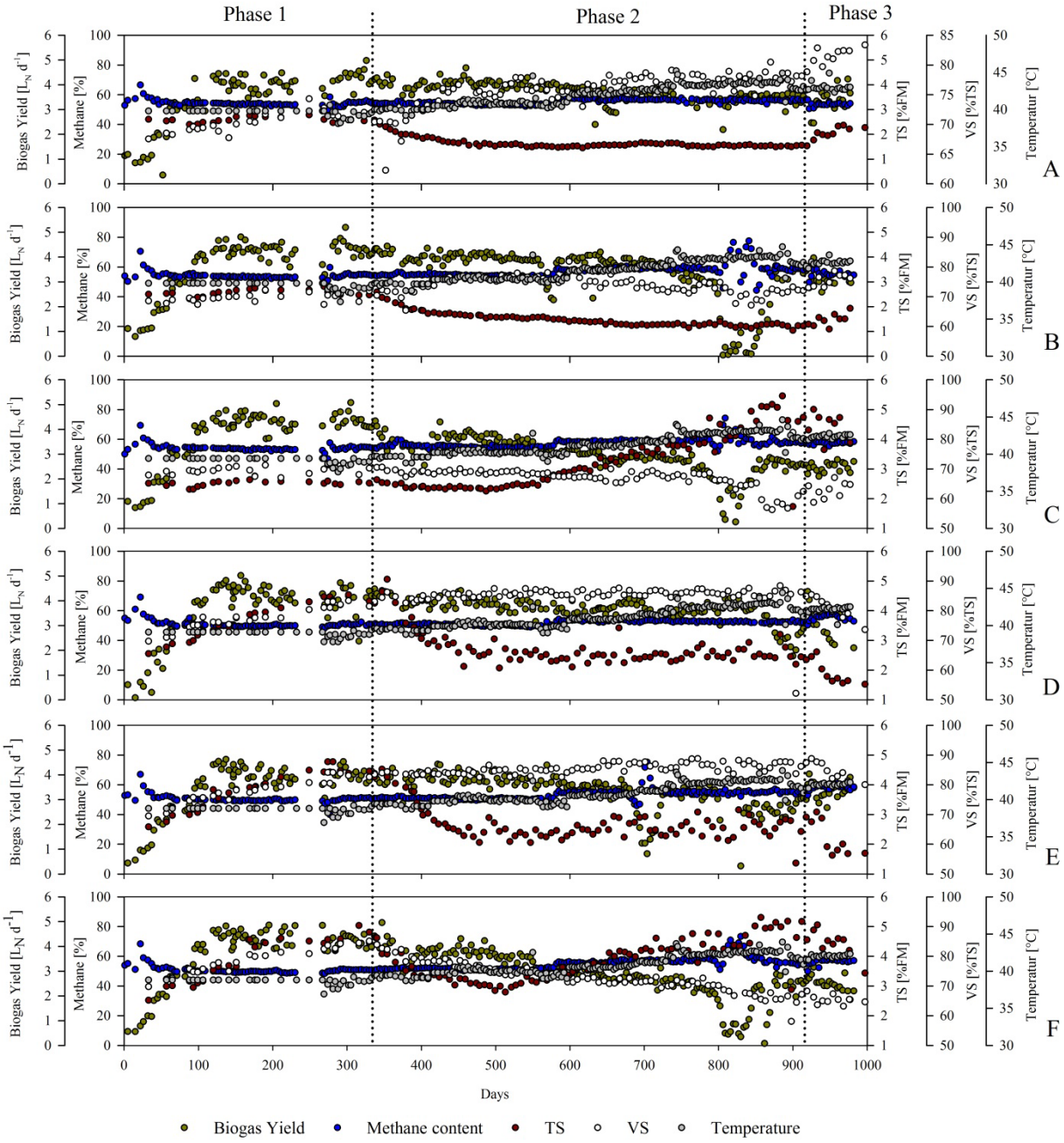

Figure 5-4: Development of the biogas yield and methane content, total solids (TS) and volatile solids (VS) as well as the process temperature in the reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E), M3 (F) over the course of time.

5.2 Relationship between process chemistry and microbiology

In Figure 5-5 the ten most relevant bacterial TRFs as well as the most significant archaeal TRFs for the NMDS clustering of the chemical parameters are shown.

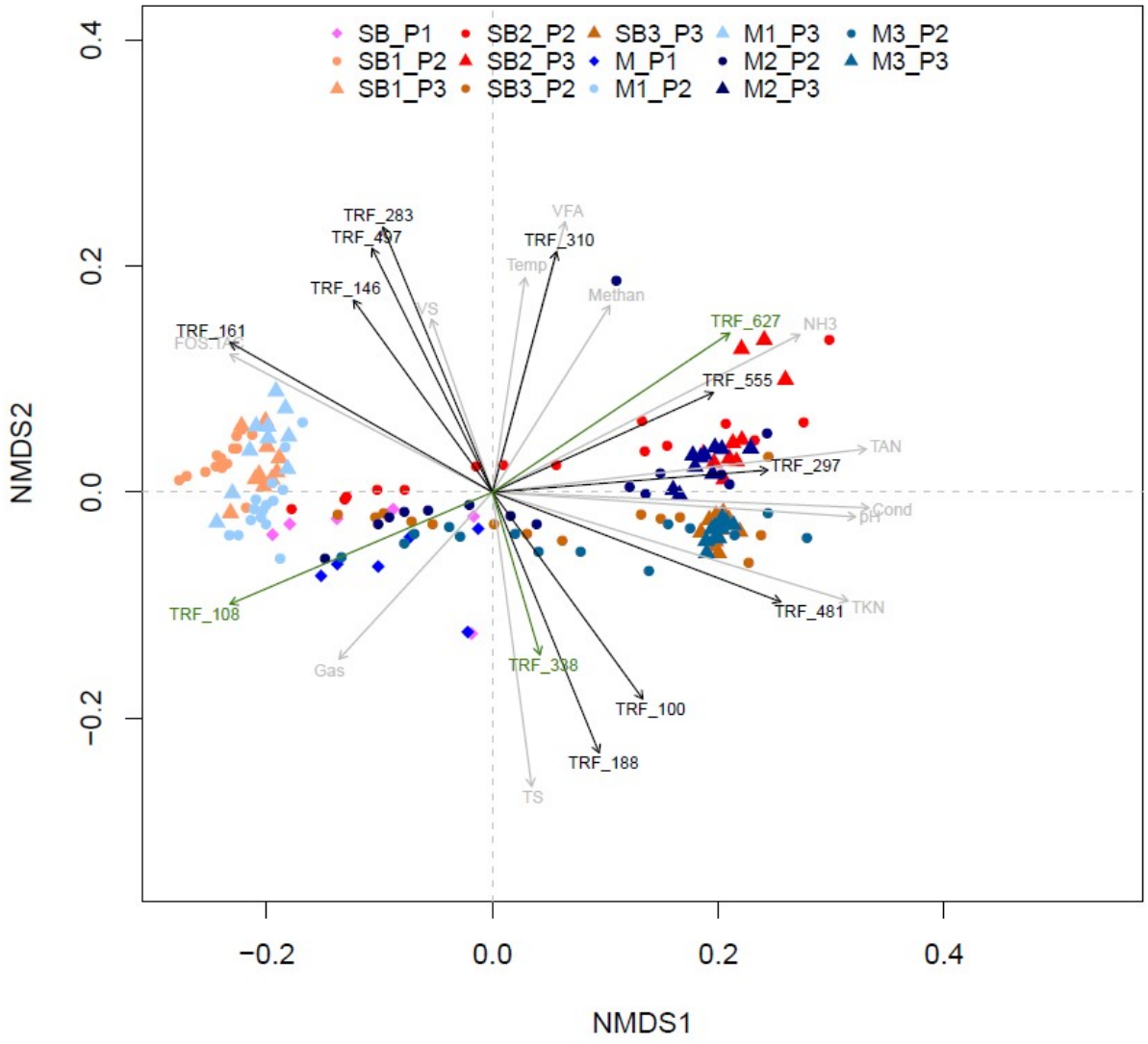

Figure 5-5: Non-metric multidimensional scaling NMDS of the inverstigated reactor system SB1, SB2, SB3, M1, M2 and M3 based on the chemical and operational parameters combined with the microbial community structure. The length and directions of the vectors indicates the influence of the biotic and abiotic parameter on the clustering (p=0.001). Only the time points used for the microbial analysis are shown. SB = sugar beet silage, M = maize silage.

The most significant TRF for the reactors SB1 and M1 is the TRF 160 (161, $r^2 = 0.6$) that was assigned to the yet not cultivable phylum *Cloacimonetes.* In chapter 3 it was discussed that the disappearance of members of this phylum might be an indicator for an upcoming process disturbance due to increasing TAN concentrations. This possibility is strengthened through the analysis of the whole experimental period. Figure 5-6 shows the structure of the bacterial community over the course of time, while Figure 5-7 highlights the TRF assigned to the phylum *Cloadimonetes,* which decreases to under detection limit in the reactors with increasing TAN concentrations. This assumption must be further validated in order to develop rapid and economical detection methods to be used by plant operators and consultants (Theuerl et al., 2019b).

The significant TRFs for the reactors given either ammonium carbonate or animal manure were TRF 297, 481 and 555. TRF 481 showed the highest significance (r^2=0.6) and the vector direction indicates high importance for the reactors getting animal manure. None of these TRFs could be assigned to sequences found in the sequence library; hence no phylogenetic or ecological assignment was possible.

Considering the archaeal community the TRF 108, assigned to the genus *Methanothrix*, showed the highest significance for the community in the reactors SB1 and M1 (r^2=0.5), hence toward the reactors with low VFA concentrations and no visible disturbances. The TRF 627, assigned to the genus *Methanosarcina*, were important for the reactors SB2, SM3, M2 and M3, hence the more inhibited reactors, whereas the vector showed in direction of higher concentrations of free ammonia (r^2=0.5).

5.3 Importance of members of the phylum *Bacteroidetes*

The most obvious change in the bacterial communities was the dominance of the TRF 84 to the dominance of TRF 93 in the reactors given sugar beet silage (Figure 5-6A, B and C). These two TRFs belong to the phylum *Bacteroidetes*, order *Bacteroidales* and could not be further assigned, so that no conclusions about the ecological function can be made. However, it can be assumed that the TAN concentration influenced the change: until day 260 the TAN concentration decreased from around 2 g L^{-1} to 0.2 g L^{-1}, and in this time also the relative abundance of TRF 84 decreased. To prevent a further decrease and a possible nutrient-deficit, ammonium carbonate was regularly added to the reactor, keeping the TAN concentration at around 0.3 g L^{-1}. At this concentration relative abundance of TRF 93 increased and after day 386 this TRF dominated the community in the SB reactors. The reactor SB1 was operated with low TAN concentrations for the rest of the experiment and the TRF 93 remained dominant until the feedstock was changed from sugar beet silage to maize silage (starting at day 918). At the same time the feedstock in reactor M1 was changed from maize silage to sugar beet silage. Here was a small increment of TRF 93 noticed (Figure 5-6D) indicating that the TRF-related microorganism have an affinity to convert more easy degradable compounds provided by the sugar beet silage. In the reactors SB2 and SB3 the abundance of TRF 93 decreased with increasing TAN concentrations showing that the related microorganisms are TAN-sensitive. In these reactors a small increase of the TRF 84 was observed, however the

abundances were not comparable to the abundances in the beginning of the reactor operation, possibly because the TAN concentration increased to fast.

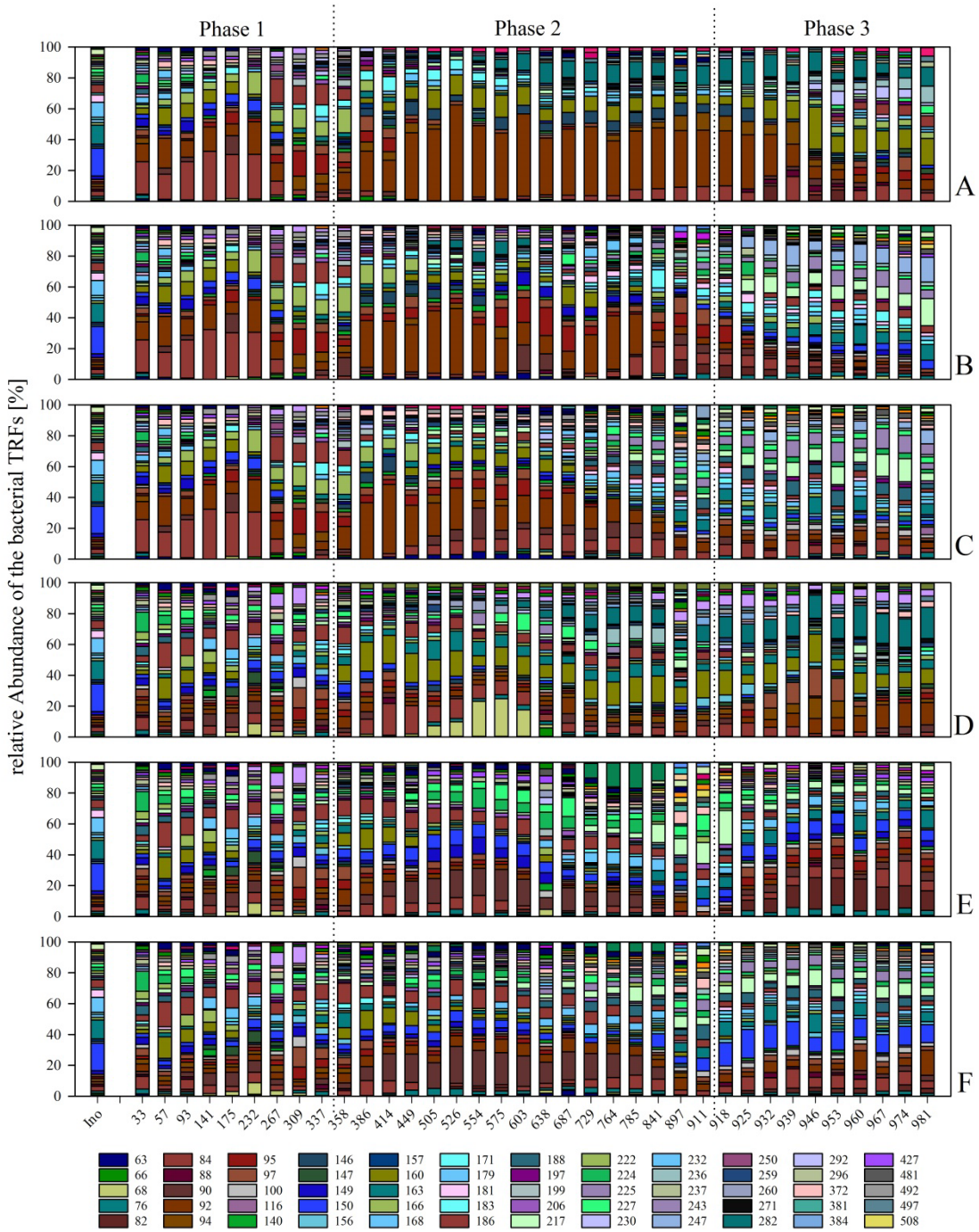

Figure 5-6: Structure of the bacterial community in reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E) and M3 (F). Coloured bars symbolize the detected terminal restriction fragments (TRFs) in base pairs (bp) and their relative abundance. Only TRFs with a relative abundance over 3% in at least one sample are shown. Each sampling point is given as median value of three technical replicates. Ino stands for inoculum and the numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

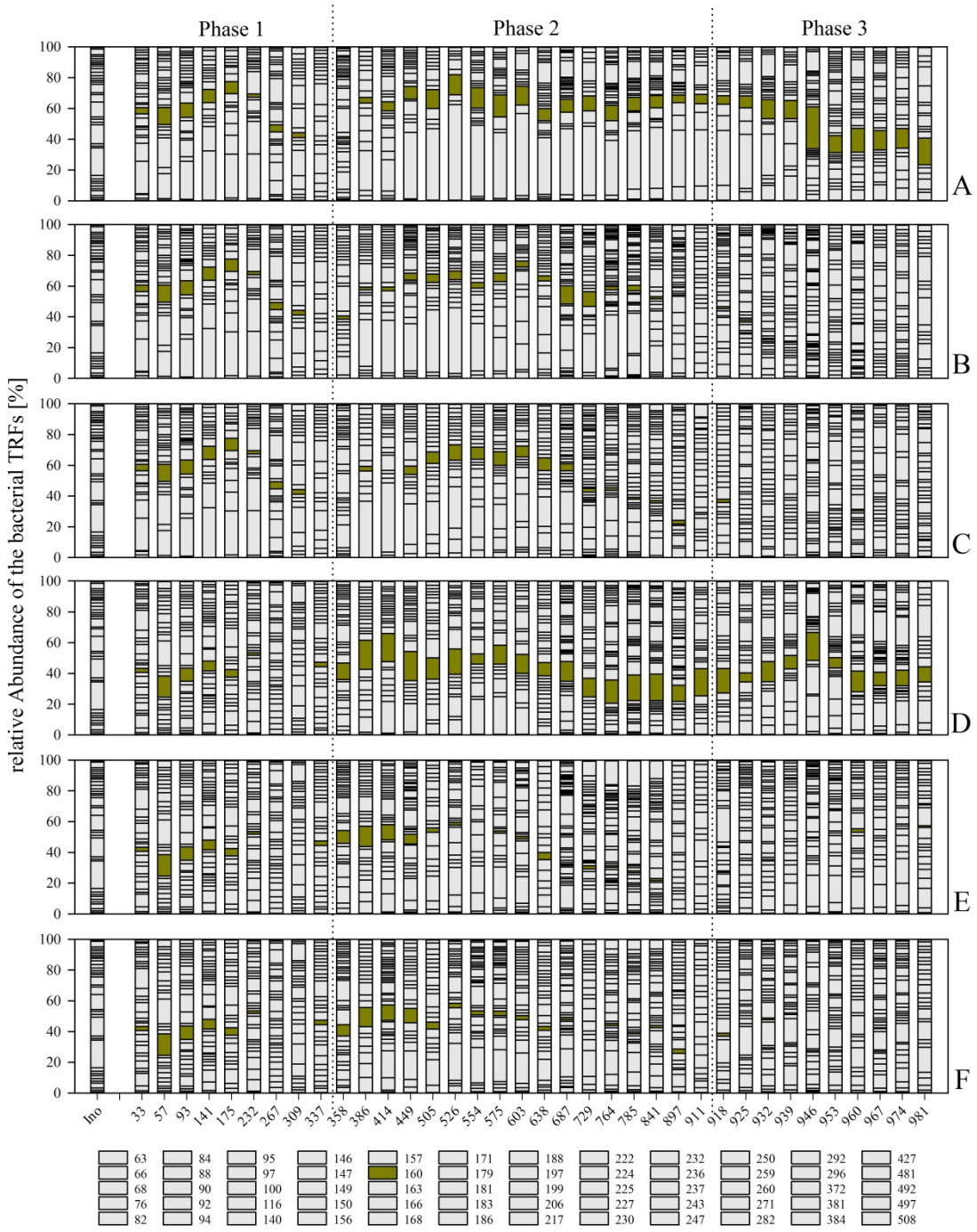

Figure 5-7: Structure of the bacterial community in reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E) and M3 (F). The coloured bar symbolize the terminal restriction fragments (TRFs) assigned to the phylum *Cloadimonetes.* Only TRFs with a relative abundance over 3% in at least one sample are shown. Each sampling point is given as median value of three technical replicates. Ino stands for inoculum and the numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

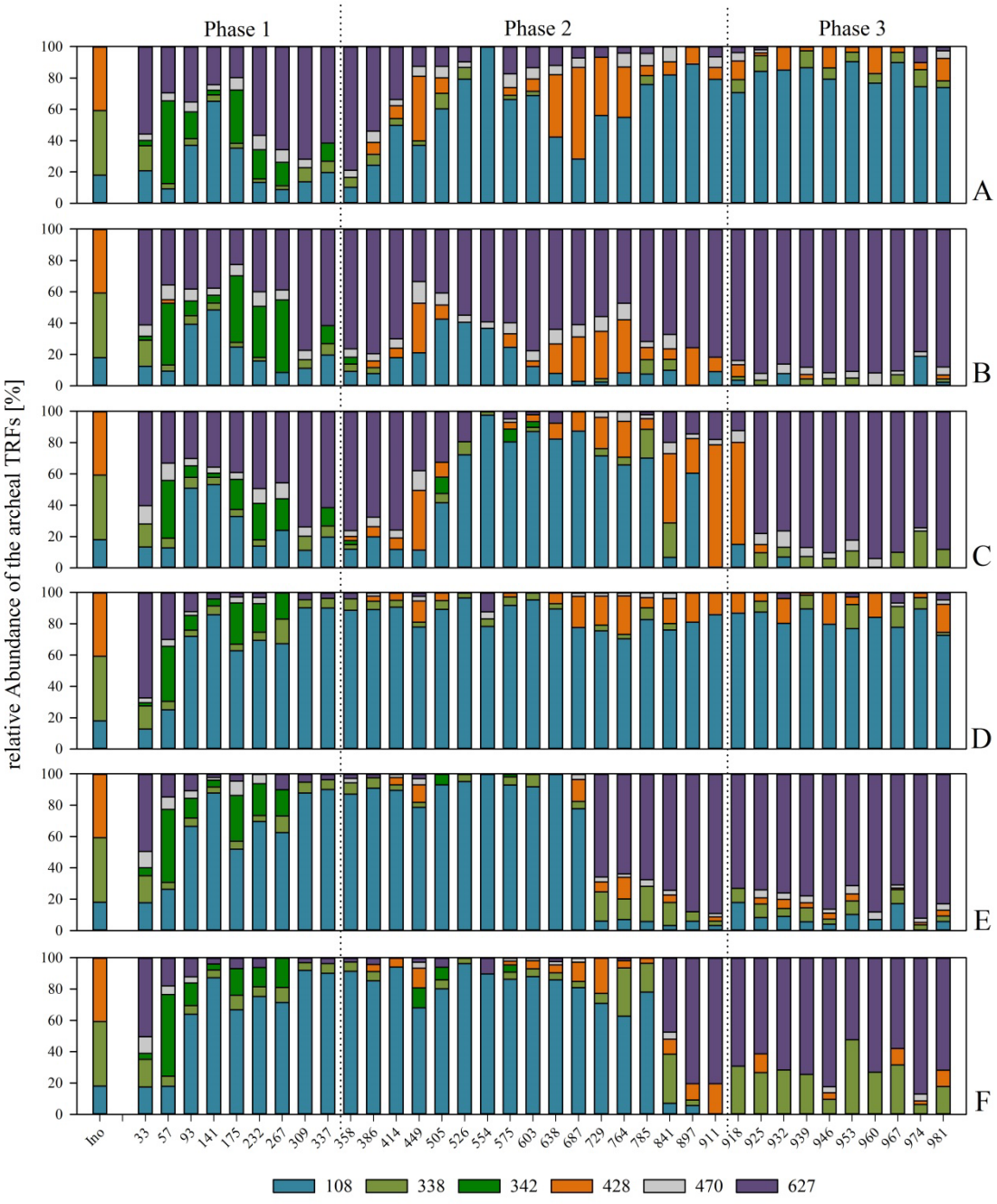

Figure 5-8: Structure of the archaeal community in reactors SB1 (A), SB2 (B), SB3 (C), M1 (D), M2 (E) and M3 (F). Coloured bars symbolize the detected terminal restriction fragments (TRFs) in base pairs (bp) and their relative abundance. Each sampling point is given as median value of three technical replicates. Ino stands for inoculum and the numbers in sampling point descriptors indicate the duration of continuous fermentation in days since start-up.

5.4 Temporal development of the microbial communities

The bacterial communities showed a different development in the six reactors over the course of time (Figure 5-6A and Figure 5-9), with a clear differentiation between the reactors with low TAN (SB1 and M1) and with increasing TAN (SB2, SB3, M2 and M3) concentration. The bacterial communities of the reactors with increasing TAN concentrations, either through addition of ammonium carbonate or through exchange of the feedstock composition into nitrogen-rich animal manure, develops in the same direction. Also the inoculum sample is found in the cluster containing samples with high TAN concentration. This shows that the TAN concentration impacts the community composition stronger than other chemical compounds of the supplied feedstocks as the reactors 1 and 2 were given 100% sugar beet silage or maize silage and reactors 3 are operated with only 25% silage and 75% animal manure at the end of the experiment.

In contrast to the bacterial communities, the archaeal community showed no clear differentiation between the reactors with low and high TAN concentration (Figure 5-9B). The reactors M1 and SB2 showed the lowest diversity and dynamic variations, whereas TRF 108 (*Methanothrix*) dominated in reactor M1 and TRF 627 (*Methanosarcina*) in reactor SB2. Furthermore, at the archaeal level no similarity with the inoculum was detected, neither in the beginning of the experimental phase, nor at the end. The detected archaeal community in the inoculum consisted of members of the genera *Methanothrix*, *Methanobacterium* and *Methanoculleus*, whereas the abundances of the two latter were higher and hence the community mainly consisted of organisms performing the hydrogenotrophic pathway of methane formation. This is not surprising considering that the TAN concentration of the digestate used as inoculum was around 4 g L^{-1}. During the first part of the experiment the mixothropic genus *Methanosarcina* became predominant. As already discussed is previous chapters, *Methanosacina* is rather robust to changes and disturbances (De Vrieze et al., 2012) and although it was under the detection limit in the inoculum it was able to prevail during start-up, as the microbial community of the inoculum adapted to the new feedstocks. Shortly after the experimental start-up the genus *Methanosarcina* remained dominant in the reactors given sugar beet silage whereasthe acetoclastic genus *Methaothrix* became dominant in the maize silage reactors. This in combination with a less dynamic bacterial community indicates a well-operating system with low VFA values.

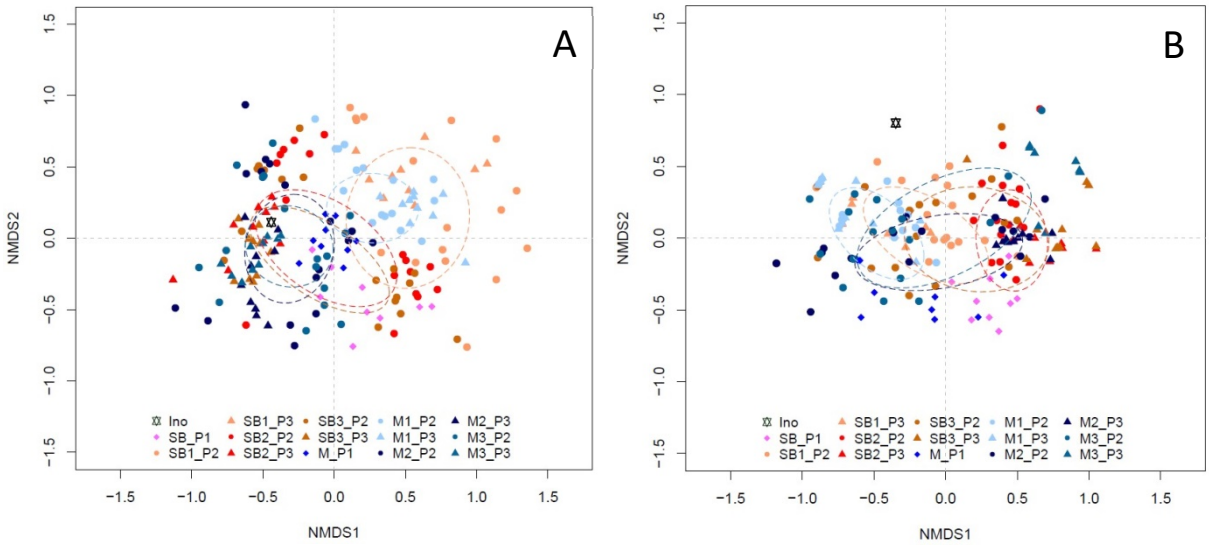

Figure 5-9: Non metric dimensional scaling (NMDS) of the bacterial (A) and archaeal (B) community. The ellipses symbolise the dispersion over the entire experiment of each reactor.

The NMDS of the reactors SB1 and M1 shows that the bacterial community in both reactors develops away from the inoculum (Figure 5-10A). The community in reactor SB1 shows a higher dynamic variation then in reactor M1 in all three phases. None of the detected TRFs was highly specific for the clustering ($r^2 < 0.6$). The archaeal community differed during phase one and two, as the TRF 627 (*Methanosarcina*) was significant for the community in the sugar beet reactor during phase one and the TRFs 428 and 470, assigned to the genus *Methanoculleus* and *Methanomassiliicoccus,* during phase two. The abundance of TRF 470 was comparable low as the highest abundance was 10% compared with the highest abundance of *Methaosarcina* with 80%. The only known species of this genus, *Methanomassiliicoccus luminyensis,* produces methane through reduction of methanol (dis Dridi et al., 2012). The most significant archaeal TRF in the maize reactor was TRF 108, assigned to the acetoclastic genus *Methanothrix* (Figure 5-10B).

Also the NMDS of the reactors SB2 and M2 showed a different development of the bacterial communities (Figure 5-10C). During phase two the dynamic in the maize reactors was slightly higher than in the sugar beet reactors and during phase three, when the feedstock was exchanged, the communities became more similar. The TRF 481 (phylogenetically unassigned) showed the highest significance for the community development during phase three (r^2=0.6). Furthermore, the communities showed a higher similarity to the inoculum towards the end. Also the archaeal community was more dynamic in the maize reactor than in the sugar beet reactor. During phase two a change from TRF 108 to TRF 627 was recorded, hence from the acetoclastic *Methanothrix* to the mixotrophic *Methanosarcina* (Figure 5-10D).

In the reactors SB3 and M3, were the feedstock was gradually changed into animal manure during phase two, the communities became more similar over the course of time (Figure 5-10E). In phase three, as the remaining 25% silage was changed, the community compositions remained rather unchanged. Similar to the reactors SB2 and M2 the most significant TRF for phase three was the unassigned TRF 481 (r^2=0.8), but the number of relevant TRFs (p=0.001, r2>0.6) for the communities was higher, as also TRF 225, 188, 236 and 297 were significant. The archaeal communities in the reactors were dominated by TRF 627 (*Methanosarcina*) (Figure 5-10E).

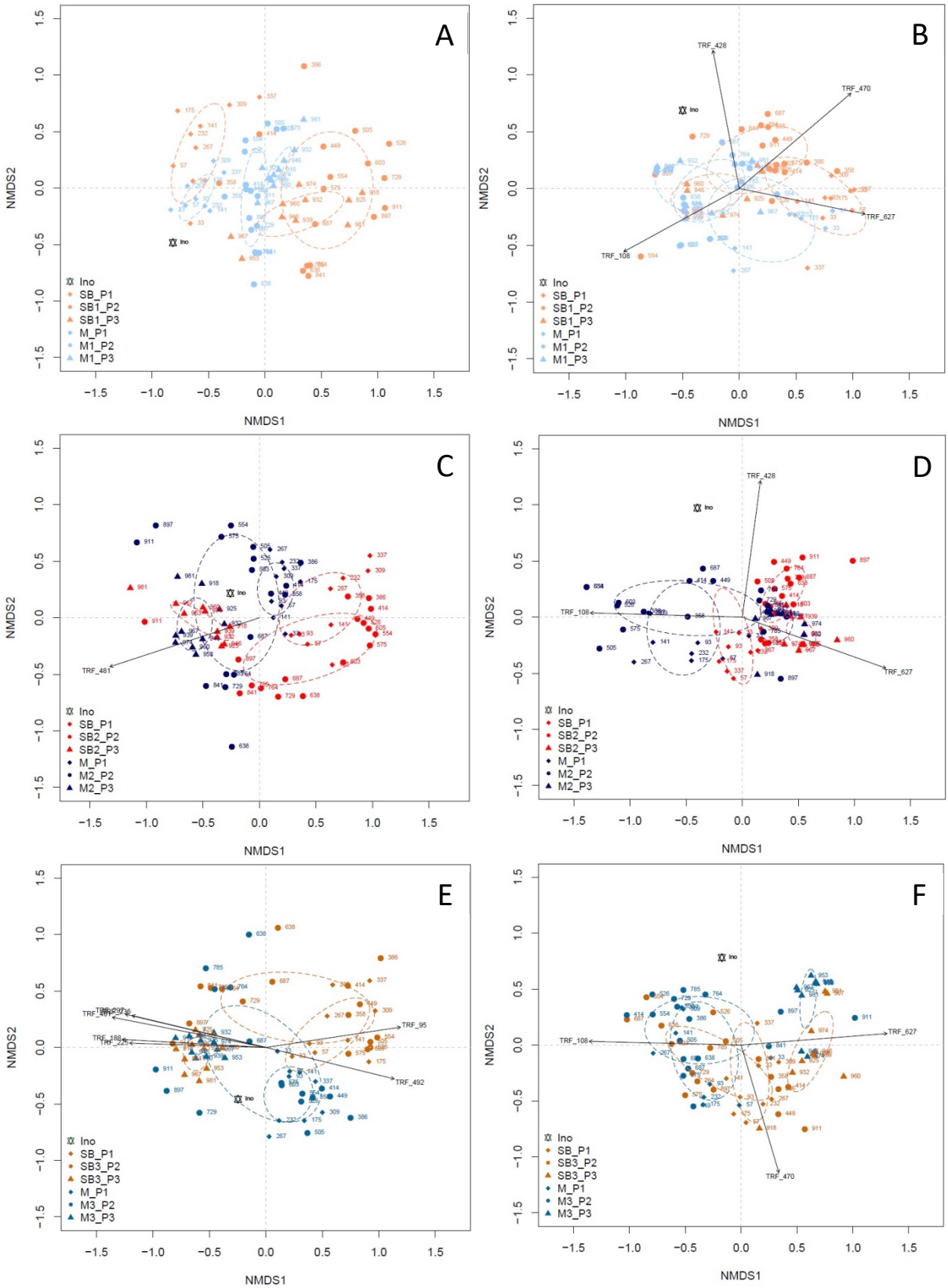

Figure 5-10: Non metric dimentional scaling (NMDS) of the bacterial (A, C, E) and the archaeal (B, D, F) in the reactors SB1 and M1 (A-B), the reactors SB2 and M2 (C-D) and the reactors SB3 and M3 (E and F). The ellipses symbolizes the dispersion of each experimental phase and the vectors symbolizes the most significant TRFs (r^2>0.6, p=0.001).

The NMDS calculation with the three reactors given sugar beet as basic feedstock shows a clear differentiation between the reactors with low TAN concentration (SB1) and increasing TAN concentrations (SB2 and SB3). Significant for the reactors with low TAN concentrations were the TRFs 283, 497 and 607 and for the ones with high TAN to the TRFs 297 and 481 (r^2>0.6, p=0.001). The TRF 283 was assigned to the phylum *Spirochetes*, the rest were unassigned. As already discussed is little known about this phylum but this study indicates that members of this phylum might have a higher affinity towards feedstock with high sugar content.

Also the reactors given maize silage showed a clear differentiation between low and high TAN concentrations. The TRFs 160, 275 and 605 were significant for the community in the reactor M1 and only the TRF 481 was significant for the reactors M2 and M3. The TRF 160 was assigned to the already discussed candidate phylum *Cloadimonetes*, which was found both in the sugar beet and the maize reactors with low TAN concentrations but apparently have a higher significance in the reactor given maize silage.

In both the reactors given sugar beet silage as well as the reactors given maize silage was the TRF significant for high TAN concentrations 627, hence *Methanosarcina*.

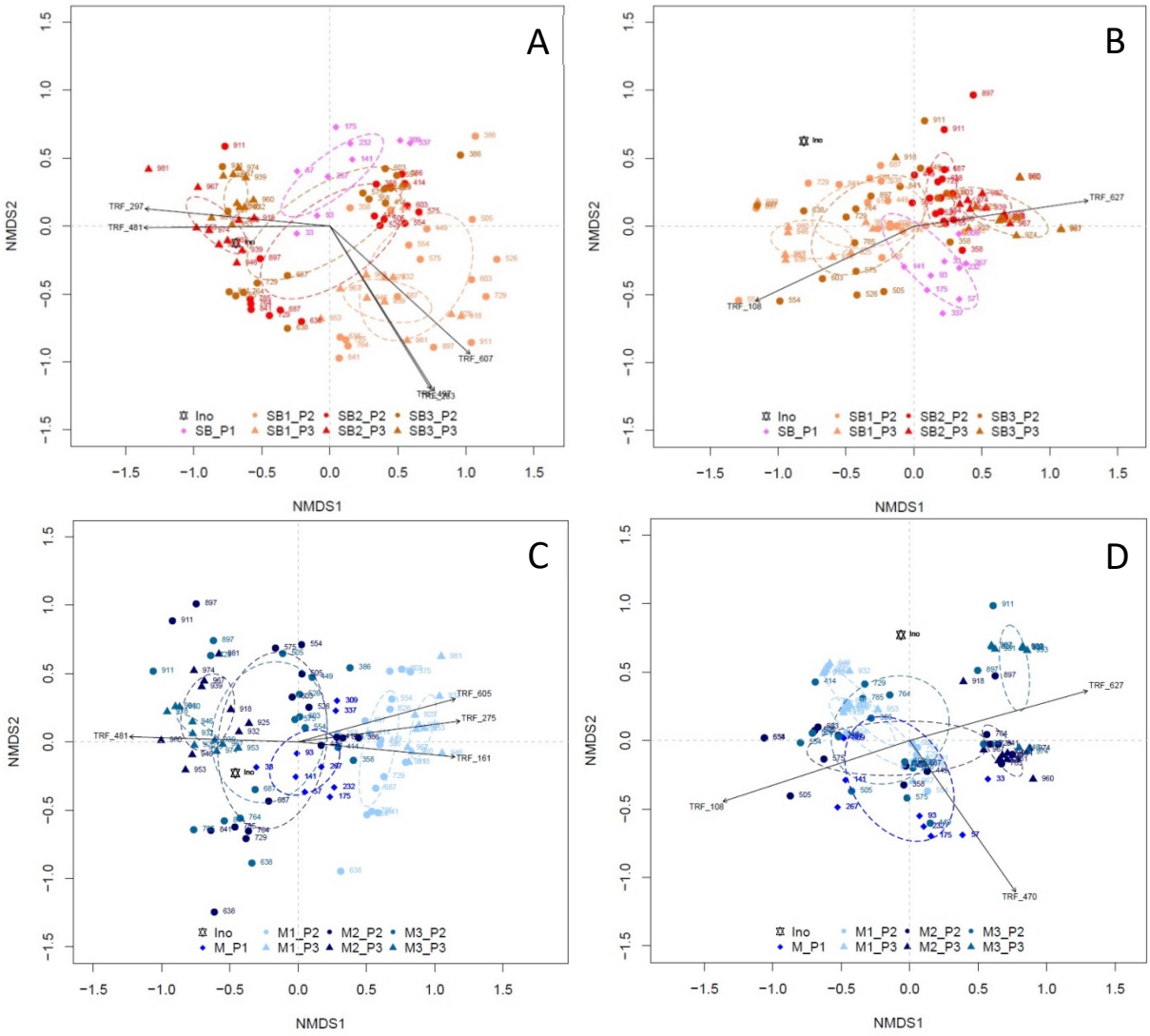

Figure 5-11: Non metric dimentional scaling (NMDS) of the bacterial (A, C) and the archaeal (B, D) in the reactors SB1, SB2 and SB3 (A-B) and the reactors M1, M2 and M3 (C-D). The ellipses symbolizes the dispersion of each experimental phase and the vectors symbolizes the most significant TRFs (r^2>0.6, p=0.001).

5.5 Concluding remarks

The digestate used to inoculate the reactors were operated with a mixture of energy crops and animal manure and had a TAN concentration of around 4 g L^{-1}. With decreasing TAN concentrations, caused by a feedstock change to sugar beet or maize silage respectively, the bacterial community in the reactors became less similar with the inoculum. However, as the TAN concentration was increased again a higher similarity to the inoculum was noted. As the TAN concentration in this study was increased both through addition of ammonium carbonate as well as through a changed feedstock composition, the results indicates that mainly the TAN

and less other chemical composition of the animal manure were decisive for the community development.

The used feedstocks sugar beet silage and maize silage differ in their chemical composition (Table 2-1). The sugar beet silage contains higher amounts of easy degradable compounds and sa shown in chapter 2 is this feedstock quickly degraded into biogas (Figure 2-1). The corresponding bacterial community had an uneven community organization with high relative abundances of mainly one TRF. According to (Marzorati et al., 2008) is an uneven community organization more fragile to external changes. As shown in Figure 5-10A is the bacterial community in the reactor SB1 more dynamic then the bacterial community in reactor M1, although the reactor M1 shows a higher diversity. This rather unstable community in the sugar beet silage reactor system might indicate a higher susceptibility to disturbances compared with the community in the maize reactor system. However it is the other way around within the archaeal communities: In the reactor SB1 members of the archael genra *Methnaosarcina*, *Methanothrix*, *Methanoculleus* and *Methanomassiliicoccus* occured with changing relative abundances while the community in the maize reactor showed very high relative abundances of the acetoclastic genus *Methanothrix.* Although a high abundance of *Methanothrix* is suggested to indicate a well-functioning and uninhibited system (Regueiro et al., 2012), the genus *Methanotrix* is also known for their sensitivity to e.g. increasing TAN, especially NH_3 concentrations (De Vrieze et al., 2012). Consequently, the question that remains was: Which reactor system, respectively which specific adapted microbiomes is more stable against disturbances? The in chapter 3 described disturbance, induced through increasing TAN concentrations, occurred in reactors M2 as the TAN concentration was around 3 g L^{-1} and in the other three reactors as the concentration was around 4 g L^{-1}. Here the reactor M3 needed more time to recover then reactor SB3. Hence it seems as if the maize silage degrading microbiome was more sensitive to the increasing TAN concentrations than the sugar beet degrading microbiome. Several studies have showed that the archaeal community and mainly the genus *Methanothrix* is highly sensitive to elevated TAN concentrations, most likely the free ammonia part. So with increasing TAN concentrations the genus *Methothrix* was inhibited. As its relative abundance was very high and the growth rates of syntrophic acetate oxidizing bacteria and hydrogenotrophic archaea are slow, the produced intermediate acetic acid accumulated in the reactor. In this case, when mainly the archaeal community was inhibited, the reactors given maize silage were the most sensitive, while the more flexible archaeal community of the sugar beet silage reactor, with members able to

perform different pathways of methane formation, were more resilient or functional redundant.

One thing that is clear throughout the whole study is the high amount of either unassigned or only at phylum or order level assignable bacterial TRFs. The most obvious and most abundant of these TRFs were TRFs 86 and 93. Both TRFs belong to the same phylum, *Bacteroidetes*, but their related 16S rRNA gene sequences showed only a similarity of around 88% to the next known species. The TRF 283, assigned to the phylum *Spirochetes* were found mainly in the reactors with low TAN concentrations, whereas the unassigned TRFs 481 and 297 were found mainly in reactors with high TAN concentrations. Despite the intensive efforts of deciphering the anaerobic digestion microbiomes, these findings shows that possibly important members are still undetected, even less described.

Without more knowledge about these unknown bacteria it is only possible to speculate about their metabolism and ecological function. In chapter 2 it was discussed that members of the phylum *Bacteroidetes* seems to be important secondary fermenters whereas Hahnke et al. (2015) reports that a complete genome sequencing of a novel member of this phylum is involved in the hydrolysis of the feedstock. Hence is the assignment at the phylum-level far from sufficient.

Independent from the microbiome studies, the analysed chemical parameters are apparently not enough as only some produced intermediates are analysed and they cannot explain the differences in for example the reactors M2 and M3 and why M2 is inhibit prior to M3 as the TAN- and VFA concentrations are similar.

6 Concluding discussion and outlook

Currently there are almost 9.500 full-scale biogas plants in Germany which are mainly operated with energy crops and residues from livestock husbandry (Daniel-Gromke et al., 2018). As shown in the presented work, a variation of the supplied feedstocks and hence a variation of the chemical composition directly affects the diversity of the biogas microbiome. In order to ensure an overall stable process with low susceptibility to process disturbances it must be taken into account that every population (all individuals of a species in a given habitat) of the biogas microbiome has different living requirements (e.g. nutrients, temperature, pH, co-occurring microorganisms) and different tolerances to environmental factors (Theuerl et al., 2019b). In this study it was shown for example members of the phylum *Bacteroidetes* were more abundant in the reactors operated with easy degradable substrate as well as for members of the phylum *Cloacimonetes* that decreased their abundant with increasing TAN-concentrations.

Although intensive research on the biogas-microbiome have been conducted during the last couple of years most of the process-involved microorganisms and hence their ecological functions are still unknown (Campanaro et al., 2019; Hassa et al., 2018; Kundu et al., 2017). Therefore are research aiming to understand of how populations, groups of microorganisms or entire biogas microbiomes respond to management measures (e.g., feedstock input) and how these reactions affect the process efficiency still of great importance. This will become even more important in Germany in the future, considering that German biogas plants should be able to produce energy (electricity and heat) flexible and on-demand and the digestate should be used as high-quality fertilizers. This production should be made from a wide range of input materials, such as Agricultural residues (solid manure, straw, flowering strips, catch crops), residues from landscape management (green waste, leaves) as well as biogenic municipal and industrial waste, that are chemically very heterogeneous and temporally and quantitatively variable available (Theuerl, 2019; Theuerl et al., 2018).

To investigate the microbial diversity, 16S rRNA gene amplicon sequencing has become a common tool during the last couple of years (Hassa et al., 2018; Zhang et al., 2019). Although this method offers the possibility to analyse a high number of samples most studies so far

have used this method mainly to compare different habitats (e.g. different biogas reactors) and not time series (e.g.Calusinska et al., 2018; Campanaro et al., 2019; De Vrieze et al., 2017; Mei et al., 2017; Ziels et al., 2017). Despite this are established methods, such as terminal restriction fragment length polymorphism (TRFLP) which was used in this study, still important for the detection of the microbial diversity in biogas plants. TRFLP is useful especially to study the microbial community dynamics over time in relation to the impact of changing environmental conditions (De Vrieze et al., 2018). The disadvantage of this method is a low phylogenetic resolution, which hampers the identification of microbial process indicators for specific process conditions. But also 16S rRNA gene amplicon sequencing which have a much higher phylogenetic resolution than TRFLP (De Vrieze et al., 2018) is probably not sensitive enough for microbial process indicator detection as a reliable assignment is only possible until genus level. The results of the Earth microbiome project showed that distinct habitat differences are first significantly confirmed at species level (Thompson et al., 2017). Consequently is there a necessity to identify the process-involved microorganisms at the species level, including their specific metabolic potentials, their actual realized functions as well as the regulatory ecological mechanisms, (Bouchez et al., 2016; Castellano-Hinojosa et al., 2018; de Vrieze et al., 2017; Schnürer, 2016; Theuerl et al., 2019a; Zhang et al., 2019). New sequencing technologies, such as Nanopore sequencing, are expected to offer the possibility to capture the microbial diversity with high phylogenetic resolution down to the species level by full-length sequencing of the 16S rRNA gene or even the whole *rrn*-operon (Cuscó et al., 2018; Kerkhof et al., 2017; Shendure et al., 2017).

Moreover, metagenome, metatranscriptome and metaproteome analyses are increasingly used as they provide an in-depth insight into the entire community. Metagenome analyses enable to detect the genetically determined potential metabolism and lifestyle, while metatranscriptome and metaproteome analyses identify the actively realized functions of hundreds to thousands (Campanaro et al., 2019; Hassa et al., 2018; Heyer et al., 2019, 2015; Zhang et al., 2019). However the ‘omic’ techniques only reflect snapshots of the microbial diversity at a certain time, under certain conditions and provide limited information about the microbial community response to varying environmental factors over time (Alivisatos et al., 2015). Nevertheless, Campanaro et al. (2019) showed the value of metagenome analyses. They compared 134 metagenome data sets and recovered 1,635 metagenome-assembled genomes (MAGs) whereby only 69 genomes could be assigned to known cultivated, physiological and/or genetically described species. The 1,635 MAGs were assigned to 53 different phyla,

whereof 25 belong to phyla with a candidate status such as the phylum *Cloacimonetes*. Thus not a single species within these candidate phyla have been cultivated and hence cannot be characterized regarding their physiological and ecological properties. Additionally this shows that this high abundant uncultured taxa probably have a high level of phylogenetic novelty and therefore a high level of undiscovered functions that are important for the overall functioning of the process (Campanaro et al., 2019; Lloyd et al., 2018).

Within the presented study, members of the candidate phylum *Cloacimonetes* were found to be sensitive to elevated TAN concentrations which assign their disappearance as a potential microbial process indicator for an upcoming process disturbance. The existence of this phylum in anaerobic digestion systems was discovered 10 to 15 years ago (Chouari et al., 2005; Pelletier et al., 2008) while the study by Campanaro et al. (2019) recorded the existence of 15 different species (or MAGs) within this phylum. Considering the current state of knowledge there are two mayor tasks/options for future research: Firstly, based on the discovery of newly yet-uncultured microorganisms by MAGs and a subsequent prediction of the potential metabolic capacities, new cultivation conditions might be deduced (Gutleben et al., 2018). This in turn provides the opportunity to comprehensively decipher the physiology and ecological behaviour of microorganisms, to improve database annotations for newly discovered (isolated and described) microorganisms and to establish novel applications in biotechnology (Gutleben et al., 2018). Such approach is not only applicable for the members of the phylum *Cloacimonetes*, but also for many other recorded yet-uncultivated microorganisms of the anaerobic digestion process. The second task, regarding the use of members of the phylum *Cloacimonetes* as a potential microbial process indicator, would be the development of a fast, affordable and easy applicable detection method. In this context, studies that systematically induce typical stress situations for the biogas microbiome are highly valuable as they provide the opportunity to identify thresholds for critical process conditions and to verify the yet-potential as a real-usable microbial process indicator (Theuerl et al., 2019a). Based on the available 16S rRNA gene sequences a specific TaqMan qPCR assay could be developed which prospectively can be used as a standard method to assess the process status.

Besides the future approaches for microbiome studies, the presented work also revealed that the currently measured and analysed chemical process data are often not adequate enough to completely explain the process status in relation to the occurring or establishing microbial

diversity. This is most probably related to the fact, that not all metabolites produced by microorganisms and that can be expected to impact other microorganisms are recorded by the common chemical analysis. Genome (transcriptome or proteome) studies on cultivated and yet-uncultivated microorganisms show a large number of metabolic pathways and of different metabolites that are (potentially) produced during the process (e.g. Manzoor et al., 2018; Sedano-Núñez et al., 2018; Tomazetto et al., 2018), while the standard chemical analyses focus only on a small number of the supposed most important compounds (e.g. Drosg, 2013). To overcome this issue fluxomics and metabolomics approaches could be beneficial (Beale et al., 2016; Cortassa et al., 2015).

For a knowledge-based microbiome management and to efficiently control the complex anaerobic digestion process, where all members of the microbiome have their own specific living conditions, there is still great necessity for further research.

7 Literature

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8 Supplement

8.1 Supplemental information to chapter 2

Table S1: Main chemical characteristics of the maize reactor and sugar beet reactor digestates. All values are given as mean values of the three reactors (biological replicates) including the standard deviation.

Days of operation	Digestate of the maize reactors				
	TS [%FM]	VS [%TS]	η_{VS} [%]	pH [-]	VFA [g L^{-1}]
0	3	74.4	-	7.6	0.56
33	2.6 ± 0.0	69.6 ± 0.1	90 ± 0.1	7.2 ± 0.0	0.0 ± 0.0
57	2.8 ± 0.1	71.2 ± 0.8	89 ± 0.6	7.3 ± 0.0	0.1 ± 0.0
93	3.0 ± 0.1	75.2 ± 0.4	87 ± 0.5	7.2 ± 0.0	0.1 ± 0.0
141	3.6 ± 0.1	75.1 ± 0.2	85 ± 0.5	7.1 ± 0.0	0.1 ± 0.0
175	4.0 ± 0.2	78.5 ± 1.0	82 ± 1.0	7.1 ± 0.1	n.d.
232	4.4 ± 0.2	80.0 ± 0.9	80 ± 0.9	7.1 ± 0.0	0.2 ± 0.0
267	4.4 ± 0.0	81.3 ± 0.8	80 ± 0.2	7.0 ± 0.0	0.0 ± 0.0
309	4.6 ± 0.3	82.8 ± 1.2	79 ± 1.8	7.0 ± 0.1	0.2 ± 0.0
337	4.6 ± 0.1	84.1 ± 0.2	78 ± 0.3	7.0 ± 0.0	0.1 ± 0.1
Days of operation	Digestate of the sugar beet reactors				
	TS [%FM]	VS [%TS]	η_{VS} [%]	pH [-]	VFA [g L^{-1}]
0	3	74.4	-	7.6	0.56
33	2.5 ± 0.1	68.0 ± 0.4	87 ± 0.2	7.3 ± 0.0	0.0 ± 0.0
57	2.5 ± 0.0	68.5 ± 0.6	87 ± 0.1	7.4 ± 0.0	0.2 ± 0.1
93	2.3 ± 0.0	69.2 ± 0.3	88 ± 0.2	7.3 ± 0.0	0.1 ± 0.0
141	2.6 ± 0.0	69.4 ± 1.2	87 ± 0.2	7.2 ± 0.0	0.1 ± 0.0
175	2.7 ± 0.0	68.7 ± 1.2	86 ± 0.2	7.2 ± 0.0	n.d.
232	2.7 ± 0.1	70.4 ± 2.0	86 ± 0.7	7.2 ± 0.1	0.2 ± 0.0
267	2.7 ± 0.1	68.1 ± 1.0	87 ± 0.4	7.2 ± 0.0	0.0 ± 0.0
309	2.5 ± 0.1	69.1 ± 1.2	87 ± 0.5	7.2 ± 0.0	0.2 ± 0.0
337	2.6 ± 0.0	70.5 ± 0.1	87 ± 0.2	7.2 ± 0.0	0.1 ± 0.0

TS = total solids, FM = fresh mass, VS = volatile solids, η_{VS} = VS degradation degree, VFA = volatile fatty acids, n.d. = not determined.

Table S2: Characteristics of the bacterial and archaeal community structure in the analysed lab-scale biogas reactors fed with maize resp. sugar beet silage indicated by the number of detected TRFs and the community organisation expressed as Gini coefficient.

Days of operation	Richness (no. of TRFs)				Community organization (Gini coefficient)			
	Bacteria		Archaea		Bacteria		Archaea	
	Maize	Sugar beet	Maize	Sugar beet	Maize	Sugar beet	Maize	Sugar beet
Inoculum	54	54	5	5	0.54	0.54	0.41	0.41
33	47	51	7	7	0.42	0.46	0.44	0.44
57	46	39	8	8	0.42	0.46	0.52	0.49
93	44	42	8	8	0.41	0.51	0.59	0.43
141	41	30	5	8	0.43	0.52	0.67	0.58
175	41	25	5	8	0.34	0.54	0.52	0.5
232	53	33	7	8	0.37	0.54	0.62	0.54
267	27	44	3	7	0.31	0.51	0.41	0.5
309	38	34	5	6	0.43	0.42	0.66	0.52
337	42	30	3	3	0.36	0.43	0.57	0.43

8.2 Supplemental information to chapter 4

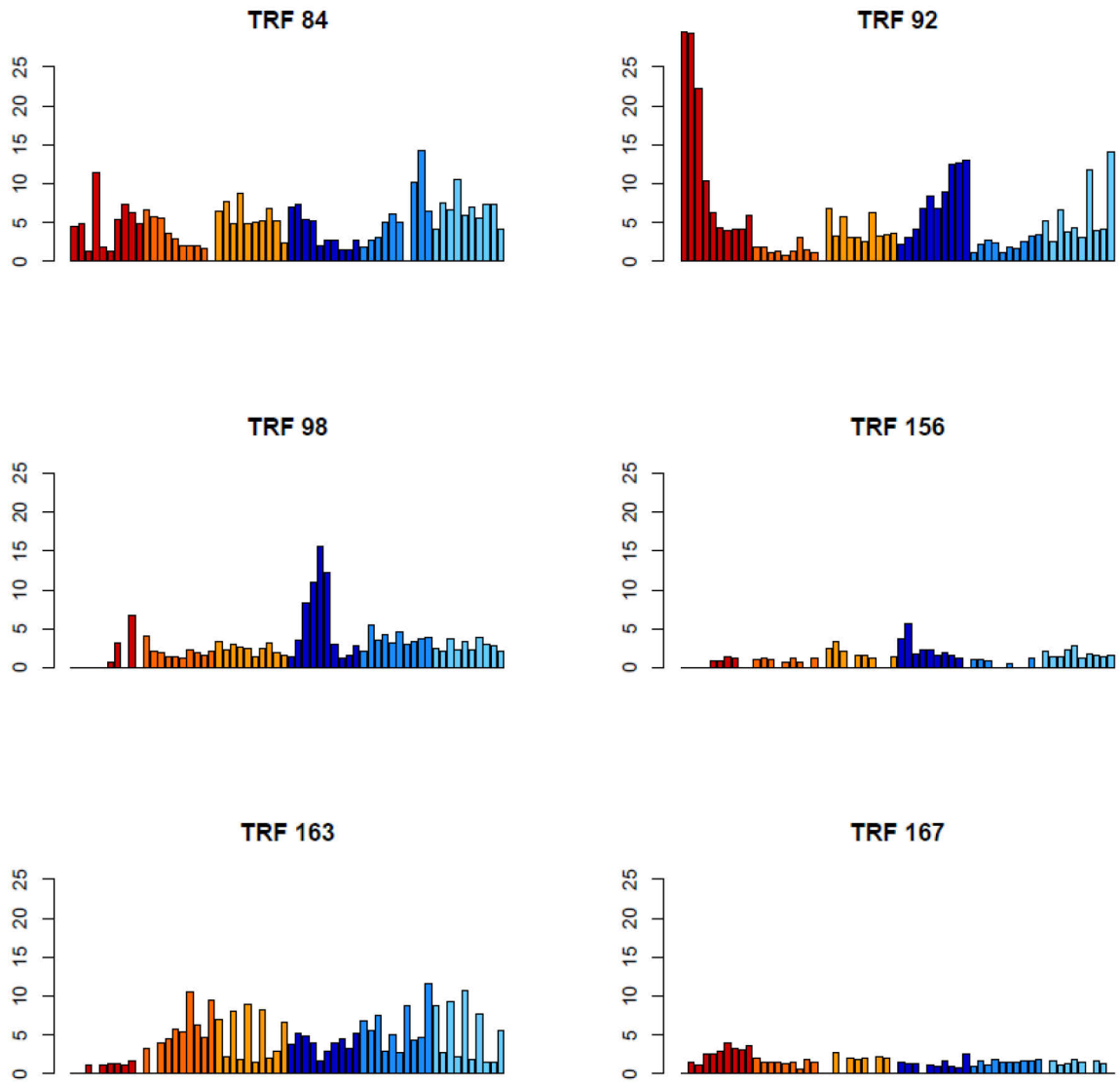

Figure S1 (continued on next page)

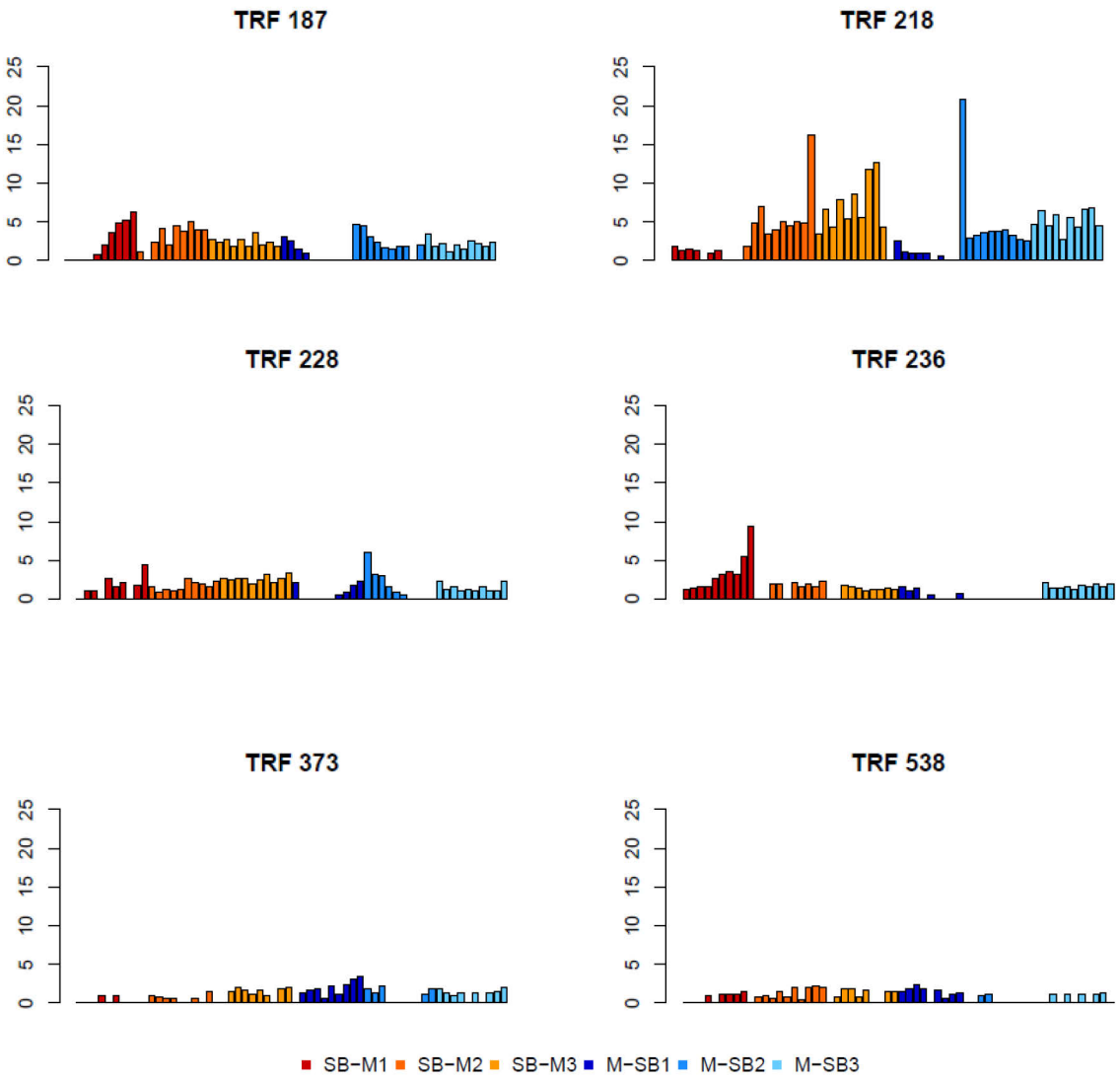

Figure S1: Abundance of the twelve TRFs found in all six reactors at least at more than one time point.

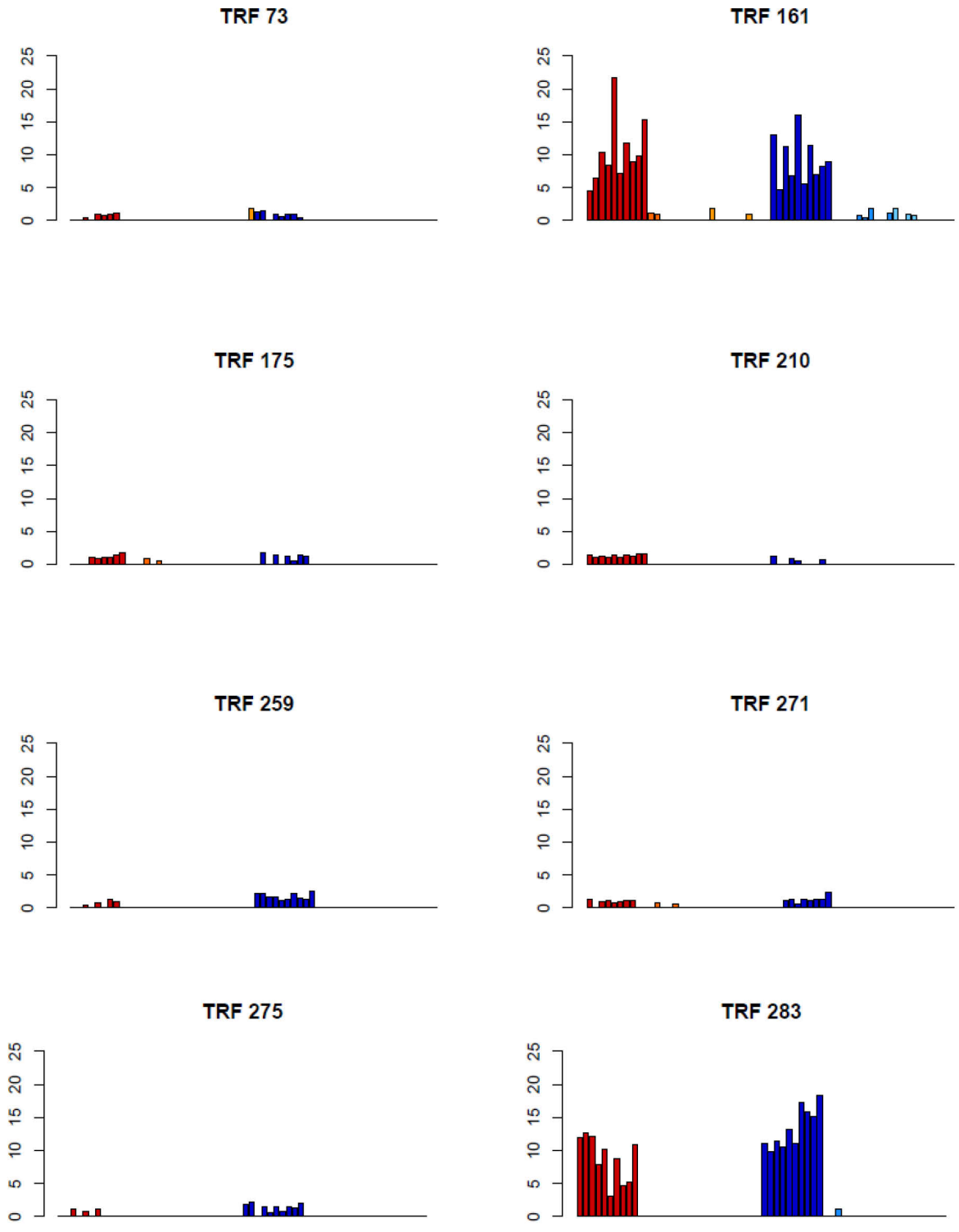

Figure S2 (continued in next page)

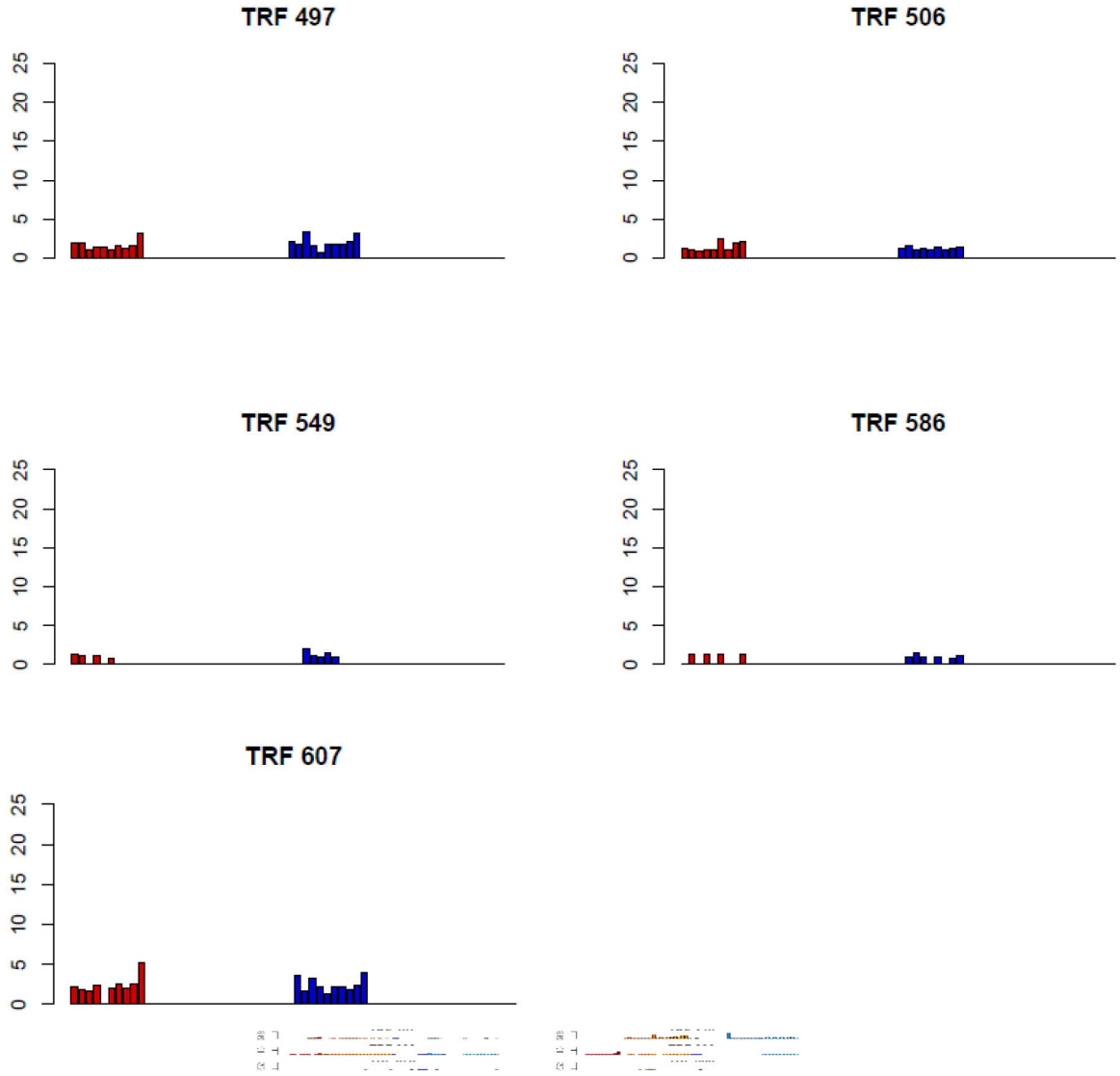

Figure S2: Abundance of the 13 TRFs found mainly in the reactors with low TAN concentration.

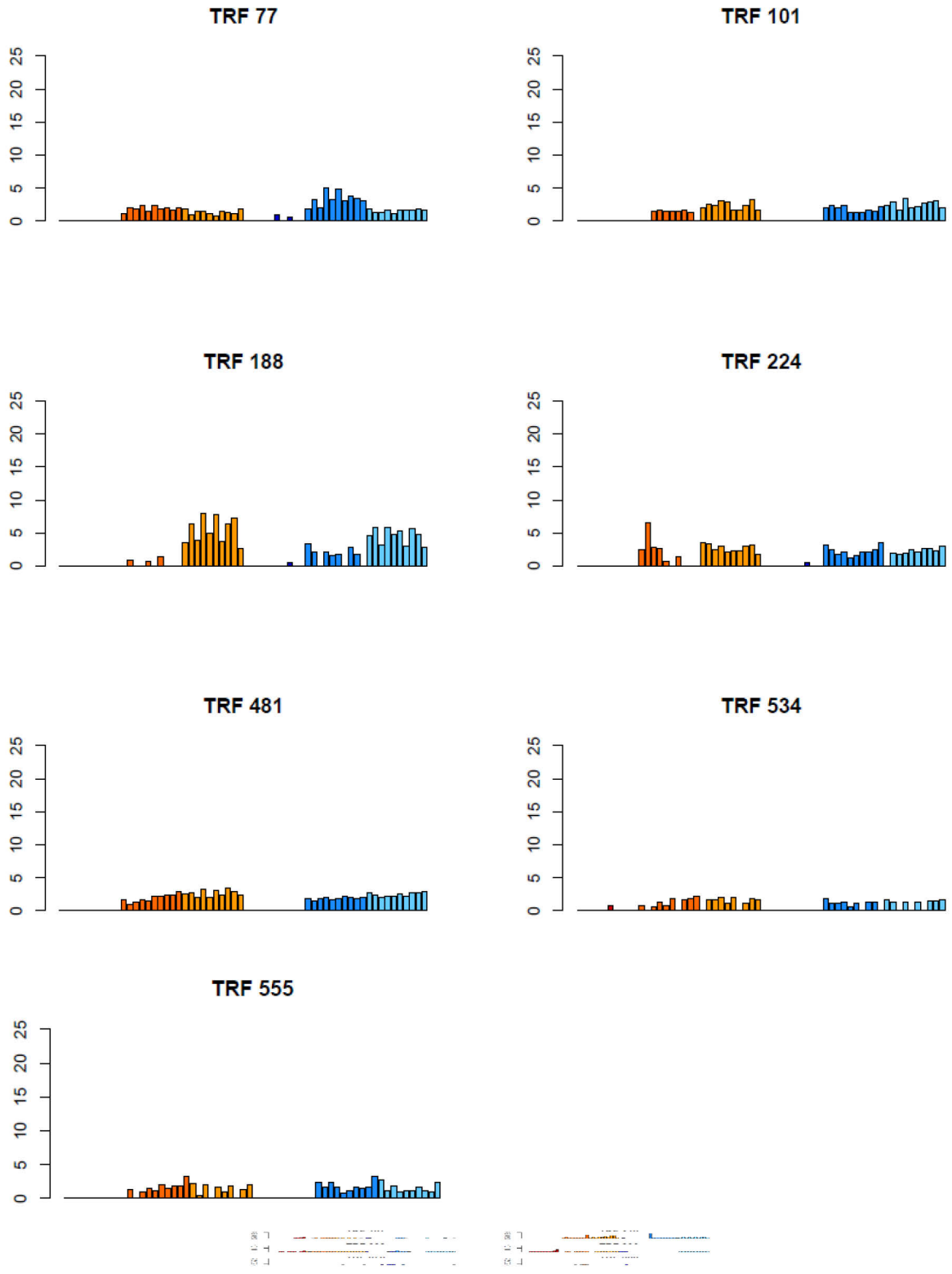

Figure S3: Abundance of the seven TRFs found mainly in the reactors with high TAN concentration.