Potential Impact of Increasing Ammonia Concentrations upon Microbial Population Dynamics in Anaerobic Meso- and Thermophilic driven Fermenters

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Khulud Alsouleman

born in Syria

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D7

Head of the Committee: Prof. Dr. Frank Beneke

Supervisor and Reviewer: Jun.-Prof. Dr. Michaela Dippold

Co-Supervisor and Co-Reviewer: Dr. Michael Klocke

To my father…

None of my dreams would have been possible without the love you gave me.

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Abbreviations

16S rRNA	16S ribosomal RNA of the prokaryotic 30S small subunit
AD	Anaerobic digestion
ATP	Adenosine triphosphate
ATB	Leibniz-Institut für Agrartechnik und Bioökonomie e.V. (Leibniz Institute for Agricultural Engineering and Bioeconomy)
Bp	Base pair
C	Conductivity
CO_2	Carbon dioxide
CS	Cattle slurry
CR	Control reactor
CSTR	Continuously respectively completely stirred tank reactor
DANN	Deoxyribonucleic acid
EEG	Renewable Energy Sources Act / Erneuerbare-Energien-Gesetz
EP	Experimental phase
EP1	First experimental phase 25% poultry manure+ 75% cattle slurry (based on VS)
EP2	Second experimental phase 50% poultry manure+ 50% cattle slurry (based on VS)
EP3	Third experimental phase 75% poultry manure+ 25% cattle slurry (based on VS)
EP4	Forth experimental phase 100% poultry manure (based on VS)
ER	Experimental reactor
EU	European Union
FIT	Feed-in tariff
FM	Fresh substrate
FNR	Fachagentur nachwachsende Rohstoffe (Agency for Renewable Resources)
GHG	Green house gas
HRT	Hydraulic retention time
Meso	Mesophilic
NH_3	Free ammonia

NH_4-N	Ammonium nitrogen
NMDS	Non-metric multidimensional scaling
OLR	Organic loading rate
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
pH	Pondus hydrogenii
PM	Poultry manure
rRNA	Ribosomal ribonucleic acid
SAO	Syntrophic acetate-oxidizing
Thermo	Thermophilic
TRF	Terminal restriction fragment
TRFLP	Terminal restriction fragmentlength polymorphism
TS	Total solids
VDI	Verein Deutscher Ingenieure (The association of German engineers)
VFA	Volatile fatty acids
VS	Volatile solids / substances

1 Summary

Anaerobic digestion is the process of decomposition of organic matter by a microbial consortium in an oxygen-free environment. The produced biogas from this process is composed of methane, carbon dioxide, nitrogen, oxygen, hydrogen sulfide and traces of other gases.

Long-term mesophilic and thermophilic anaerobic digestion experiments were investigated to evaluate the reactor performance and the response of the microbial community under consideration of the structure variations due to an increasing content of NH_4^+-N caused by stepwise addition of nitrogen-rich substrates, in this case studies poultry manure (PM).

Therefore, laboratory-scale continuously respectively completely stirred tank reactors (CSTR) with a working volume of eight liter and steady organic loading rate (OLR of 3.0 gVS L^{-1} d^{-1}) in mesophilic (37°C) and thermophilic (55°C) conditions were operated.

The gradual increasing of NH_4^+-N caused by stepwise addition of nitrogen-rich substrates (poultry manure) will lead to an increase in the free ammonia NH_3 concentration. Free ammonia is considered a common inhibitor for the anaerobic digestion process due to its cytotoxic effects, resulting from deprotonation of ammonium (NH_4^+). As the free ammonia (NH_3) concentration depends on the concentration of NH_4^+-N, the pH-value and the reactor temperature, therefore a NH_4^+-N and NH_3 values of > 3 g kg_{FM}^{-1} respectively > 0,4 g kg_{FM}^{-1} which has no impact on the anaerobic digestion process under mesophilic condition caused a serious disturbance and inhibition under thermophilic condition.

The anaerobic microbiome acclimated to low PM levels in mesophilic and thermophilic conditions which resulted in a stable anaerobic digestion process. After that, with the consecutive application of medium PM level in mesophilic condition, a process disturbance was induced which was characterized by a shift from a *Bacteroidetes*-dominanted to a *Clostridiales*-dominated bacterial community accompanied by a change from the acetoclastic to the hydrogenotrophic pathway of methane formation. However, the “new” microbial community in mesophilic condition was functionally redundant as the overall process rates in terms of biogas yield methane content and volatile fatty acids VFA content were similar to the former one. A further increase of

poultry manure (high PM level) resulted in complete process failure due to the ongoing increasing in the total ammonium nitrogen and volatile fatty acid content.

Compared to a mesophilic experiment, the thermophilic anaerobic microbiome was much more sensitive for process disturbances. The application of medium PM level resulted in a process disturbance and a final process failure. The microbial community was able to compensate the high cytotoxic ammonia contents only for a short time. The ongoing increase in the total ammonium nitrogen NH_4^+-N content in combination with an increase of the salt content (quantified as electrical conductivity) are assumed to be the main reasons for the final process failure.

Overall, the microbial community structure in this study might be the key factor explaining the adaption capacity, as it highlighted how an anaerobic microbiome in mesophilic condition was enabled to adapt to changing environmental conditions while the thermophilic ones with less diversity was much more sensitive and failed to overcome the prevalent environmental conditions. Thus, these results serve as a basic to understand and monitor the different microbiome responses to a specific environmental disturbance and to contribute to further optimization of biogas production process based on nitrogen rich substrates. Also, the results of this study may facilitate the application of anaerobic digestion of process-risk feedstock (nitrogen-rich manure) as a management technology and bioenergy resource on the full–scale in the future.

2 Introduction

Reducing greenhouse gas emissions resulting from open storage and uncontrolled spreading of animal slurries and manures are major challenges faced in the agricultural sector (Barret *et al*., 2015). One of the most important and commonly applied technologies to achieve this goal is the bioconversion of animal wastes into energy-rich biogas by anaerobic digestion (AD). Therefore, the implementation of AD within the animal waste management is a promising technology as it provides a sustainable, renewable energy resource and reduces the negative environmental impacts. However, the AD of animal wastes such as cattle, swine and poultry manure, which are usually rich in nitrogen compounds, is related to the risk of process instability.

The accumulation of ammonium nitrogen (NH_4^+-N) and especially the undissociated form (free ammonia, NH_3), which are the end-product of anaerobic degradation of nitrogen-rich substrates such as proteins and peptides, is considered to be toxic for the occurring microbial community.

In order to investigate the impact of increasing amounts of NH_4^+-N due to the consecutive poultry manure level addition on the reactor performance and especially on the occurring microbiome, a long-term, mesophilic (37°C) and thermophilic (55°C), lab-scale AD experiments were performed and monitored. The characterization of the microbial community structure and its response to changing environmental condition was assessed by a DNA-based community profiling method (terminal restriction fragment length polymorphism, TRFLP) in combination with a cloning/sequencing approach targeting either the bacterial or archaeal 16S rRNA genes. Multivariate statistical analyses were performed to correlate the prevalent environmental conditions with the corresponding microbiome.

3 Review of literature

3.1 The challenge of the reduction of global GHG emissions

3.1.1 Global GHG emissions and strategies for renewable energy production

In the last decades, the worldwide climatic perturbations have increased due to the continuously increasing population and industrialization (Nelles *et al*., 2011). This increase in the worldwide population led to a constant growth of the global energy demand and hence greenhouse gas (GHG) emissions from anthropogenic activities especially from the fossil fuels consumption (Shah *et al*., 2016). Driven by the higher energy demand in 2018, the global energy-related CO_2 emissions rose for 1.7% which was the highest rate of increase since 2013, and 70% higher than the average increase since 2010 (IEA, 2019).

The primary sources of global GHG emissions are the increasing consumption of fossil fuels (coal, oil, and gas) which reach to 76% of the total GHG emission while the AFOLU (agriculture, forestry and other land use) contribute the remaining 24% of the total GHG emission with 12% from the agricultural sector alone (WRI, 2012; UBA, 2013; Bruckner *et al*., 2014; Smith *et al*., 2014; Scheftelowitz and Thrän, 2016).

Consequently, the GHG emission reduction was considered a major challenge faced not only by the energy sector but also by the agricultural sector worldwide.

To achieve the predict reduction target of worldwide GHG emissions, an alteration of the energy system towards the use of renewable energy such as wind power, hydropower, solar energy and bioenergy, is one of the most important recommendation (Scarlat *et al*., 2015; Scheftelowitz and Thrän, 2016). In the European Union the share of renewable energy in the gross final energy consumption has increased from 8.5% in 2005 to almost 14% in 2016 (IEA, 2018; Scarlat *et al*., 2018).

Biogas is considered as one of the indispensable sources in the energy transition system towards renewable energy production (Martinot *et al*., 2002; Szarka *et al*., 2013). Methane, which is the main component of the biogas, can be used as alternative to the fossil fuel to generate heat, electricity (Weiland, 2010). The production of biogas prevents an emission of 549 g CO_2 equivalent per kWh in electricity generation and 171 g CO2 equivalent per kWh in heating supply (BMU, 2012). Also, the biogas can also be upgraded to biomethane which could be injected directly in the natural gas grid after a specific purification steps or used as gaseous vehicle fuel (Theuerl *et al*., 2019).

The biogas production can be categorized depending on the source of biogas in three main categories; biogas produced from AD using agricultural waste, manure, and energy crops, with about 74% of the primary biogas energy output, a biogas derived from landfill gas recovery with about 17% of the primary biogas energy output and, as smaller extent, from sewage sludge treatment plants and other sources, with 9% of the primary biogas energy output (Scarlat *et al*., 2018).

3.1.2 **Biogas production in Europe: Germany as example**

Germany, as example of the most developed countries in biogas energy production, is considered nowadays the European leader of biogas production. In Germany, approximately 81% of the energy produced in 2017 being based on fossil fuels (BMU, 2018; FNR based on ZSW/AGEB, 2018). The gross consumption of the fossil fuels for energy supplies (provision) amount to 83% of total GHG emissions (Bruckner *et al*., 2014). While the agricultural sector contribution accounted for 7.7% of the total GHG emissions (UBA, 2013), and more than 10% of the later GHG emissions were caused by the open storage and uncontrolled spreading of animal residues (Scheftelowitz and Thrän, 2016). In regard to these data, Germany has set its targets to increase the quota of the renewable energy up to 14% in the heating sector, up to 30% in the electricity sector and about 10% in the transport sector by 2020 (BMU, 2009; FNR, 2013). As a consequence, the share of the renewable energy sources in the primary energy consumption reached in 2017 up to 13.1% whereby the use of the biomass alone covered 7.1% (FNR, 2019).

During the last years, and due to the EEG law (Erneuerbare-Energien-Gesetz/ Renewable Energy Sources Act) which provides guaranteed feed-in tariffs (FIT) program for renewable energy sector, the biogas sector faced clear development. Therefore, an increase in the number of the biogas plants from 7215 plants in 2011 to 9494 in 2018 was recorded (FNR, 2012; FNR, 2019). The German contribution of total biogas production in the EU reached to 50% in 2015 (Scarlat *et al*., 2018). More than 50% of the biogas potential in Germany results from AD of energy crops. Together with animal manure and harvesting residues, more than 80% of the potential feedstocks were produced by the agricultural sector (FNR, 2008; Weiland, 2010).

Due to the estimated continuous increase in the human population from 6.9 billion people in 2010 to 9.15 billion people in 2050 (Alexandratos and Bruinsma, 2012), the

livestock industries are growing rapidly worldwide. This trend yields in large amounts of animal waste products, especially in developing countries (Sakar *et al*. 2009). The EU ranks third in world's poultry meat production after USA and Brazil, but more than 70% of the EU's poultry meat is produced in six countries: Poland, UK, France, Germany, Spain, and Italy (Eurostat, 2014). In 2016, the animal excrements (slurry, manure) in Germany formed 44.5% of the total substrate input in biogas plants (mass related) with 72% of cattle slurry and 3% of poultry manure (FNR, 2019).

3.1.3 Biogas production in the Middle East Region: Syria as example

Syria is one of the developing and Middle East countries which characterized by long hot summer and mild wet winter. Middle East countries have enormous potential for renewable energy resources; wind, solar in addition to the biomass. But at the same time the renewable energy applications in these countries have not been widely promoted yet. The main objective of choosing Germany (EU leader in biogas production) and Syria (a developing country with immature experience in biogas production) as key countries, is to transfer the current state of knowledge, policies, facilitates from Germany to Syria. This in turn will help to elaborate recommendations and future plans for efficient application of the biogas production in Syria. The total primary energy supply in Syria was dominated by 71.3% of crude oil/petroleum products, 21.8% of natural gas, 4.1% of hydro energy and 2.8% of biomass energy (Country Report Syria, 2009). During the last years, the Syrian government has also been setting new legislation and regulations for renewable energy development, which aims to encourage the use of renewable energy. Therefore, the Syrian government has set its target to provide 4.3% of primary energy demand from renewable energies by 2030 (RCREEE, 2019).

Different (AD) units and small biogas plants were established in cooperation with other countries in Syria to support biogas production from the most available and cheap organic wastes. So that, Syria has now several pilot projects which use biogas to produce electricity, including biogas production from the animal wastes and treatment of wastewater in Damascus (Al-Mohamad, 2001).

In regard to Al-Mohamad (2001), the daily municipal and agricultural wastes are higher than 300 million cubic meters per year, which in turn forms a continuous source for biogas production.

In Syria as one of developing and agricultural countries, the livestock industry - including the poultry industry - increases obliviously to meet the food needs of growing population (FAO, 2008). The demand on poultry is high in Syria as it is considered the cheapest source of meat protein; in addition to its relatively short production cycle time which make it profitable under the industrialized production system.

3.2 Engineering the biogas production

3.2.1 The principles of the anaerobic digestion process

The AD process is a highly complex chemical microbial-mediated process in terms of functionality and community diversity (Vanwonterghem *et al*. 2014). This process is achieved by the interaction between different microbial taxa within the superkingdom *Bacteria* and *Archaea*, involving several consequent degradation phases, typically hydrolysis/cellolysis, acidogenesis, acetogenesis, and methanogenesis as shown in **Fig. 1** (Angenent *et al*. 2004; Vanwonterghem *et al*., 2014; Hassa *et al*., 2018). The efficiency and stability of this process is entirely dependent on the concerted and syntrophic activity of microorganisms belonging to different functional guilds (Li *et al*., 2009).

The first step of the AD process is the hydrolysis. In this step, the hydrolytic bacteria break down the polymeric substances such as carbohydrates, proteins, and lipids into oligo-, di-, and monosaccharides, amino acids, as well as fatty acids by the excretion of hydrolytic extracellular enzymes such as proteases, amylases, cellulases, or lipases (Boone and Mah, 1987; Bergmann, 2010; Weiland, 2010).

In the second subsequent acidogenesis step, the obtained metabolic products from the first stage are degraded by a large variety of fermentative bacteria into volatile fatty acids, alcohols, formate, carbon dioxide (CO_2), some organic nitrogen compounds, some organic-sulfur compounds, and molecular hydrogen (H_2) (Gerardi, 2003; Bergmann, 2010; Cabezes, 2015).

The third step in the AD process is the acetogenesis. The acetate-forming bacteria or acetogenic bacteria convert mainly volatile fatty acids and alcohols into acetate and H_2. The oxidation of intermediate fermentation products to acetate is performed by hydrogen producing acetogenic bacteria.

Most of the representatives of these bacteria grow in a syntrophic relationship with hydrogen utilizing methanogens under low hydrogen concentration which results in

energetically favorable metabolic pathway of methane production (Gerardi, 2003; Talbot *et al*., 2008). The Syntrophic acetate oxidation involves the conversion of acetate to hydrogen and carbon dioxide by syntrophic acetate-oxidizing bacteria (SAO) which is energetically unfavorable. This unfavorable reaction can proceed if hydrogen-utilizing methanogens eliminate the hydrogen keeping the hydrogen partial pressure low enough to make the reaction sufficiently exergonic.

Otherwise, Siriwongrungson and colleagues (2007) have indicated under thermophilic conditions that the H_2 produced after butyrate oxidation was directly used together with CO_2 by homoacetogenic bacteria for the production of acetate. It was found that such homoacetogenic bacteria have a competitive advantage over hydrogen-utilizing methanogens due to their ability to use a wide range of substrate in unfavorable conditions for example in slightly acidic and low temperature (Phelps and Zeikus, 1984; Conrad and Wetter, 1990). On the other hand, other studies showed that homoacetogenic bacteria have also a competitive advantage over aceticlastic methanogens (which converts acetate to methane and CO_2) under thermophilic conditions (Schink, 1997) and mesophilic conditions with high ammonia concentrations (Angenent *et al*., 2002; Schnurer and Nordberg, 2008).

The last phase of the AD process is the methanogenesis which is considered to be the rate-limiting step of the biogas process due to the very slow growth rates of methane producers and their sensitivity to inhibitory substances (Chen *et al*., 2008; Liu and Withman, 2008). In this step, CO_2 and H_2, acetate, or methyl-group containing compounds can directly be converted into methane (CH_4) by methanogenic archaea. All methanogens belonged to the archaeal phylum *Euryarchaeota* and until now were classified into seven orders: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales* and *Methanomassiliicoccales* (Thauer *et al*., 2008; Thauer *et al*., 2010; Lang *et al*., 2015).

Methane can be produced by three principal groups of methane-forming archaea:

- The acetolastic methanogens converts acetate to methane and CO_2. This pathway is the predominant source of atmospheric methane and only members of the *Methanosarcinales* are capable of acetoclastic methanogenesis (Fournier and Gogarten, 2008; Lang *et al*., 2015).
- The hydrogenotrophic methanogens which use H_2 or formate as electron donor to convert CO_2 to CH_4. This hydrogenotrophic methanogenesis is the most widespread and is considered the most favorable methanogenesis pathway in

terms of energy gains even it is slower than the acetoclastic pathway. The known groups of methanogens that use H_2 are all members belonging to the previous orders with the exception of *Methanomassiliicoccales*.

- Methylotrophic methanogens which utilize methyl-group containing compounds such as methanol, methylated amines and methylated sulfides to produce methane. These methanogens are found in the orders *Methanosarcinales, Methanobacteriales* and *Methanomassiliicoccales* (Vanwonterghem *et al*., 2016).

The classic hypothesis that methane metabolism originated early in the evolution of the *Euryarchaeota* (Gribaldo and Brochier-Armanet, 2006) has recently been changed. It has been proposed depending on the metagenomic reconstruction of environmental samples that certain microbial species of phyla *Bathyarchaeota* and *Verstraetearchaeota* phyla are also capable to conduct methanogenesis (Evans *et al*., 2015; Borrel *et al*., 2016; Vanwonterghem *et al*., 2016). The recently proposed *Bathyarchaeota* phylum represented an evolutionarily diverse group of microorganisms (Kubo *et al*., 2012; Gagen *et al*., 2013; Lazar *et al*., 2014; Meng *et al*., 2014) which found in a wide range of environments. In addition, He and colleagues (He *et al*., 2016) indicated that *Bathyarchaeota* also have the potential to fix inorganic carbon in the form of CO_2 to produce acetate. Otherwise Maus and colleagues (2018) found in their work that the *Bathyarchaeota* in the analyzed biogas reactor biofilms are not able to produce methane via the hitherto known methanogenesis pathway (Maus *et al*., 2018), which in turn indicates a diverse metabolism within this phylum. In contrast, (Berghuisa *et al*., 2019) found that these non-euryarchaeal methanogens have been found to be exclusively methylotrophic.

It could be assumed depending on the previous contradictory results that the member of the phylum *Bathyarchaeota* has genetic potential diverse metabolic activities. Also, the accurate role or function of the members of this phylum in anaerobic digestion remains until now unclear.

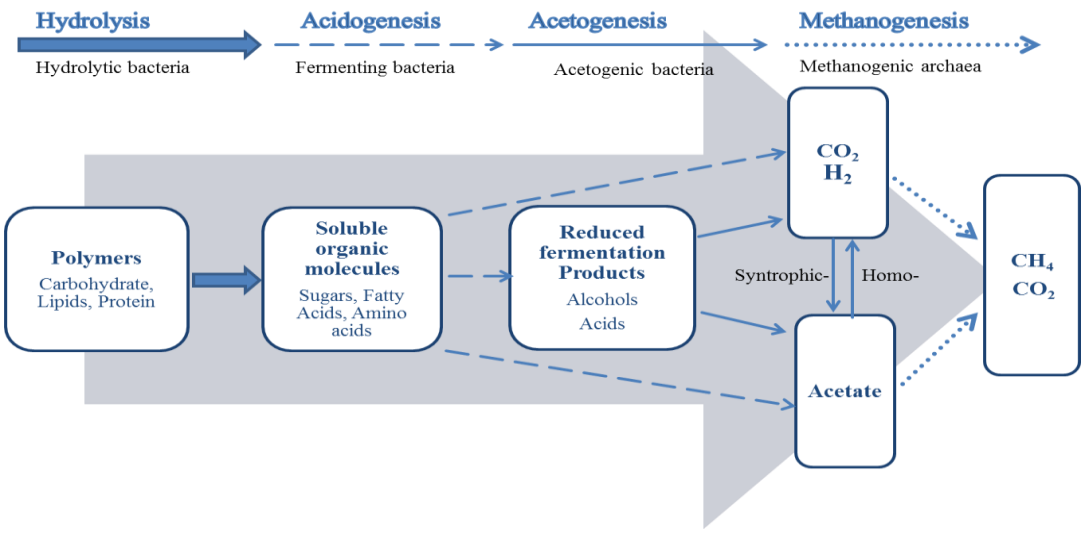

Figure 1: The four-stages of the anaerobic digestion process to produce biogas (modified after Weiland, 2010).

3.2.2 The anaerobic digestion of nitrogen rich manures

3.2.2.1 The importance of anaerobic digestion of nitrogen rich manures

As known, liquid and solid manures are usually considered and used as very important fertilizers (Scheftelowitz and Thrän, 2016) as they contribute to the closing of the nutrient cycles and hence substitutes mineral fertilizer (Arthurson, 2009; Weiland, 2010). But at the same time, the application of pure manure as fertilizer forms a big challenge to the sustainable development as can lead to eutrophication of water bodies due to the large amounts of pathogens and excess organic matter, as well as the release of climate relevant gases in terms of methane, ammonia, CO_2 or N_2O, and odorants from the natural degradation during storage (Jongbloed and Lenis, 1998; Dagnall *et al*., 2000; Kelleher *et al*., 2002; Moeller *et al*., 2004 ; Sakar *et al*., 2009 ; Thompson *et al*., 2013).

As the main risk of nitrate leaching in water bodies represents the main limitation to the direct application of not pre-treated livestock manure to soil. The anaerobic degradation of the organic matter (animal manure) ensures the formation of high amount of ammonium (the N-form which is more rapidly assimilated by the crops) without incurring in the subsequent oxidation into nitrate (Arthurson, 2009).

Therefore, the implementation of the AD of animal manure has become a promising alternative treatment technology for animal waste management as it considered a sustainable waste disposal system (Weiland, 2010). Also the AD of the animal manure

contributes to the GHG emission reductions as the produced biogas displaces the use of the fossil fuel (Dämmgen and Webb, 2006; Sakar *et al*., 2009; Bekkering *et al*., 2010; Rademacher *et al*., 2013, Lv *et al*. 2014; Scheftelowitz and Thrän, 2016), and can also reduce the GHG emissions from the natural decomposition of the manure during the storage. In addition to the previously mentioned environmental benefits, the produced digestate can be used as fertilizer as it has higher extent nutrients in inorganic plant-available forms (more easily leachable) compared to untreated waste due to the large input of organic nutrients that are mineralized during the digestion process (Field *et al*., 1984; Larsen *et al*., 1986; Plaixats *et al*., 1988Möller *et al*., 2008; Kirchmann and Witter, 1992). This in turn brings additional economic and environmental benefits by reducing the use of chemical fertilizers (Dagnall *et al*., 2000; Moeller *et al*., 2004; Thompson *et al*., 2013; Scarlat *et al*.,2018).

3.2.2.2 Limitations of anaerobic digestion of nitrogen rich manures

Animal wastes, a widely used substrate for biogas production, are rich in organic nitrogen (proteins and urea-uric acid in birds) (Krylova et al., 1996; Bujoczek *et al*., 2000: Kelleher *et al*., 2002; Sakar *et al*., 2009; Abouelenien *et al*., 2010; Singh *et al*., 2010; Zhang *et al*., 2011). Proteins are complex, high molecular-weight compounds. Proteins are long chains of amino acids (such as alanine, arginine, glycine, lysine etc.) which joined by peptide bonds. All amino acids contain an amino group ($–NH_2$) and a carboxyl group (–COOH). The peptide bonds joint the hydroxyl group (–OH) in the carboxyl group (–COOH) of one amino acid with the amino group ($–NH_2$) of other amino acid. The exoenzymes proteases and peptidases hydrolyze the peptide bond between amino acids. These amino acids can be taken up into bacterial cells by transporters and can be converted by the intra-cellular endoenzymes to a variety of organic acids depending on the converted amino acid (Kirchmann and Witter, 1992; Möller *et al*., 2008).

The conversion of the amino acids to organic acid is showed in the later equation:

$$4H_2NCH_2COOH + 2H_2O \rightarrow 4NH_3 + 2CO_2 + 3CH_3COOH \qquad \text{(Equation I)}$$

In this study the ammonium nitrogen NH_4^+-N and the total ammonium nitrogen TAN referred to the same compound to be able to compare the results of this study with other studies (Niu *et al*., 2013; 2014). The reduced nitrogen is thereafter present as

ammonium nitrogen NH_4^+-N and exits in two forms in the anaerobic digester, the ammonium ion NH_4^+ and free or undissociated ammonia NH_3. The two forms are in equilibrium, and the relative concentration of each form is dependent on the digester pH as illustrated in (Equation II) (Gerardi, 2003).

$$NH_4 \leftrightarrow NH_3 + H^+ \quad \text{(Equation II)}$$

The free ammonia (NH_3) concentration can be calculated based on the concentration of NH_4^+-N, the pH-value and the reactor temperature using the formula (Equation III) (Hansen *et al*., 1998).

$$\frac{NH_3}{N{H_4}^+ - N} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}}\right)^{-1} \quad \text{(Equation III)}$$

Whereby: NH_3= Free ammonia concentration

NH_4^+-N = Ammonium nitrogen

T= Temperature (kelvin)

Free ammonia or the undissociated ammonia (NH_3) has a positive impact on the anaerobic digestion process as it provides the alkalinity to the system. Due to (Gehardi, 2003), the released ammonia NH_3 reacts with the carbon dioxide and water to form ammonium carbonate which maintains the system's alkalinity as follow:

$$NH_3 + CO_2 + H_2O \rightarrow NH_4HCO_3 \quad \text{(Equation IV)}$$

At the same time free ammonia is considered a common inhibitor for methanogens especially for the acetoclastic methanogens due to its passive diffusion ability through the cell membranes into the cells (Kroeker *et al*., 1979; de Baere *et al*., 1984; Sung and Liu 2003; Chen *et al*., 2008; Rajagopal *et al*., 2013; Yenigün & Demirel, 2013; Lv *et al*., 2014). The most widely accepted mechanisms explaining the inhibition of methanogenesis by free ammonia is the direct inhibition of the methane synthesizing enzymes by free ammonia. The second mechanism is related to the ability of hydrophobic free ammonia molecules to diffuse passively into the cell and convert there to ammonium which alters the intracellular pH of the cell, or can effect on the concentration of other cations (proton imbalance) such as K^+ (important ion to maintain the pH balance) or Mg^{2+} (important ion in the action of many enzymes that catalyze

ATP-dependent reactions) (Sprott *et al*., 1984; Henrichs *et al*., 1990; Kadam and Boone, 1996).

Numerous studies had been conducted to evaluate the potential of several animal residues such as cow respectively cattle, swine and poultry manure as feedstock to produce biogas (Yenigun and Demirel, 2009; for review: Nasir, 2012; Niu *et al*., 2013, 2014; Regueiro *et al*., 2015; Toumi *et al*., 2015; Akyol *et al*., 2016; Usack and Angenent, 2016).

Anaerobic digestion of cattle slurry (CS) has been assessed over the last 25 –30 years and is now an established waste management technique

The cattle slurry was used in this study as considered an excellent "carrier" substrate for the anaerobic digestion of concentrated waste such as poultry manure, which would be difficult to treat separately. The reasons for choosing the cattle slurry as co-digestion substrate in this study are:

- The high moisture content of this substrate which acts as solvent for wastes of high dry content (poultry manure).
- The high buffering capacity of this substrate which in turn prevents the process disturbances arising from the pH fluctuations due to the temporary accumulation of the volatile fatty acids (VFA).
- The richness of this substrate with the necessary nutrients for an optimal bacterial growth.
- The wide availability of this substrate (Angelidaki and Ellegaard, 2003; Callaghan *et al*., 2002).

The high solid content of poultry manure (Kelleher *et al*., 2002), and thus the corresponding higher biogas yields (Zhang *et al*., 2011; Niu *et al*., 2013) make poultry manure a very valuable co-feedstock for anaerobic digestion.

Table 1 showed the chemical and physiochemical characterization of the used poultry manure and cattle slurries in this study.

Tab. 1: The chemical and physiochemical characterization of the used poultry manure and cattle slurry

Chemical and physiochemical characterisation	Poultry manure	Cattle slurry
Dry matter content (TS% FM)	60	7-10
Organic matter content (TS% FM)	63	80
pH	7	7
Conductivity (mS cm^{-1})	4	11-15
TAN (g Kg^{-1} FM)	3	1-2
TKN (g Kg^{-1} FM)	34	3-5
Total VFA (g Kg^{-1} FM)	6	5-8

FM: Fresh material; TAN: Total ammonium nitrogen; TKN: Total Kjeldahl nitrogen; VFA: Volatile fatty acids; FM: Fresh material; TS: Total solid

However, the major concern of applying the AD technology on animal manure, especially on poultry manure, is related to the risk of accumulation of ammonium nitrogen (NH_4^+-N), the end-product of anaerobic degradation of nitrogen-rich substrates (Kayhanian, 1999; Liu *et al*., 2012; Yenigün and Demirel, 2013; Niu *et al*., 2014). Several studies investigated the effect of NH_4^+-N accumulation on the reactor performance. They reported that process inhibition threshold varies widely, from 1.7 to 14 g NH_4^+-N L^{-1}(Niu *et al*., 2013; Rajagopal *et al*., 2013; Shi *et al*., 2013; Yenigün *et al*., 2013; Westerholm *et al*., 2016).

In addition, further studies have investigated the use of poultry manure or poultry litter as feedstock for AD with different technical procedures. Some of these studies investigated the process stability with respect to process parameters such as organic loading rate OLR, hydraulic retention time HRT, total solid content TS, temperature, reactor design and another operational parameters (Webb and Hawkes, 1985; Kalyuzhnyi *et al*., 1998; Bujoczek *et al*., 2000; Atuanya and Aigbirior, 2002; Chamy *et al*., 2011; Dalkilic and Ugurlu, 2015; Latifi *et al*., 2019; Zahan and Othman, 2019). Other studies focused on the anaerobic digestion of poultry manure as co-substrate and various mixtures were investigated (Gungor-Demirci and Demirer, 2004; Anozie *et al*., 2005; Zhang *et al*., 2011; Carlini *et al*., 2015; Bayrakdar *et al*., 2017; Chao *et al*., 2017). Also, some studies were published focusing on the microbiological aspects of the anaerobic digestion process of poultry manure (Zhang *et al*., 2011; Niu *et al*., 2013; Niu *et al*., 2014; Alsouleman *et al*., 2016: Alsouleman, 2019). These studies illustrated clear

shifts in the microbial community structure as a response to the elevated ammonium nitrogen content and the prevalent operational parameters. In all previous studies, the recovered and inhibited microbial community was dominant with members of the phylum *Firmicutes* on the bacterial level and with hydrogenotrophic methanogens on the archaeal level.

3.2.2.3 Technical solutions for anaerobic digestion of nitrogen rich manures

During the last years, several studies have been done to reduce the impact of the ammonia accumulation during the anaerobic digestion of nitrogen rich substrate. The most applied methods are: the anaerobic digestion in semi-solid form (Bujoczek *et al*., 2000) or in wet form (Bujoczek *et al*., 2000; Gangagni Rao *et al*., 2008; Yetilmezsoy and Sakar, 2008); the co-digestion with other substrate (Carlini *et al*., 2015, Zhang *et al*., 2011); the acclimation of the microbial community to the high concentration of the ammonia (for review: Rajagopal *et al*., 2013; Güngör-Demirci and Demirer, 2004; Abouelenien *et al*., 2009b); additives with adsorptive capacity such as zeolites (Milán *et al*., 2001; Tada *et al*., 2005); the application of activated carbon (Cuetos *et al*., 2017) or biochar (Mumme *et al*., 2014); and bioaugmentation which is the addition of specific microbial cultures to improve the operational performance (Fotidis *et al*., 2013; Li *et al*., 2017).

Other efforts were focused on the ammonia removal techniques in combination with AD such as: ammonia stripping in which a fluid is percolated with gas (Bousek, 2016; Walker *et al*., 2011; Abouelenien *et al*; 2010); membrane extraction (Fuchs *et al*., 2018); struvite precipitation by magnesium phosphate compounds (Romero-Güiza *et al*., 2014); biological removal through Anammox (for review: Magrí *et al*., 2013); ultrasonication (Chao *et al*., 2014); and microwave irradiation which depends as was proposed by Lin *et al*. (2009) on the formation of molecular ammonia (NH_3) and the subsequent evaporation of NH_3 by MW radiation. Both thermal effect of microwave irradiation which is related to the heat generated by the absorption of microwave energy by water and other polar molecules and non-thermal effect which is claimed to change the chemical, biochemical, or physical behaviors of systems were responsible of this removal (Lin *et al*., 2009). In addition to the previous methods, there are still other nitrogen removal techniques which are applied on the side streams of municipal effluent and could be used also in AD processes (for review: Fuchs *et al*., 2018).

3.3 Characterization of the microbial community

3.3.1 The importance of investigating the process microbiology

As was described previously, a diverse and complex interacting microbial community respectively network comprising hydrolytic, acidogenic and acetogenic bacteria as well as methanogenic archaea convert biomasses into energy-rich biogas through consequent degradation phases. It is well known that the performance of an anaerobic digestion system is primarily linked to the structure and functionality of this diverse and complex interacting microbial network. Therefore, the management and engineering of this microbial community enhanced the development of the optimization strategies of the anaerobic digestion process (Carballa *et al*., 2015; Koch *et al*., 2014).

During the last years, the understanding of the factors that determine the anaerobic digestion process stability, as an example of ecosystem, has been one of the main challenges. Hence, the knowledge of the conditions that affect the process stability is needed to determine the effects of external parameters on the microbial community structure. For example, the abundance of the bacterial phyla *Firmicutes* and *Bacteroidetes* varying in the biogas community depending on the prevalent process conditions. While the diversity of the methanogenic archaea affected mainly by the substrate composition and hence by the availability of nutrients and ammonium/ammonia contents (Alsouleman *et al*., 2016, Alsouleman, 2019)

It was proved previously that, the disturbances in the microbial populations or the change in the prevalent microbial community structure from one trophic level affect the entire community and might cause a change in the functionality of this microbial community. Alsouleman and colleagues (Alsouleman *et al*., 2016) recorded that, the addition of 50% poultry manure led to a reconstruction of the prevalent *Bacteroidetes*–*Methanosaetaceae* microbiome. The resulted microbiome -which was functional redundant- was *Clostridiales*–*Methanobacteriaceae*- dominated. This disturbance in the microbial community structure and hence in the functionality of the microbial community might be reflected in the reactor performance by accumulation of intermediates, pH changes, or reduced efficiency (Schink, 1988).

Therefore, detailed and accurate information on the diversity and identity of the key microorganisms capable of carrying out specific metabolic processes in anaerobic digestion are very important to understand bioreactor functioning especially when concerning new metabolic processes. For example, the discovery of microorganisms

involved in the anaerobic oxidation of ammonium (Anammox process) (Jetten *et al*., 1999; Ni and Zhang, 2013), and in the syntrophic oxidation of organic acids (McInerney *et al*., 2008). Also the operational and chemical parameters of the process itself such as the substrate composition, applied organic loading rate, hydraulic retention time and the operating temperature affect the structural composition, the organization, the functionality as well as the ecological behavior of the microbial community (Demirel and Scherer, 2008; Carballa *et al*., 2011). Different environment pressure levels on the entire microbial community of the AD process may affect the efficiency of the whole process and may lead to a process imbalance or disturbance (Fernandez *et al*., 1999). On the other hand, these disturbances which caused by the physico-chemical factors may also be a feasible development strategy in shaping the profile of microbial community of the anaerobic digestion process and improve the efficiency of this process, since it could inhibit certain species and promote the growth of others that are resistant to the disturbance (for review: Theuerl *et al*., 2019; Alsouleman *et al*., 2016). Shaw and colleagues (Shaw *et al*., 2019) proved that an increase in COD and TS removal efficiency and methane content was recorded after a long-term temperature shock. This result agreed with the results of the previous studies that recorded an increase in COD removal rate or a decrease of total volatile fatty acids15 after temperature shock (Ahn and Forster, 2002).

Hence, the resulted microbial community structure from the artificial disturbance arising from the stepwise increasing in PM content (increasing in the ammonia concentration) might be also a feasible strategy to shape the structure and functionality of the prevalent microbial community and hence to improve the efficiency of the anaerobic digestion process of nitrogen-rich substrate.

There are four ways in which the microbial community in term of structure and functionality responses to the changing in the environmental parameters or to the process imbalances or disturbances. Firstly, the microbial community might be resistant to the disturbance on the engineering level and maintains its original composition after a disturbance. Secondly, the microbial community composition can be resilient by meaning that the microbial community changes due to the changing in the environmental conditions, but still has the ability to recover quickly and return to the original one. Thirdly, the microbial community composition changes and differs from the original one but has the ability to perform as the original one; in this case the functional redundancy in the microbial community structure is the applied mechanism

to maintain the functional stability during the disturbance. And lastly, the microbial community composition changes and performs completely different (Allison *et al*., 2008; Spirito *et al*., 2018).

The whole anaerobic digestion process can be disturbed when a single degradation step of the consequent degradation steps is out of balance (Gerardi, 2003). This disturbance can occur due to one (Shaw *et al*., 2019) or mix of physio-chemical factors (for review: Theuerl *et al*., 2019). For example, a process disturbance resulting from medium content of ammonium nitrogen (NH_4^+-N) led to change in the structure and functionality of the microbial community. The prevalent microbial community structure after this disturbance was able to maintain the stability of the anaerobic digestion process and perform efficiently under the new conditions (Alsouleman *et al*., 2016).

On the other hand, a process disturbance, resulting from high content of ammonium nitrogen (NH_4^+-N) and volatile fatty acids, was characterized by a big decline in the activity of the hydrogentrophic methanogens and acetogenic bacteria causing a process failure (Westerholm *et al*., 2016; Alsouleman *et al*., 2016).

The deeper understanding of the fundamental structure and metabolic interactions within biogas microbial consortia in different environmental conditions is very essential in order to control the whole process and at the end to determine the optimal operation conditions (Zakrzewski *et al*., 2012; Niu *et al*., 2014; Cabezas *et al*., 2015). Hence several studies have assumed that biomonitoring of the microbial community characteristics and the identification of key organisms related to specific process conditions could lead to an early detection of operational problems, making preventive action possible which could be used at the end as basis for microbiological monitoring, control and management (Verstraete *et al*., 2007; Lee *et al*., 2008; Malin and Illmer, 2008; Talbot *et al*., 2008; Theuerl *et al*., 2015).

3.3.2 Physico-chemical process analyses

There is a variety of the anaerobic digestion systems and configurations. The proper design of the reactor is dependent on the feedstock characteristics (content, quality), the investment costs, and the principle functioning of the anaerobic digestion process (Ward *et al*., 2008). Different reactor designs are commonly used for the AD of livestock manure waste such as: continuously respectively completely stirred tank reactors (CSTR) with continuous or periodic influent feeding (Ahring *et al*., 2001; Omar *et al*.,

2008; Zhang *et al*., 2011, Niue *et al*., 2013; Niu *et al*., 2014; Alsouleman *et al*., 2016), upflow anaerobic sludge blanket (UASB) reactors (Marañón *et al*., 2001; Castrillon *et al*., 2002), anaerobic batch reactors (Kalia and Singh, 2001; Adebayo *et al*., 2015), and plug flow reactors (PFR) (Ramaswamy and Vemareddy, 2015). Out of these, the most commonly used reactor types to investigate the biogas production from poultry manure are: the batch system (e.g. Dahunsi *et al*., 2019; Carlini *et al*., 2015), the continuously respectively completely stirred tank reactor (CSTR) (Niu *et al*., 2014; Niu *et al*., 2013; Zhang *et al*., 2011), and the upflow anaerobic sludge blanket (UASB) reactors (Yetilmezsoy and Sakar, 2008). In this study, laboratory-scales continuously respectively completely stirred tank reactors (CSTRs) with a working volume of 8 Liter were operated in mesophilic and thermophilic conditions as shown in **Fig. 2**. To ensure a high diversity of a well-performing starter. The start-up phase was carried out based on the VDI 4630 (The association of German engineers, 2006). To avoid process inhibition through a lack of micronutrient, 10 µl per g volatile substances (VS) trace element solution DSMZ 144 was added during the whole experimental period (German collection of microorganisms and cell cultures, Braunschweig, Germany) as recommended by Schattauer *et al*. (2011). Schattauer and his colleagues (2011) found in their investigation of 10 biogas plants that, the biogas plant which was fed with manure and energy crops recorded a depletion of the content of these trace elements over a longer time span.

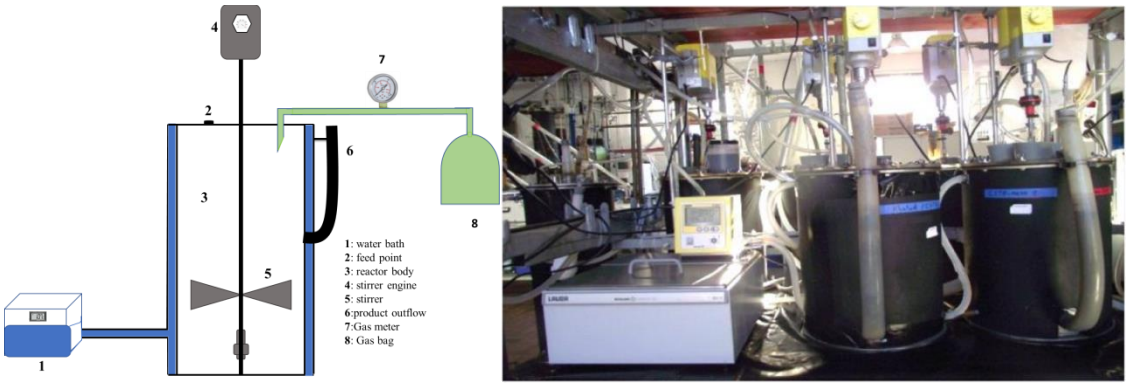

Figure 2: Construction of the CSTR biogas reactor

Afterwards, the OLR was maintained at 3.0 g $_{VS}$ L^{-1} d^{-1} for further 65 days and both reactors were operated at stable conditions indicated by pH, VFA as well as biogas yield and methane content. During the experimental phase (EP), the experimental reactors (ER) in two temperature condition were fed with an increasing amount (based on VS) of poultry manure, whereby the OLR was kept at 3.0 g_{VS} L^{-1} d^{-1}: low PM level = 75% CS and 25% PM, medium PM level = 50% CS and 50% PM and high PM level= 25% CS and 75% PM. While over the entire EP, the parallel operated control reactor (CR) was fed with cattle slurry as sole substrate (OLR of 3.0 g $_{VS}$ L^{-1} d^{-1}).

Biogas production from the anaerobic digesters was daily monitored and the biogas content was analyzed detecting the content of carbon dioxide (CO_2), methane (CH_4), hydrogen sulphide (H_2S) and oxygen (O_2).

During the anaerobic digestion process, various process parameters were determined: pH, total solids (TS), volatile solids (VS), total ammonium nitrogen (NH_4^+-N), soluble volatile fatty acids (VFA) in terms of acetate, propionate, iso- and n-butyrate, iso- and n-valerate, and capronate in addition to the conductivity, according to the Association of the German Agricultural Investigation and Research Institutes VDLUFA (1997). The free ammonia nitrogen (NH_3) content was calculated by using the formula previously described by Hansen *et al*. (1998) (Equation III).

3.3.3 DNA-based analysis of the microbial community structures

Different approaches are available now to investigate the process microbiology of the anaerobic digestion process (for review: Hassa *et al*., 2018; for review: Cabezas *et al*., 2015; Vanwonterghem *et al*., 2014; Su *et al*., 2012; for review: Talbot *et al*., 2008). Mainly, these approaches can be divided into culture-dependent and culture-independent methods, whereby each method has its advantages and disadvantages.

As generally known, most of our knowledge about microorganisms nowadays, their physiological capacities and the possibilities to use them in biotechnological applications has derived from the traditional isolation, cultivation and characterization of pure strains and species (Stewart, 2012). Classically, the application of culture-dependent techniques is required to identify microorganisms which are responsible for specific metabolic processes and to deeply understand their physiological potential of these microorganisms (Su *et al*., 2012; Amann *et al*., 1995).

But on the other side, these techniques are restricted by the use of the chosen cultivation media, which favor the growth of a limited number of community members and therefore limit the validity of the obtained results (Marzorati *et al*., 2008; Wagner *et al*., 1993). Wagner *et al*. (1993) reported that only 1-15% of the total microbial community could be detected in activated sludge samples by using culture-dependent methods. Therefore a "microbial dark matter" (uncultured microbial majority) is identified as the most important priority for biologist. This term refers to the sum of the taxonomically and functionally unassigned sequences in environmental genomics data sets, in addition to the uncultured microbes (for review: Brian *et al*., 2014).

Moreover, our knowledge and understanding of the anaerobic digestion process, a very complex microbial process in terms of functionality and community diversity, would be insufficient depicted due to the fact that the environmental factors which influence the microbial community structure, activity and interactions would not be taken into consideration (Kleerebezem and van Loosdrecht, 2007; Yoshiguchi *et al*., 2012; Zarraonaindia *et al*., 2013; Vanwonterghem *et al*., 2014). Thus, a combination of cultivation-independent methods is essential to investigate and study the complex anaerobic microbiome.

A commonly used cultivation-independent approach to investigate and profile the microbial community depends on the analysis of the 16S respectively small subunit ribosomal-RNA (rRNA) gene. The 16S rRNA gene is the most widely used marker gene because this gene is present in all bacteria and archaea, its function over time has not changed, the 16S rRNA gene is with 1500 bp long which is enough for informatics purposes, has the most extensive reference databases, and the presence of variable regions in this gene allows sufficient diversification while the presence of conserved regions enabled the design of suitable PCR primers (Godon *et al*., 1997; Sekiguchi *et al*., 1998; Patel, 2001; Talbot et al., 2008; Su *et al*., 2012; Sundberg *et al*., 2013; Veˇtrovsky´ and Baldrian, 2013; Cabezas *et al*., 2015; Theuerl *et al*., 2015). Hence, a 16S rRNA (gene)-based approach can provide a broad overview of community presence, activity in form of fluorescence in situ hybridization FISH and potential performance (depending on the prevalent microbiome), which could serve as a valuable overview and basis for several molecular techniques.

3.3.3.1 The terminal restriction fragment length polymorphism (TRFLP)

The terminal restriction fragment length polymorphism (TRFLP) is a fingerprinting technique to monitor the main spatial and temporal changes in the microbial community composition in response to the environmental perturbations (Lukow *et al*., 2000; Marsh, 2005; Talbot *et al*., 2008; Enwall und Hallin, 2009; Sboner *et al*., 2011; van Dorst *et al*., 2014; Cabezas *et al*., 2015; Alsouleman *et al*., 2016; Weise *et al*., 2016; De Vrieze *et al*., 2018). This method has been introduced firstly by Liu *et al*. in 1997. After that, huge efforts were done to optimize this technique in order to limit the drawbacks in applying this technique in the investigation of the microbial communities even in anaerobic digestion processes (Osborn *et al*., 2000; Engebretson and Moyer, 2003; Abdo *et al*., 2006; Osborne *et al*., 2006; Schütte *et al*., 2008 ; Rademacher *et al*., 2012).

During the last years, the TRFLP analysis has been widely applied in microbial community investigation of biogas production process (e.g., Feng *et al*., 2010 ; Wang *et al*., 2010; Carballa *et al*., 2011; Pycke *et al*., 2011; Ziganshin *et al*., 2011; Rademacher *et al*., 2012; Klang *et al*., 2015; Alsouleman *et al*., 2016 ; De Vrieze *et al*., 2018; Alsouleman, 2019). The Traditional T-RFLP technique relies on the use of at least one fluorescently labelled PCR primer to amplify the 16S rRNA gene. After the DNA amplification, the fluorescently labeled PCR product was digested by a restriction enzyme (endonuclease). Afterwards, the fluorescently labeled fragments were separated together with an internal length standard - allowing a size calculation of the terminal restriction fragments (TRFs) - by an automated capillary gel electrophoresis system **Fig. 3**.

Further analysis and comparison of the TRFLP profiles can be conducted by using appropriate software solutions, e.g., BioNumerics (Applied Maths, Belgium). The TRFLP profiles of each sampling point were evaluated separately in the fingerprint curve-processing window. The identification of “true” terminal restriction fragments (TRFs) by distinguishing background and baseline “noise” or false positives (bleed through peaks) from signals of correctly fluorescent-labelled fragments as well as the alignment (band matching) of detected terminal restriction fragments (TRFs) was done. Finally, TRFs were visualized by their relative distribution within tables (Appendix 1; Alsouleman, 2019).

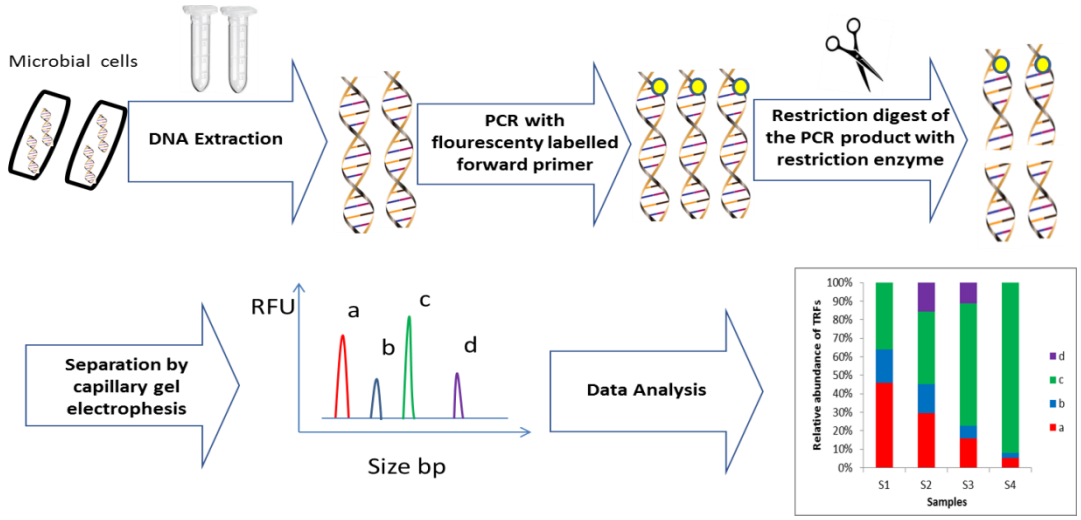

Figure 3: Short workflow of the TRFLP analysis

Different studies in different environments have compared the TRFLP with next generation amplicon sequencing based on the 16S rRNA gene, e.g., by using the Illumina sequencing platforms. These studies proved the potential of the TRFLP as robust and reliable technique for fast community screening (for review: De Vrieze *et al*., 2018; Witzig *et al*., 2015) and the capability of this technique to be used as pre-analysis before the application of the next generation sequencing (Brugger *et al*., 2012). Recently, De Vrieze *et al*. (2018) revealed with a comparison of the Illumina amplicon sequencing (next generation sequencing technique) and bacterial TRFLP and archaeal TRFLP profiles of 25 full-scale AD plants a high degree of similarity in the β-diversity profiles. The β-diversity index gives the value of the dissimilarity between communities between samples. While they found a clear dissimilarity between the Illumina archaeal profile and TRFLP archaeal profile at α-diversity levels which give information about the number of the species and their relative abundance in each sample. Also, they concluded that the TRFLP technique may be easier and cheaper and alternative to 16S rRNA gene amplicon sequencing to monitor the overall structure of the microbial communities.

In this study, as the major aim is to investigate the microbial structure, its dynamics over time and how the prevalent operational and environmental conditions could affect the microbial community structure, the terminal restriction fragment length polymorphism (TRFLP) was used. The TRFLP analyses were carried out following the optimized protocol published by Rademacher *et al*. (2012). Bioinformatic evaluation of

the obtained microbiological data was performed according to Klang *et al*. (2015) using the software package BioNumerics 7.1 (Applied Maths, Belgium).

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

The 16S rRNA gene sequence libraries were constructed to identify and characterize the detected TRFs and hence the microbial community structure during the course of fermentation. The PCR amplification of the 16S rRNA gene was conducted using the same primer set of the TRFLP approach but in this case without fluorescent labeling. Cloning of 16S rRNA gene amplicons was performed according to Rademacher and colleagues (2012).

The sequences of the selected clones as determined by GATC Biotech AG (Germany) were then clustered 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). Then the taxonomic position of the representative sequences and the identification of the detected TRFs were determined according to the Klang *et al*., (2015). This cloning and sequencing approach was firstly reported by Giovannoni *et al*. (1990) in an analysis of the diversity of bacterioplankton in Sargasso Sea. After that a huge number of studies applying this approach to identify and characterize the complex microbial community composition of the anaerobic microbial community were conducted (Godon *et al*., 1997; Sekiguchi *et al*., 1998; Roest *et al*., 2005; Nettmann *et al*., 2008; Goberna *et al*., 2009; Nelson *et al*., 2011; Rademacher *et al*., 2012; Klang *et al*., 2015; Alsouleman *et al*., 2016; Alsouleman, 2019).

3.4 Conception and aims of this study

The main aim of this study was to evaluate the impact of a stepwise increase of ammonium nitrogen due to the addition of different poultry manure levels on the mesophilic and thermophilic anaerobic reactors performance and especially the structure and dynamic variations of the occurring microbial community. In other words, this study aimed to investigate how much poultry manure, and respectively how much ammonium nitrogen, can be tolerated by the AD microbial community without any negative effects on the overall process performance in two different temperature ranges, and to follow the response of the occurring microbial community to the environmental disturbance arising from the changing in the feedstock supply and nutrient availability in terms of stability, functional redundancy and resilience.

The composition and dynamics of microbial communities during long term bioreactors operation were investigated by molecular methods targeting 16S rRNA genes. T-RFLP fingerprinting in combination with 16S rRNA gene sequence libraries were performed covering the whole experimental period and all putative community changes correspond to the increasing amount of poultry manure.

In detail, the main aim of this study was achieved by:

- The evaluation of the effect of the consecutive addition of three poultry manure levels on the long-term mesophilic and thermophilic reactors performance; three experimental phases were defined depending on the added PM level: EP1 with low PM level = 75% CS and 25% PM, EP2 with medium PM level = 50% CS and 50% PM, EP3 with high PM level = 25% CS and 75% PM.

- The characterization of the bacterial and archaeal community of the control reactors (feeding with cattle slurry as sole feedstock) in mesophilic and thermophilic conditions.

- The investigation of the changes in the microbial community structure as a consequence to poultry manure addition. Therefore, the bacterial and archaeal community structure was analyzed using TRFLP technique at different time points during the experimental phases depending on the reactor performance.

- The correlation between the microbial community structure and the prevalent process parameters (NH_4^+-N, NH_3, VFA, VS and conductivity)**.**

Out of these parameters it will be possible to draw conclusions on: how much poultry manure can be applied without any disturbance of the overall AD process performance on the experimental level; the efficiency of the thermophilic AD of the added PM levels, which is a preferable option from the economical point of view in the countries with long and hot summer like Syria, and the efficiency of the microbial community structure which is robust against the disturbances arising from the added PM level. The previous aspects will help to run the AD of nitrogen-rich manure as efficient as possible and to apply this technology in future on full scale as an animal waste treatment technology and bioenergy resource.

4 Publications

4.1 Reorganisation of a mesophilic biogas microbiome as response to a stepwise increase of ammonium nitrogen induced by poultry manure supply

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Khulud Alsouleman, Bernd Linke, Johanna Klang, Michael Klocke, Niclas Krakat, Susanne Theuerl

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Short Communication

Reorganisation of a mesophilic biogas microbiome as response to a stepwise increase of ammonium nitrogen induced by poultry manure supply

Khulud Alsouleman [a,b], Bernd Linke [a], Johanna Klang [a], Michael Klocke [a], Niclas Krakat [a], Susanne Theuerl [a,*]

[a] Leibniz Institute for Agricultural Engineering Potsdam-Bornim (ATB), Department Bioengineering, Max-Eyth-Allee 100, D-14469 Potsdam, Germany
[b] Faculty of Agricultural Science, Georg-August-University Göttingen, Büsgenweg 5, D-37077 Göttingen, Germany

HIGHLIGHTS

- Process-risk feedstocks such as poultry manure have to be used with caution.
- Low amounts of NH_4^+-N and VFA favour a *Bacteroidetes–Methanosaetaceae* microbiome.
- The addition of 50% poultry manure led to a reconstruction of the microbiome.
- The functional redundant microbiome was *Clostridiales–Methanobacteriaceae*-dominated.
- A natural-regulated microbial diversity management was recorded.

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ABSTRACT

An anaerobic digestion experiment was investigated to evaluate the impact of increasing amounts of ammonium nitrogen due to poultry manure addition on the reactor performance, especially on the microbiome response. The microbial community structure was assessed by using a 16S rRNA gene approach, which was further correlated with the prevalent environmental conditions by using statistical analyses. The addition of 50% poultry manure led to a process disturbance indicated by a high VFA content (almost 10 $g_{HAc\text{-}Eq}$ L^{-1}) in combination with elevated concentrations of ammonium nitrogen (5.9 g NH_4^+-N kg_{FM}^{-1}) and free ammonia (0.5 g NH_3 kg_{FM}^{-1}). Simultaneously the microbiome, changed from a *Bacteroidetes*-dominated to a *Clostridiales*-dominated community accompanied by a shift from the acetoclastic to the hydrogenotrophic pathway. The "new" microbial community was functional redundant as the overall process rates were similar to the former one. A further increase of poultry manure resulted in a complete process failure.

1. Introduction

The implementation of anaerobic digestion (AD) on animal waste has become a promising alternative treatment technology as it reduces the negative environmental impacts and offers a sustainable renewable energy resource. However, applying AD on animal manure is related to the risk of ammonium nitrogen (NH_4^+-N) accumulation, whereby the undissociated form (free ammonia, NH_3), is considered to be toxic for the microbial community, especially for the acetoclastic methanogens, which may lead to process disturbances/failures (Chen et al., 2008; Lv et al., 2014). It has also been shown that a process imbalance can naturally be prevented by a shift from acetoclastic to hydrogenotrophic methanogenesis in combination with syntrophic acetate oxidation (Schnürer and Nordberg, 2008). Hence one of the most important factors to ensure a stable biogas production is a highly efficient microbiome which is resilient against process disturbances (Theuerl et al., 2015). Consequently, the scientific challenge is to expand the knowledge of the complex ecological network within the biogas reactor.

In order to investigate the process microbiology, a broad range of methods is available, whereby it is recommended to use a combination of different methods and link the community structure information to its role in its respective habitat (Cabezas et al., 2015; Carballa et al., 2011; Verstraete et al., 2007). So far only a

* Corresponding author. Tel.: +49 331 5699 900; fax: +49 331 5699 849.
E-mail address: susanne.theuerl@googlemail.com (S. Theuerl).

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few long-term studies are available dealing with the effects of a gradual increase of NH_4^+-N due to the addition of poultry manure on the reactor performance, especially under consideration of the structure and dynamic variations of the microbiome. Therefore, the aim of the present study was to investigate how much poultry manure, respectively how much NH_4^+-N can be tolerated by the AD microbiome without any negative effects on the overall process performance.

2. Methods

2.1. Experimental setup, biogas reactor operation and chemical analyses

To investigate the response of the microbial community due to an increasing concentration of NH_4^+-N caused by addition of poultry manure (PM), two mesophilic (37 °C) laboratory-scale (eight litre working volume) continuously stirred tank reactors (CSTRs) were operated at an organic loading rate (OLR) of 3.0 g_{VS} L^{-1} d^{-1}. Over the experimental phase (EP), the control reactor (CR) was operated with cattle slurry (CS) as sole feedstock, whilst the experimental reactor (ER) was fed with an increasing amount of PM (based on volatile solids, VS) as followed: EP1 = 75% CS and 25% PM for 143 days with a hydraulic retention time (HRT) of 30 days; EP2 = 50% CS and 50% PM for 165 days (HRT = 37 days); EP3 = 25% CS and 75% PM for 171 days (HRT = 52 days); and finally EP4 = 100% PM for 22 days (HRT = 134 days). Over the entire trail period, the produced amount of biogas was measured continuously, whilst the gas composition was analysed twice a week. Digestate samples were analysed regarding their main chemical characteristics (total Kjeldahl nitrogen (TKN), NH_4^+-N content, volatile fatty acids (VFA) in terms of acetate, propionate, *iso*- and *n*-butyrate, *iso*- and *n*-valerate, and capronate) according to Schönberg and Linke, (2012).

2.2. Microbial community analyses

To investigate the process microbiology 14 different time points (depending on the reactor performance) were chosen from the ER (day 98, 137, 155, 185, 207, 230, 274, 305, 319, 337, 372, 479, 490, 514) and three from the CR (day 98, 207, 479). Genomic DNA was extracted using the FastDNA® SPIN Kit for soil (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's guidelines.

To characterise the microbial community structure and its dynamic variation the genomic fingerprinting method terminal restriction fragment length polymorphism (TRFLP) targeting either the bacterial or the archaeal 16S rRNA gene was used as described by Rademacher et al. (2012) and Klang et al. (2015). Bioinformatic evaluation of the obtained data was performed as published by Klang et al. (2015) using the software package BioNumerics 7.1 (Applied Maths, Belgium).

Additionally, 16S rRNA gene sequence libraries were constructed from samples ER-98, ER-137, ER-185, ER-305, ER-372, ER-514, and CR-98, covering all putative community changes. Cloning of PCR products was performed according to Rademacher et al. (2012), followed by DNA sequencing (GATC Biotech AG, Konstanz, Germany). The obtained sequences were processed, grouped into operational taxonomic units (OTUs) and virtually cut according to Klang et al. (2015) using the software package BioNumerics 7.1 (Applied Maths, Belgium). Subsequently, OTUs were taxonomically assigned using the Ribosomal Database Project RDP Version 2.6 (Wang et al., 2007). All new sequences obtained in this study have been deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers LN849462–LN849688 (*Bacteria*) and LN874153–LN874209 (*Archaea*).

In order to get information about the microbial system ecology, the community organisation expressed by the Gini coefficients was calculated (Carballa et al., 2011; Klang et al., 2015; Theuerl et al., 2015; Verstraete et al., 2007). Additionally, the software package of PC Ord Version 6 (McCune and Mefford, 2011) was used to perform a non-metric multidimensional scaling (NMS) (Clarke, 1993) to conduct a correlation amongst and between the operational and microbiological parameters.

3. Results and discussion

3.1. Performance of anaerobic co-digestion of cattle slurry with stepwise increased addition of poultry manure

During the experimental period, the control reactor (CR) showed no significant changes neither in the produced biogas (376 ± 72 L_N kg_{VS}^{-1} with a CH_4 content of 62 ± 2%; Fig. 1A) nor in the main chemical parameters (NH_4^+-N = 1.8 ± 0.2 g kg_{FM}^{-1}, NH_3 = 0.07 ± 0.02 g kg_{FM}^{-1} and VFA concentration $\leqslant 0.5$ g L^{-1}).

The increasing NH_4^+-N concentration caused by the addition of PM showed no significant effects on the process parameters until 4.2 g kg_{FM}^{-1} were reached in EP2; a values which exceeded the reported inhibition levels of the AD, especially of the acetoclastic methanogenesis (Drosg, 2013; Schnürer and Nordberg, 2008). Subsequently, a serious process imbalance occurred as indicated by a VFA accumulation of almost 10 $g_{HAc\text{-}Eq}$ L^{-1} (mainly acetic acid), and a drop in the biogas production (Fig. 1). After a certain time (without active counteracting) the system recovered symbolized by a decrease in the VFA concentration and a subsequent increase of the biogas, respectively methane contents. Hence, it can be supposed, that an adaption from the acetoclastic to hydrogenotrophic pathway of methane formation took place.

A further increase of PM (75% in EP3, respectively 100% in EP4) and, hence, a continuous increase of NH_4^+-N of up to 9.6 g kg_{FM}^{-1} resulted in a complete process failure indicated by a constant increase in the VFA concentration accompanied by a strong decrease of the biogas amount and methane content (Fig. 1).

3.2. The response of the microbiome to increased amounts of poultry manure

The microbiome of the three samples from the CR consisted mainly of members from the phylum *Bacteroidetes* (35%) followed by *Firmicutes* (17%), and the WWE1 candidate division (10%) at the bacterial level and members of the genus *Methanosaeta* (phylum *Euryarchaeota*) with an abundance of around 60% at the archaeal level. The Gini coefficients in the CR were 0.41 (*Bacteria*), respectively 0.69 (*Archaea*), values which are reported to symbolise well-established communities (Theuerl et al., 2015). Hence, it can be assumed that the methane formation was mainly carried out by the acetoclastic pathway which in turn indicated a good performing reactor system as previously reported by Regueiro et al. (2012).

Although no significant changes in the reactor performance were observed during EP1 (see Section 3.1), the microbial community, especially the archaeal community was affected by changes in the feedstock supply and, therefor, the nutrient availability as the abundance of members belonging to the family *Methanobacteriaceae* increased slightly. This led to a more even distributed community organisation combined with an increasing importance of the hydrogenotrophic pathway of methane formation.

Compared to EP1, the sample ER-155 taken 11 days after increasing the PM amount to 50% (EP2), showed significant differences in the bacterial community structure whereby members from the family *Porphyromonadaceae* (phylum *Bacteroidetes*)

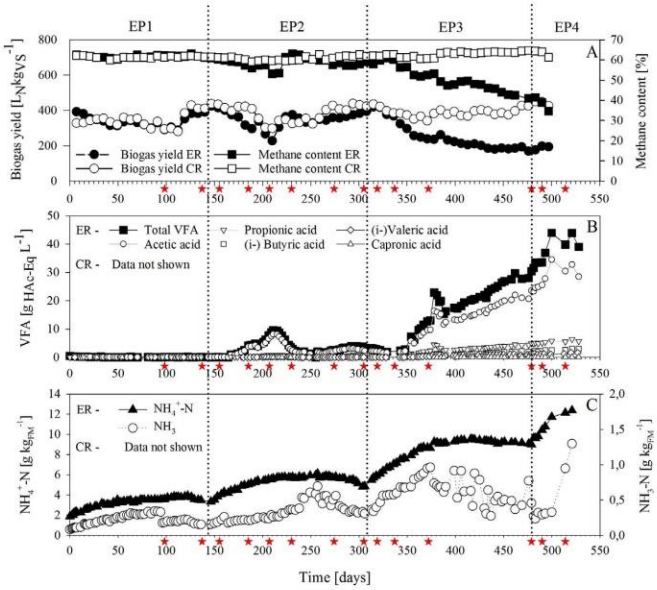

Fig. 1. Process performance in terms of the biogas yield and methane content for the control and experimental reactor (A), the volatile fatty acids (VFA) produced in the experimental reactor (B) as well as the ammonium nitrogen (NH_4^+-N) and the free ammonia (NH_3) nitrogen content of the experimental reactor (C) over the entire experimental period. Stars at *X*-axis indicate the sampling points for the molecular biological analyses. EP = experimental phase, ER = experimental reactor, CR = control reactor.

accounted for around 44% of the entire community (calculated as a sum of all group-related TRFs). This increased abundance may explain the subsequent VFA accumulation as several members from the order *Bacteroidales* are reported as acid producers with acetic acid as main end product (e.g., Hahnke et al., 2016). Over the course of the process disturbance a reorganisation of the bacterial community occurred as the *Bacteroidetes*-dominated microbiome was gradually replaced by members of the order *Clostridiales* (phylum *Firmicutes*) as they reached about 47% of the entire bacterial community at day 274 (Fig. 2A). This finding is in accordance with De Vrieze et al. (2015) who reported a negative correlation between the order *Bacteroidales* and the orders belonging to the phylum *Firmicutes*, with higher abundances of the *Bacteroidales* order at low NH_4^+-N and VFA concentrations. At this point it is questionable whether the VFAs produced by *Bacteroidales* led to self-inhibition or whether the increasing NH_4^+-N concentrations suppressed their growth. Moreover, De Vrieze et al. (2015) further pointed out that *Bacteroidales*-dominated AD microbiomes are also dominated by the archaeal family *Methanosaetaceae* which was also found in this study. As it is well-known that *Methanosaetaceae* are significantly negative correlated with the both mentioned parameters NH_4^+-N and VFA, it can be assumed that the increased amount of acetic acids in combination with rising NH_4^+-N concentration led to an inhibition of the acetoclastic pathway of methane formation which in turn forced the community reorganisation.

The order *Clostridiales* consists not only of several species which possess hydrolytic and acido-/acetogenic capacities (substrate degradation and acid production) but also of species which are able to perform syntrophic acetate oxidation (Schnürer and Nordberg, 2008). In case of a syntrophic relationship a close contact between hydrogen producers and consumers is necessary which means that the reorganisation at the bacterial level should be accompanied by a shift to hydrogenotrophic methanogens which was recorded by the TRFLP results (Fig. 2B). In the beginning of EP2 a high abundance of TRF-107 bp was detected; a TRF which was assigned to the genus *Methanosaeta* based on the conducted sequence libraries of ER-98 and ER-137. However, the sequence libraries of ER-185 revealed a different picture as sequences from the family *Methanobacteriaceae* (symbolized *inter alia* also by TRF-107 bp and further by TRF-340 bp) became more abundant. Consequently, the prevalent chemical conditions arising from the increasing amount of NH_4^+-N and NH_3 resulted in a shift from the acetoclastic pathway of methane formation to the hydrogenotrophic pathway (Fig. 2B) which is well known to be the more stable/robust metabolic pathway considering the risk of ammonia toxicity (Chen et al., 2008; Fotidis et al., 2014). Concluding, the addition of 50% PM resulted in a reorganisation of the occurring microbial community. According to the reported ecological theories by Allison and Martiny (2008) it can be assumed that the "new" microbial community is functional redundant compared to the former one as the overall process rates were similar after the disturbance phase. This is a very good example for a natural-regulated, highly efficient microbial diversity management system.

According to the ongoing increase of the NH_4^+-N content during EP3 (75% PM) the trend of EP2 (Fig. 2A) was moving on towards a *Clostridiales-Methanobacteriaceae*-dominated microbiome. Although EP3 lasted for 170 days, the induced process disturbance could not be compensated by the occurring microbial community. Regarding that the biogas yield and the methane content showed a significant decrease resulting in a complete process failure (Fig. 1), it has to be concluded that the methanogenic activity was seriously inhibited. Hence, it is reasonable to assume that the molecular hydrogen (H_2) level increased which further inhibited the growth of acid converting bacteria that can only grow if the H_2, (which is produced by themselves), is consumed by their syntrophic

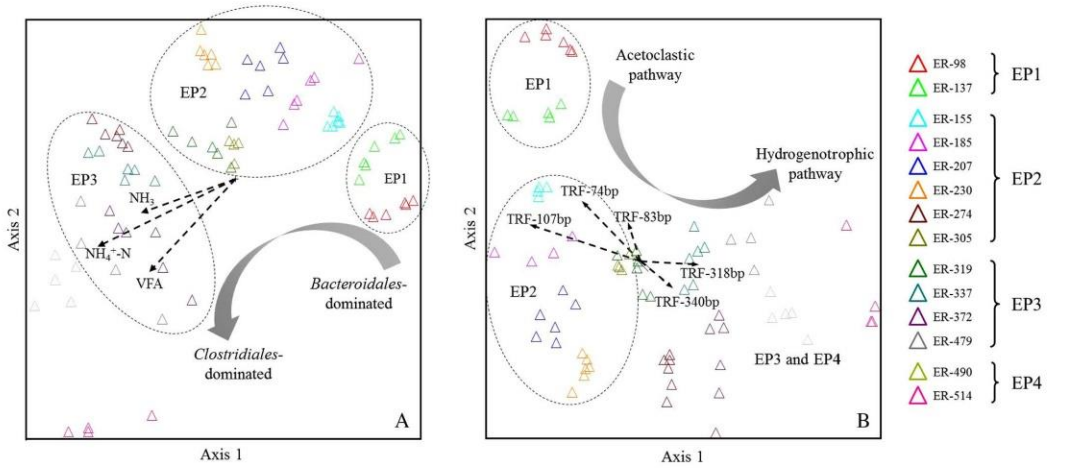

Fig. 2. Non-metric multidimensional scaling (NMS) regarding three selected prevalent process parameters in terms of ammonium nitrogen (NH_4^+-N), free ammonia nitrogen (NH_3), and total volatile fatty acids (VFA) and their effects on the bacterial community structure (A), respectively regarding the bacterial community structure and the corresponding archaeal community with special emphasis on the major pathway of methane formation (B).

archaeal partners. Consequently, the hydrolytic and acidogenic bacterial community members of the microbiome still converted the supplied feedstock/substrates into acids but the successive steps of conversion were completely inhibited which explains the VFA accumulation (see Section 3.1).

4. Conclusion

This study revealed that the present AD microbiome was able to tolerate a certain amount of poultry manure. The addition of 50% PM induced a process disturbance which was characterised by a shift from a *Bacteroidetes*-dominated to a *Clostridiales*-dominated bacterial community accompanied by a change from the acetoclastic to the hydrogenotrophic pathway of methane formation. This study is a good example for a natural-regulated microbial diversity management system, as it highlighted how an anaerobic microbiome was enabled to adapt to changing environmental conditions. In regard to configure the biogas process as efficiently as possible, it can be assumed that the use of process-risk feedstocks is possible but it has to be used with cautions and especially with respect to the demands of the microbiome.

Additional information

The relative distribution of the detected bacterial and archaeal terminal restrictions fragments (TRFs) highlighted by colours according to an increasing abundance and their phylogenetic assignment is given as supplement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.02.104.

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4.2 Effect of increasing amounts of ammonium nitrogen induced by consecutive mixture of poultry manure and cattle slurry on the microbial community during thermophilic anaerobic digestion

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Khulud Alsouleman

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Effect of Increasing Amounts of Ammonium Nitrogen Induced by Consecutive Mixture of Poultry Manure and Cattle Slurry on the Microbial Community during Thermophilic Anaerobic Digestion

Khulud Alsouleman[1,2*]

[1]*Department Bioengineering, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany*
[2]*Faculty of Agricultural Sciences, Georg-August-Universität Göttingen, Göttingen, Germany*

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**Corresponding author*
Phone: +49-162-9828140
Email: khulud1981@gmail.com

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Thermophilic anaerobic digestion (TAD) is characterized by higher biogas production rates as a result of assumedly faster microbial metabolic conversion rates compared to mesophilic AD. It was hypothesized that the thermophilic microbiome with its lower diversity than the mesophilic one is more susceptible to disturbances introduced by alterations in the operating factors, as an example, the supply of nitrogen-rich feedstock such as poultry manure (PM). Laboratory scaled TAD experiments using cattle slurry and increasing amounts of PM were carried out to investigate the (in-) stability of the process performance caused by the accumulation of ammonium and ammonia with special emphasis on the microbial community structure and its dynamic variation. The results revealed that the moderate PM addition, *i.e.*, 25% (vol/vol based on volatile substances) PM, resulted in a reorganization of the microbial community structure which was still working sufficiently. With 50% PM application, the microbial community was further stepwise re-organized and was able to compensate for the high cytotoxic ammonia contents only for a short time resulting in consequent process disturbance and final process failure. This study demonstrated the ability of the acclimated thermophilic microbial community to tolerate a certain amount of nitrogen-rich substrate.

Keywords: Ammonia inhibition, microbiome, process disturbance, biogas

Introduction

Reducing greenhouse gas emissions resulting from open storage and uncontrolled spreading of animal slurries and manures is a major challenge faced by the agricultural sector [1]. One of the most important and commonly applied technologies to achieve this goal is the bioconversion of animal wastes into energy-rich biogas by anaerobic digestion (AD) [2, 3].

The thermophilic AD (TAD, 50–60°C) has generally higher metabolic rates and hence higher overall process efficiency than mesophilic AD performed at 37°C [4–7]. On the other hand, TAD is very sensitive to changes in operating factors such as total solids (TS), mixing rate, organic loading rate (OLR), pH, volatile fatty acid (VFA) content, and total ammonium nitrogen content (TAN) which in turn reduce the process stability [8–11].

Due to the cytotoxic effects of ammonia (NH_3), resulting from deprotonation of ammonium (NH_4^+), many efforts have been previously made to determine the TAN thresholds in mesophilic and thermophilic AD processes which ranged between 1.8 and 5.0 g NH_4^+-N/L. These inhibition thresholds varied widely due to the direct or indirect effect of many other factors such as the substrate composition, the initial nitrogen concentrations, the temperature, the pH, the OLR, the acclimation period, and the acclimation of the inoculum [12–20].

The efficiency and stability of the AD process are entirely dependent on the concerted and syntrophic activity of the involved microorganisms. Several studies investigated basically the effect of increasing TAN on the thermophilic microbial community structure [11, 21–25]. For example,

Hao *et al.* [21] showed in batch thermophilic acetate fed experiments at two TAN (low, *i.e.*, 0.26 g/l, and high, *i.e.*, 7.00 g/l) - that the microbial communities were similar for both TAN conditions. While in a previous work the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was shown as the dominant pathway in both mesophilic and thermophilic full-scale anaerobic digesters with high ammonia levels (2.8–4.57 g NH_4^+-N/L), whereas the acetoclastic methanogenic pathway dominated at low ammonia content (<1.21 g NH_4^+-N/L) [22]. Other studies showed that the mesophilic microbial community shifted significantly as members belonging to the *Bacteroidetes* and *Methanosaeta* were gradually disappeared with elevated TAN and hence elevated NH_3 contents [20, 26, 27].

However, with main focus on the reactor performance and the microbial community structure of the TAD of nitrogen-rich substrate, only a few is known about the relationship between TAD microbial community shifts and performance variation resp. disturbances due to the accumulation of TAN.

We hypothesized that the potential impact of the stepwise increase of the TAN, as a consequence of the application of nitrogen-rich fermentation substrates, and hence the consequent prevalent operational process conditions will lead to more serious disturbances in the TAD process compared to mesophilic ones as previously published by Alsouleman *et al.* [20]. So that, this study focused mainly on the (in-) stability of the long-term TAD process in terms of biogas and methane yield, VFA content and accumulation of TAN as a response to the environmental perturbations arising from increasing amounts of poultry manure (PM). Also the response of the thermophilic microbial community was determined by 16Sr RNA gene targeted terminal restriction fragment length polymorphism (TRFLP) fingerprinting which allows the direct screening and comparison of microbial communities' dynamics in different samples [28–36]. Subsequent determination of respective nucleotide sequences enables the identification of representatives for the most abundant taxa [37–39].

Materials and Methods

Experimental Setup and Biogas Reactor Operation

Two thermophilic (55°C) continuously resp. completely stirred tank reactors (CSTRs) with a working volume of 8 L were operated in parallel for (385 days), allowing the adaptation of the microbial community to the apparent process conditions. The start-up of the CSTRs was conducted according to [40]. To ensure a high diversity of a well-performing starter community, both reactors were initiated with anaerobic inoculum from previous experimental reactors which were fed with cattle and pig slurry as well as maize silage. This inoculum was diluted 1:2 with tap water before inoculation. During the first 75 days, both reactors were fed with CS starting with an organic loading rate (OLR) of 0.5 g_{VS}/l/d and gradually increased (using 0.5 g_{VS}/l/d steps) until the final OLR of 3.0 g_{VS}/l/d was achieved. Afterwards, the OLR was maintained at 3.0 g_{VS}/l/d for the further 65 days.

To avoid process inhibition through a lack of micronutrient, 10 µl of the trace element solution DSMZ 144 was added per g volatile solids (VS) (German collection of microorganisms and cell cultures, Germany) as recommended by [41]. Both reactors were operated at stable conditions indicated by pH, VFA as well as biogas yield and methane content.

The experimental CSTR (= ER) was fed with an increasing amount (based on VS) of PM as followed whereby the OLR was kept constantly at 3.0 g_{VS}/l/d: first experimental period (= EP1) with 75% CS and 25% PM for 176 days and hydraulic retention time (HRT) of 26 days; second experimental period (= EP2) with 50% CS and 50% PM for 165 days and HRT of 39 days. Over the entire EP, the control reactor (CR) was operated with CS as sole substrate with a constant OLR of 3.0 g_{VS}/l/d equal to the OLR of the ER.

Biogas production was monitored daily using a drum-type gas meter (Meter TG 05, Ritter Apparatebau, Germany). The biogas composition was analysed three times per week using the gas analyser SSM 6000 (Pronova Analysentechnik, Germany).

Chemical Characterization of the Used Feedstocks and Digestate Samples

The used feedstocks as well as the digestate samples (sampling twice a week), were analysed as follows: Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), the NH_4^+-N content, the amounts of VFA in terms of acetate, propionate, iso- and n-butyrate, iso- and n-valerate, and capronate were analysed according to [42]. The pH-value was measured three times a week using pH/cond 340i with the pH-Electrode SenTix41 (WTW, Germany).

The free ammonia (NH_3) content was calculated based on the NH_4^+-N content, the pH-value and the reactor temperature using the formula previously described by Hansen [17].

Microbial Community Analysis

To investigate the process microbiology, samples were taken from the ER at following process-relevant time points; day 81, and 162, *i.e.*, the beginning and the end of EP1; day 199, *i.e.*, the beginning of the EP2: day 250, *i.e.*, the beginning of short-termed stable biogas yield period; day 300, *i.e.*, the beginning of the biogas yield decrease period; day 340, *i.e.*, the end of EP2. From the CR, samples were analysed for days 81 and 340, *i.e.*, the beginning and the end of ER, as the CR showed an almost stable anaerobic digestion process during the whole ER as indicated by VFA content, biogas yield, and methane content. From two sub-

samples of each sample (*i.e.*, technical replicates), the microbial genomic DNA was extracted using the FastDNA SPIN Kit for soil (MP Biomedicals, Germany) according to the manufacturer's guidelines.

To characterize the microbial community structure, terminal restriction fragment length polymorphism (TRFLP) analysis targeting the bacterial or the archaeal 16Sr RNA gene was applied as described in [23].

16Sr RNA gene sequence analysis was conducted as described before [23]. The obtained sequences were processed, grouped into operational taxonomic units (OTUs) and virtually cut according to [43] using the software package BioNumerics 7.6 (Applied Maths, Belgium).

All sequences obtained in this study have been deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers LT718731 - LT718850 (*Bacteria*) and LT718710 - LT718730 (*Archaea*).

Statistical and Ecological Analysis

Correlation coefficients among and between the operational and microbiological parameters, an indicator species analysis (ISA) [44], a non-metric multidimensional scaling (NMS) [45, 46], Gini coefficients [47, 48] in addition to Shannon [49] and Richness [47] diversity indices were estimated to show the correlation amongst and between the operational and microbiological parameters and to get more information about the microbial system ecology and the diversity of the microbial community. Detailed information on the whole materials and methods section is provided as supplemental material appendix 1.

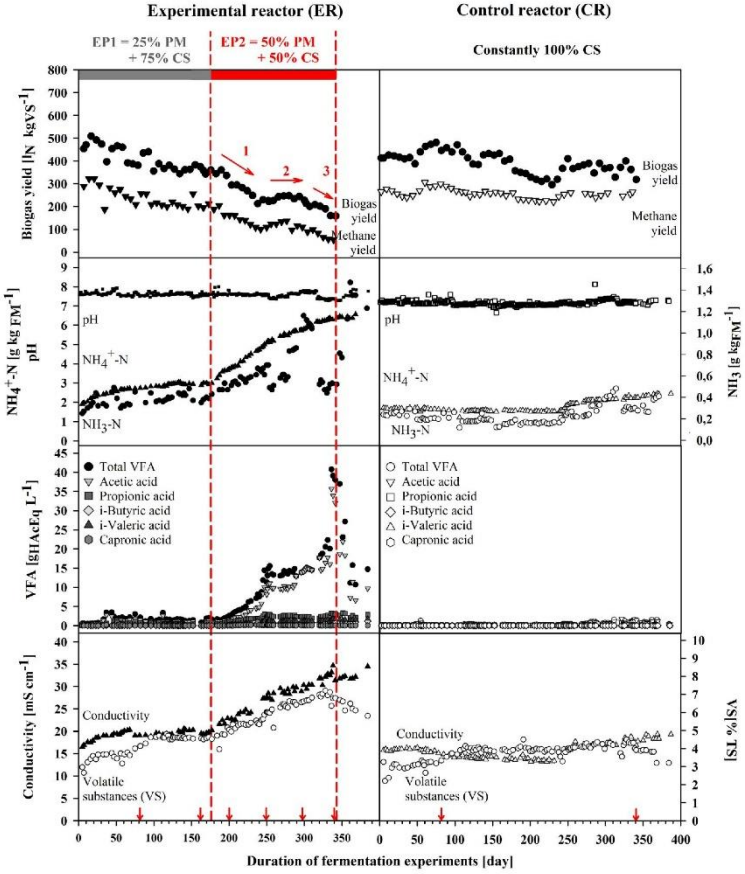

Fig. 1. Process performance parameters for the experimental (ER) and control reactor (CR) during experimental phases1 (EP1) and 2 (EP2).
The arrows on the X-axis indicate sampling points for molecular analyses. The arrows 1, 2, 3 indicate three-stage process disturbance.

Results

Operation of CSTRs

Changes in control and experimental TAD performance during the entire experimental period are shown in (Fig. 1). During both experimental periods (EP1 and EP2), the control reactor (CR) supplied exclusively with cattle slurry showed no significant changes in the produced biogas yield ($R2 = 0.27$; $p < 3.7 \times 10^{-25}$) (Fig. 1). Thus, the almost stable chemical parameters in the CR indicated a more or less stable and metabolically active microbial biocoenosis.

In the experimental reactor (ER), as expected, the change of the feedstock composition during EP1 resulted in an increased TAN content up to 3.0 g NH_4^+-N/kg_{FM} at the end of this experimental phase with a corresponding increase in NH_3 content up to 0.4 g/kg_{FM} respectively (Fig. 1). The total VFA content which consisted mainly of acetic acid showed a minor increase in this experimental phase. The content of the other volatile fatty acids in terms of propionate, iso- and n-butyrate, iso- and n-valerate and capronate was found in minor amount and was more or less constant. The relative alteration of the VFA concentration (as acetic acid) during EP1 was 1.46 g/l compared with 1.13 g/l in control reactor. The pH value was almost stable with 7.62 ± 0.09 on average. However, the change of feedstock composition had no significant effect on the overall process performance indicated by a constant biogas content ($R^2 = 0.31$; $p = 8.8 \times 10^{-16}$).

In EP2, a gradual ongoing increase in the TAN was recorded and reached highest values of 6.3 g NH_4^+-N/kg_{FM} with a corresponding NH_3 content of 0.5 g/kg_{FM} at the end of this experimental phase. No evident alteration in the pH value was recorded during the EP2 which recorded an averaged value of 7.56 ± 0.14. Also, a gradual increase in the VFA content was recorded, which reached up to 15.5 g/l at day 253. Presumably the increased VFA content and also the increased TAN/NH_3 content led to a decrease in the biogas yield as well as in the methane content (Fig. 1, first phase showing process disturbance resp. microbiome inhibition). Afterwards (from day 254 to day 309), a gradual increase in the TAN and a constant and stable VFA content of approximately 14.0 ± 0.7 g/l were recorded. So that, a short-termed stable biogas yield- indicated by comparatively constant biogas yield of 235.1 ± 44.57 L_N/kg_{VS} over 55 days- was recorded indicating a stabilisation of the process performance and microbial activity (Fig. 1, second phase showing short-termed stable biogas yield). After that and with continuous applying of 50% PM, an ongoing accumulation of the VFA was recorded resulting in VFA content of 38.1 g/l accompanied with a decreasing in the biogas yield to 158.8 L_N/kg_{VS} with a methane content of 35.8% at the end of EP2. The previous conditions symbolized a critical resp. significant process disturbance (Fig. 1, third phase showing process disturbance resp. microbiome inhibition).

Alteration of the Microbial Community Structure in the Response of TAN Increase

The dynamic variation of the microbial community was investigated by the fingerprinting method TRFLP targeting either the bacterial or the archaeal 16SrRNA genes based on the samples ER-81, ER-162, ER-199, ER-250, ER-300, and ER-340 from the ER, and CR-81 and CR-340 of the CR, followed by taxonomic profiling of the microbial community by 16S rRNA gene nucleotide sequence analysis of the samples ER-81, ER-250, ER-340, and CR-81.

The Shannon and the Richness indices for microbial diversity were calculated and presented in (Table 1). Both

Table 1. Statistical parameters of bacterial and archaeal 16S RNA gene libraries and TRFLP analysis for reactor effluent samples.

Reactor resp. AD experiment	AD substrate[a]	Day of sampling (day)	Sample denomination	Shannon diversity		Richness resp. no. of TRFs	
				Bacteria	*Archaea*	*Bacteria*	*Archaea*
Control	100% CS (vol/volVS[b])	81	CR-81	3.12	0.977	21	8
Experimental phase 1 (EP1)	25% PM + 75% CS (vol/vol VS)	81	ER-81	3.24	0.806	22	8
		162	ER-162	-	-	20	6
Experimental phase 2 (EP2)	50% PM + 50% CS (vol/vol VS)	199	ER- 199	-	-	17	6
		250	ER-250	2.87	0.223	13	7
		300	ER-300	-	-	15	5
		340	ER-340	3.10	0.532	13	6
Control	100% CS (vol/volVS[b])	340	CR-340	3.11	1.32	16	9

[a]CS, cattle slurry; PM, poultry manure.

[b]VS, volatile substances.

Table 2. Relative distribution of the terminal restriction fragments (TRFs) detected for Bacteria as the relative abundance of particular TRFs divided on the sum of relative abundance of all TRFs in the sample (0, light green; 1 to 4, green; 5 to 9, yellow; 10 to 14, orange; ≥ 15, red).

Reactor resp. AD experiment	Control	Experimental phase 1 (EP1)		Experimental phase 2 (EP2)				Control	
AD substrate[a]	100% CS (vol/vol VS[b])	25% PM + 75% CS (vol/vol VS)		50% PM + 50% CS (vol/vol VS)				100% CS (vol/vol VS)	
Day of sampling (day)	81	81	162	199	250	300	340	340	
Sample denomination	CR-81	ER- 81	ER-162	ER-199	ER-250	ER-300	ER-340	CR-340	
Gini coefficient	0.43	0.35	0.40	0.39	0.40	0.40	0.43	0.40	
TRF [bp]	Relative abundance (%)								Phylogenetic assignment of detected TRFs[c]
75	2	2	3	2	0	0	4	3	*Firmicutes, Bacilli, Lactobacillales* (*Firmicutes, Clostridia*)
94	7	7	3	2	0	0	0	6	*Firmicutes, Bacilli, Bacillales* (*Bacteroidetes*)
99	5	5	5	6	8	3	8	0	Firmicutes, Clostridia, Natranaerobiales
141	5	5	8	7	0	0	0	0	*Firmicutes, Clostridia* (*Actinobacteria, Actinobacteria, Actinomycetales*)
150	14	14	27	23	26	21	21	22	*Firmicutes, Bacilli, Bacillales* (*Firmicutes, Clostridia*)
154	0	0	4	7	8	10	9	0	*Firmicutes, Bacilli, Lactobacillales*
159	4	4	0	0	0	0	0	0	*Firmicutes, Clostridia, Thermoanaerobacterales*
161	2	2	3	0	2	3	3	0	*Firmicutes, Clostridia* (*Actinobacteria, Actinobacteria, Actinomycetales*)
166	3	3	4	6	6	7	6	0	*Firmicutes, Erysipelotrichia, Erysipelotrichales* (*Firmicutes, Clostridia, Clostridiales*)
169	2	2	2	3	4	4	4	0	*Firmicutes, Clostridia, Clostridiales*
177	3	3	6	4	3	0	3	5	*Firmicutes, Bacilli, Bacillales*
180	8	8	10	10	9	8	10	9	*Firmicutes*[d]
190	5	5	0	6	7	8	0	10	*Firmicutes, Clostridia, Clostridiales*
194	3	3	3	3	3	3	0	5	*Firmicutes, Clostridia, Clostridiales*
198	0	0	0	2	3	3	2	0	nd[e]
205	2	2	2	2	0	2	0	4	*Firmicutes, Clostridia, Clostridiales*
213	0	0	0	3	11	2	4	3	*Proteobacteria, Gammaproteobacteria, Pseudomonadales* (*Xanthomonadales*)
216	5	5	8	7	3	3	0	6	*Firmicutes, Bacilli, Lactobacillales* (*Clostridia, Clostridiales*)
228	0	0	2	0	0	2	0	5	*Bacteria*[3]
288	2	2	4	3	0	0	0	3	*Firmicutes, Clostridia, Clostridiales*
294	3	2	2	0	0	0	0	2	*Firmicutes, Clostridia, Clostridiales*
367	2	2	2	0	0	0	0	2	*Firmicutes, Clostridia, Halanaerobiales*
376	2	2	0	0	0	0	0	0	*Firmicutes, Clostridia, Halanaerobiales*
453	3	3	0	0	0	0	0	2	*Firmicutes, Clostridia, Clostridiales*
466	0	0	0	0	0	0	2	0	nd
502	3	3	2	0	0	0	0	3	nd
558	0	0	0	0	0	3	2	0	*Firmicutes, Bacilli, Lactobacillales*
571	0	3	2	0	0	0	0	0	nd

TRFs with relative abundance >= 2% were shown.

[a]CS, cattle slurry; PM, poultry manure.

[b]VS, volatile substances.

[c]In case of alternative sequencing or taxonomic identification results, corresponing taxonomic affiliation is given in brackets.

[d]Lowest taxonomic rank.

[e]nd, not determined.

indices were considerably higher for bacterial than for archaeal communities in both control and experimental reactors. Also the bacterial community organisation in all samples was more even than the archaeal one. At the same time, the Richness indices for both bacterial and archaeal communities were lower in thermophilic condition compared to mesophilic ones as was previously shown by Alsouleman *et al*. (Data not show) [20] and also was in accordance with [50], reporting that the thermophilic microbiome has lower diversity than the mesophilic one.

In the CR, the highest diversity was recorded for the bacterial and archaeal communities compared to the experimental reactor (Table 1). The bacterial community of the CR reactor at both sample points (CR-81 and CR-340) consisted mainly of members belonging to the phylum *Firmicutes* from orders *Clostridiales*, *Bacillales*, and *Halanaerobiales* (Table 2). The methanogenic community included members belonging to genera *Methanosarcina*, *Methanobrevibacter*, *Methanothermobacter*, and *Methanoculleus* (Table 3).

In the ER, during EP1, no significant changes were detected in the overall reactor performance. Interestingly, between sampling day 81 (sample ER-81) and 162 (sample

Table 3. Relative distribution of the terminal restriction fragments (TRFs) detected for Archaea as the relative abundance of particular TRFs divided on the sum of relative abundance of all TRFs in the sample (0, light green; 1 to 4, green; 5 to 9, yellow; 10 to 14, orange; ≥ 15, red).

Reactor resp. AD experiment	Control	Experimental phase 1 (EP1)		Experimental phase 2 (EP2)				Control	Phylogenetic assignment of detected TRFs within phylum *Euryarchaeota*
AD substrate[a]	100% CS (vol/vol VS[b])	25% PM + 75% CS (vol/vol VS)		50% PM + 50% CS (vol/vol VS)				100% CS (vol/vol VS)	
Day of sampling (day)	81	81	162	199	250	300	340	340	
Sample denomination	CR-81	ER-81	ER-162	ER-199	ER-250	ER-300	ER-340	CR-340	
Gini coefficient	0.47	0.5	0.56	0.59	0.51	0.64	0.61	0.54	
TRF [bp]	Relative abundance (%)								
68	0	4	5	4	3	0	0	0	nd[c]
73	2	6	6	7	9	5	5	3	Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanosarcina
84	0	0	0	0	3	0	0	2	Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanosarcina
108	9	22	3	4	5	3	3	1	Methanomicrobia, Methanomicrobiales, Methanomicrobiaceae, Methanoculleus
173	4	7	7	7	7	5	5	5	nd
317	1	5	6	3	4	3	4	2	Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanothermobacter
342	6	5	3	0	0	0	3	3	Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanothermobacter
336	26	33	52	59	48	68	68	29	Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanobrevibacter
471	3	0	0	0	0	0	0	3	Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanosarcina
627	28	4	0	0	0	0	0	29	Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanosarcina

TRFs with relative abundance >= 2% were shown.

[a]CS, cattle slurry; PM, poultry manure.

[b]VS, volatile substances.

[c]nd, not determined.

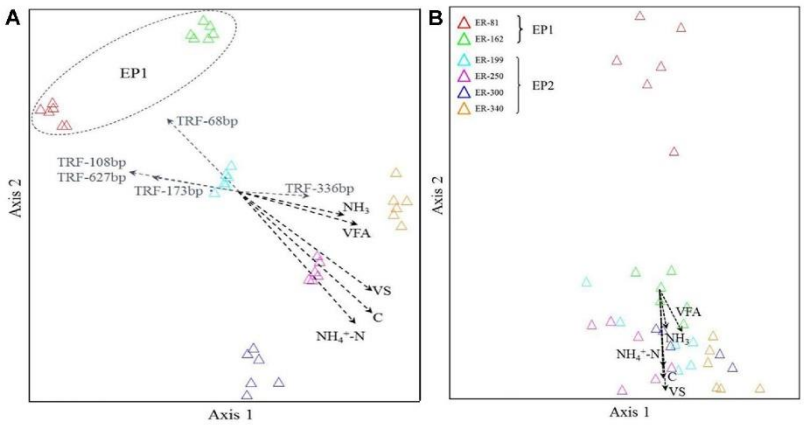

Fig. 2. Non-metric multidimensional scaling (NMS) of the effects of the prevalent process parameters in the experimental reactor (ER) in terms of ammonium nitrogen (NH_4^+), free ammonia nitrogen (NH_3), total volatile fatty acids (VFA), volatile solids (VS) and conductivity (C) on the bacterial and archaeal community structure.

The x-axis symbolizes the distance in ordinations space while the y-axis symbolizes the dissimilarity of the investigated objects. EP1, EP2 indicate exprimental phases. Sampling points are indicated by triangles. TRFs indicate detected methanogenic archaea.

ER-162), a definite shift in both the fermentative bacteria community and the methanogenic archaea community was observed (Fig. 2). The Gini coefficients of the bacterial community and the archaeal communities of ER-81 were 0.35 resp. 0.50, and for ER-162 were 0.40 resp. 0.56 which indicate a well-balanced community composition at both process stages as they were in the medium range of the community organisation.

Similar to the CR, in the ER the most abundant members of the bacterial community were also belonged to the order *Clostridiales* followed by members of the order *Bacillales* (Table 2). This result was supported by the performed indicator species analysis where the highest significant indicator values (IV = 100; $p \leq 0.05$) in this phase were found for the TRFs related to the order *Clostridiales* independent from the tested process factors (data not shown). The calculated pairwise distance between the bacterial communities of CR-81 and ER-81 resp. ER-162 showed a change in the bacterial community composition in the ER of up to 17% (Table 4).

In the ER, the archaeal community during the EP1 showed more distinct changes in their composition as the relative abundance of members belonging to the genus *Methanobrevibacter*reached increased to 52% of the entire archaeal community in the ER-162 comparing by 26% in control reactor (Table 3). This increase through the whole experimental phase was accompanied with a apparent decrease in the relative abundance of members belonging to the family *Methanosarcinaceae* TRF-627. While other members of this genus did not affect (*e.g.*, TRF-73) similar to members of genus *Methanothermobacter* (TRF-317). The calculated pairwise distance between the archaeal communities of CR-81 and ER-81 resp. ER-162 showed a change in the archaeal community composition in the ER of up to 32% (Table 5).In the ER and during the EP2 (which was divided into three phases depending on the reactor performance (Fig. 1), Shannon's diversity index for the bacterial community of the sample ER-250 reflected the lowest diversity of the microbial community during the entire experimental phase (Table 1).

A slight alteration in the structure of the bacterial community in sample ER-199, the first disturbance phases (Fig. 1), was detected (Table 2). This slight alteration was also supported by the calculated pairwise distance between the bacterial communities of ER-162 and ER-199, which reached up to 9% (Table 4).

For members of class *Euryarchaeota*, a clear decrease in the relative abundance of the genus *Methanothermobacter* accompanied with a continuous increase in the relative abundance of the genus *Methanobrevibacter* was detected (Table 3).

Between day 250 and day 300 (Fig. 1, second phase

Table 4. Similarity matrix in comparison of the bacterial communities.

Reactor resp. AD experiment	Control	Experimental phase 1 (EP1)		Experimental phase 2 (EP2)				Control
AD substrate[a]	100% CS (vol/vol VS[b])	25% PM + 75% CS (vol/vol VS)		50% PM + 50% CS (vol/vol VS)				100% CS (vol/vol VS)
Day of sampling (day)	81	81	162	199	250	300	340	340
Sample	CR-81	ER-81	ER-162	ER-199	ER-250	ER-300	ER-340	CR-340
CR-81	100	99	83	81	65	62	60	81
ER-81		100	84	81	65	62	60	81
ER-162			100	91	77	75	80	78
ER-199				100	89	88	84	79
ER-250					100	91	91	71
ER-300						100	86	71
ER-340							100	57
CR-340								100

The calculated pairwise similarity considered both, changes in the number and the relative abundance of each detected terminal restriction fragment within and between two samples depending on Pearson algorithm.

[a]CS, cattle slurry; PM, poultry manure.

[b]VS, volatile substances.

showing short-termed stable biogas yield), the calculated pairwise distance between the bacterial communities of ER-250 and ER-300 showed a change in the bacterial community composition of up to 9% (Table 4).

Regarding the archaeal community, an increase in the relative abundance of the genus *Methanobrevibacter* was recorded which formed 68% of the entire archaeal community structure, whereby members belonging to the genus *Methanosarcina* completely disappeared (Table 3).

At the end of the fermentation experiment, in sample ER-340, the most abundant members of the bacterial community were also belonged to the phylum *Firmicutes* mainly from the orders *Bacillales*, followed by members of the order *Lactobacillales* and *Clostridiales* (Table 2).

In the course of the experiment, the trend of the archaeal community organization moved in the same way as ER-199, and the most abundant members were belonged to the genus *Methanobrevibacter*, whereby members belonging to

Table 5. Similarity matrix in comparison of the archaeal communities.

Reactor resp. AD experiment	Control	Experimental phase 1 (EP1)		Experimental phase 2 (EP2)				Control
AD substrate[a]	100% CS (vol/vol VS[b])	25% PM + 75% CS (vol/vol VS)		50% PM + 50% CS (vol/vol VS)				100% CS (vol/vol VS)
Day of sampling (day)	81	81	162	199	250	300	340	340
Sample	CR-81	ER-81	ER-162	ER-199	ER-250	ER-300	ER-340	CR-340
CR-81	100	93	68	63	65	61	60	90
ER-81		100	66	77	67	65	63	79
ER-162			100	79	83	82	80	72
ER-199				100	86	85	84	70
ER-250					100	88	86	56
ER-300						100	90	53
ER-340							100	52
CR-340								100

The calculated pairwise similarity considered both, changes in the number and the relative abundance of each detected terminal restriction fragment within and between two samples depending on Pearson algorithm.

[a]CS, cattle slurry; PM, poultry manure.

[b]VS, volatile substances.

the genus *Methanosarcina* completely disappeared (Table 3). After this inhibited steady state phase, a severe perturbation in the process performance was recorded.

Discussion

In the CR, AD was performed by members of the bacterial phylum *Firmicutes* in combination with obligate or facultative hydrogenotrophic methanogens, namely of genera *Methanosarcina*, *Methanobrevibacter*, *Methanothermobacter*, and *Methanoculleus*. The predominance of the *Firmicutes* phylum is mostly explained by its capability to produce diverse enzymes performing celluloylsis, hydrolysis, acidogenesis, and acetogenesis. This microbial structure under thermophilic conditions was in accordance with previously published results and supported the assumption of more or less stable microbial biocenoses and metabolic activity in the CR [11, 23, 51–53]. The moderate alteration in the microbial community structure in the CR between sampling days 81 and 340 with constant biogas yields can be assumed to be more related to the natural development of the microbial community itself (Table 4)

In the ER, during EP1, no significant effects on the reactor performance were recorded. The TAN reached a maximum of 3 g/kg_{FM} with a corresponding NH_3 content of 0.4 g/kg_{FM}, values which are lower than the published thresholds for process inhibition [11, 14, 54]. Even a slight increase in the VFA concentrations was recorded in this experimental phase, the degradation process run stably. It is assumed that the increase in the TAN concentration led to an increase in the buffer capacity, which supports a stable process. The recorded VFAs concentrations were lower than the recorded inhibition thresholds [55, 56].

Nevertheless, it is known that the used feedstock can affect the microbial community structure [57–60]. Hence, in this study, a definite shift in the microbiome structure was observed between ER-81 and ER-162 in the EP1, according to 25% PM addition as well as the consequent abiotic parameters. In the first sample ER-81 of the EP1 a minor change in the microbial community structure was recorded as was indicated by the changes of the relative abundance of the detected archaeal and bacterial TRFs. While in the second sample ER-162 of EP1, a clear shift was detected as indicated by the similarity values (Tables 4 and 5), the relative abundance of the detected archaeal and bacterial TRFs (Tables 2 and 3) in addition to the NMS analysis. The NMS revealed no significant correlation between the investigated process parameters and the related arrangement of the bacterial community in the EP1. So that the bacterial community of EP1 (ER-81 and ER-162) formed a separate cluster (Fig. 2), while the archaeal community structure already showed a clear shift during EP1 as the archaeal community of the first sample of EP1 (ER-81) formed a separate cluster (Fig. 2).

It can be assumed that functional redundancy was the primary microbial strategy in EP1, ensuring an ongoing and stable biogas production as the compositional shifts did not affect significantly the reactor performance [61, 62]. The results of this study showed that the phylum *Firmicutes* was the most abundant phylum during the whole experimental phase which was shown previously [63, 64]

The archaeal community in EP1 seemed to have lower tolerance values for the elevated TAN. The more sensitive response of the archaeal community was related to a clear decrease in the abundance of some obligate (*Methanoculleus*) or facultative (*Methanosarcina*) hydrogenotrophic methanogens which accompanied with a substantial increase in the abundance of other and exclusively hydrogenotrophic genus *Methanobrevibacter* (Table 2). These results are in accordance with [22, 50] reporting that the obligate or facultative acetoclastic methanogens dominate the archaeal community preferentially at medium TAN, VFA, and/or salt concentrations. High concentrations of these parameters in combination with thermophilic conditions were positively correlated with a predominance of obligate hydrogenotrophic methanogens.

Hydrogenotrophic methanogens utilize carbon dioxide (CO_2) and molecular hydrogen (H_2) for methane production. Thus, the acetate produced by fermentative bacteria has to be converted via the acetate oxidation pathway performed by syntrophic bacteria [65, 66]. This fact could explain the previous slight recorded changes within the bacterial community composition of the EP1. To conclude, the shift within the microbial community was the result of a synergetic effect of physical (temperature) and chemical (partial feedstock change) factors which had no effects on the overall process performance.

In the second experimental phase (EP2; day 177- 341) the amount of PM was doubled to 50% (vol/vol based on VS). As expected, shortly after starting EP2, the reactor performance was distinctly negatively influenced symbolized by a decrease in both the biogas yield and the methane content which was significantly related to a gradual increase in the NH_4^+-N and NH_3 as well as the VFA concentrations (Fig. 1).

The high VFA and TAN concentrations led to the presence of them in their un-dissociated forms, which are more toxic for microorganisms [67, 68].

As is known, in a highly buffered system like this system, the VFA is the best indicator for indicating process imbalance and reactor failure [69]). The relative alteration of the VFA concentration in EP2 reached to 36 g/l compared with 1.13 g/l in the control reactor.

During EP2, a three-stage process disturbance was recorded: an initial disturbance (as mentioned above), a short-termed stable biogas yield stage, and a final complete process disturbance resulting in complete process failure. The initial accumulation in a VFA led to a drop in the pH

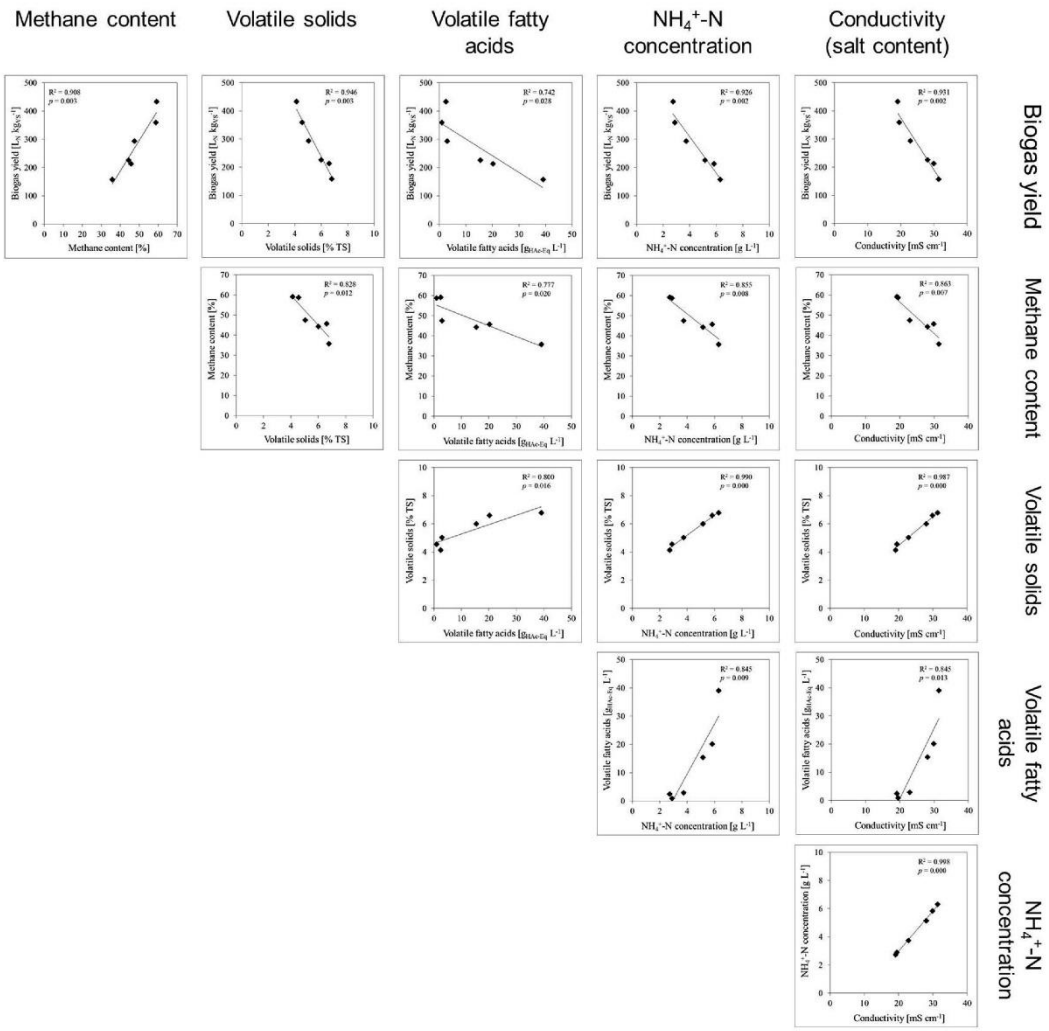

Fig. 3. Correlation matrix indicated by linear regression among and between the operational parameters.
The operational parameter values were determined for 6 sampling points of the experimental reactor. Statistical significance was established at the $p < 0.05$ level.

value and hence, a decrease in the NH_3 content (between day 250 and 270, Fig. 1). It can be assumed that these conditions encouraged the microbial communities especially the archaeal community to adapt and recover again which could be interpreted the subsequent process stability at elevated TAN but with a lower biogas yield and methane content [14].

The reason for the final reactor performance deterioration could not be related to one, but rather to different prevalent environmental factors (Fig. 3).This is reflected in the NMS analyses results which showed that the variation within the bacterial and the archaeal community structure community in EP2 could be related to different parameters; VFA, NH_4^+-N, NH_3, salt content, and/or VS (Fig. 2). The salt content was symbolized in our study by the conductivity which reached to the reported threshold value of 30 mS/cm [57, 70]. Here, it could be assumed depending on our finding that, the alteration in the microbial community structure in the ER was more related to the strong impact of the PM addition (Table 4). As the methanogenic activity is seriously inhibited, it can be assumed that the growth of acid converting bacteria is restricted as they can only grow if the H_2 is consumed by their syntrophic archaeal partners which subsequently led to a VFA accumulation of more than 38 g/l at the end of this experimental phase. Based on these findings and in comparison with an AD experiment performed under the same but mesophilic conditions [20], an anaerobic microbiome adapted to thermophilic conditions can be assumed as much more sensitive for process disturbances in consequence of biomass load with high contents of organic nitrogen.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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4.3 Members of the WWE1 candidate division and the phylum *Bacteroidetes* as indicators to forecast a subsequent process disturbance

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Theuerl, S; **Alsouleman, K**, Klang, J

Abstract

In order to achieve a stable, efficient and flexible biogas production microbiologist are faced with the challenge to understand and define the potentials and limits of the complex and highly sensitive ecosystem “biogas plant”. Hence, knowledge about the adaptability and resistance of microbial populations to specific ecological conditions are of high importance regarding the development of new control and management strategies.

Within the datasets of three independently conducted projects microbial populations which potentially indicate stable process conditions were identified. Two mesophilic lab-scale experiments investigated the effect of animal manure derived increasing ammonium nitrogen content on the reactor performance with special emphases on the microbiome response. In the third study the microbial community was monitored in a full-scale biogas plant during a change from mesophilic to thermophilic conditions, interrupted by a strong temperature drop due to technical problems. The bacterial and archaeal community was investigated using TRFLP in combination with a cloning/sequencing approach based on 16S rRNA gene analyses.

In all three studies, changing operational parameters led to an inhibition of the process, which was mainly related to an abrupt and distinct change within the archaeal community structure. In contrast the bacterial community showed no specific reaction but a subtle reorganization of the bacterial community occurred over time. Most notable was the decreased abundance, or even disappearance, of specific bacterial TRFs assigned either to the WWE1 candidate division or the phylum *Bacteroidetes* prior to the change at the archaeal level. These result leads to the assumption that these *Bacteria* are highly sensitive to changing reactor conditions and might be possible indicator-organisms for a good reactor performance as their disappearance forecasts a subsequent process disturbance.

5 Discussion

Long-term mesophilic and thermophilic anaerobic digestion experiments were performed to evaluate the amount of poultry manure, specifically how much NH_4^+-N can be tolerated by the AD microbiome in two different temperature ranges and how the microbial community reacts against this operational disturbance (Alsouleman *et al*., 2016; Alsouleman, 2019). In the following, a comprehensive comparison of the overall performance of the long–term anaerobic digestion experiments in two different temperatures due to the consecutive additive PM levels will be discussed. Special emphasis on the microbial community structure and its dynamic variation during ongoing fermentation will be explained. In addition, the advantage and the potential drawbacks respectively limitation of the main applied key methods are discussed briefly.

5.1 Methodical aspects

5.1.1 Applicability of CSTRs for anaerobic digestion of nitrogen rich manure

The well-controlled reactors enable to investigate the suitability of the new applied feedstock and also to study comprehensively the involved microbial community which in turn helps in setting up the anaerobic digestion process as efficient as possible (Holm-Nielsen *et al*., 2009). The continuously respectively completely stirred tank reactor (CSTR) was used in this study due to the simplicity of the process and the reactor operation especially as a process-risk feedstock (manure rich in nitrogen) was applied. Other advantages to use CSTRs are the general low construction and investment costs of this reactor system comparing with other types which are preferable aspects especially for its application in developing countries like Syria.

On the other hand, one main limitation of using this system is the need of the continuous stirring which was hampered due to the continuous increasing in the total solid content arising from the PM addition. A further challenge is the required long hydraulic retention time (HRT) which ranged in this study between 26 and 134 days as a short retention time is likely to cause a washout of the active microbial population (Weiland, 2010; Angelidaki *et at*., 2011). From the economical point of view, the increased cost arising from the higher energy demand for stirring and suitable dilution to have the favored total solid content to run the CSTR system as efficient as possible

are other drawbacks when a substrate with high total solid (poultry manure) content is needed to apply.

Nevertheless, from the technical point of view, the findings of this study proved that the lab-scale CSTR was suitable to digest efficiently until the medium PM level (50% PM +50% CS without water addition) in mesophilic condition. While in thermophilic condition the AD of only low PM level (25% PM +75% CS without water addition) was run efficiently. Even though, different aspects such as the design of digester specific to poultry manure digestion, the impact of separating the anaerobic digestion process of poultry manure, the optimizing of the feedstock feeding system still need to be addressed in order to run the anaerobic digestion process of poultry manure efficiently.

5.1.2 Applicability of TRFLP analysis for microbial community analysis in poultry manure AD

The TRFLP analysis is considered a rapid, high-throughput, and highly reproducible method in microbial ecology studies. The TRFLP is useful for monitoring the phylogenetic diversity and the dynamic of the involved microbial communities in various environments (Kitts, 2001). However, this technique may underestimate the microbial diversity due to different drawbacks associated with this method itself (Liu *et al*., 1997). These limitations in application of this technique range from the sampling procedure, via the selection of the primers and restriction enzymes, up to the statistical analyses of the obtained data and linking the microbial community changes to the environmental changes (Osborn *et al*., 2000; Engebretson and Moyer, 2003; Abdo *et al*., 2006; Osborne *et al*., 2006; Schütte *et al*., 2008; Rademacher *et al*., 2012).

In this study, the TRFLP analyses were carried out following the optimized protocol proposed by Rademacher *et al*. (2012). Regarding the DNA extraction, two DNA extractions kits (FastDNA® Spin Kit for Soil, PowerSoil® DNA Isolation Kit), including a beating step were applied. However, an effective DNA extraction in this study was reached by using the FastDNA® Spin Kit for Soil with a mechanical treatment step as recommended also by Bergmann, (2010) and Vanysacker *et al*., (2010).

The resulted TRFLP profiles (in terms of the number of the detected TRFs and their relative abundance) were comparable with those of other studies on microbial

communities of the biogas production process (Rademacher *et al*., 2012; Klang *et al*., 2015; Theuerl *et al*., 2015).

To overcome the limitation of the TRFLP analysis arising from the TRFs identification, subsequent 16S rRNA gene sequence libraries were constructed to determine and match the detected TRFs with the respective nucleotide sequences. Nevertheless, there were still reported TRF peaks, which were not represented in the clone libraries. For example no corresponding 16S rRNA gene sequences were identified for the archaeal TRF-174 bp, TRF-627bp and for the bacterial TRF-122bp, TRF-206bp at mesophilic conditions and also for archaeal TRF-68bp, TRF-173bp and for the bacterial TRF-198bp, TRF-466bp at thermophilic conditions which indicated putative false/Pseudo-TRFs (Schütte *et al*., 2008; Rademacher *et al*., 2012). These Pseudo-TRFs can be produced by PCR and the subsequent restriction enzymes digest. The formation of single strand DNA (ssDNA) sequences during PCR can reproducibly lead to pseudo-TRFs. These ssDNA sequences can form secondary dsDNA structures, which are recognized as target by restriction enzymes in the digestion step leading to false fragments and hence to overestimation of the genetic diversity (Egert & Friedrich, 2003; 2005). Another main limitation in this study was that, several unrelated organisms can produce the same TRF size. The partial 16S rRNA gene sequences do not always allow discrimination between species as one TRF may represent two or more species with identical partial sequence (Dunbar *et al*., 2000; Kitts, 2001). In another word a single TRF can represent several genera (Brunk *et al*., 1996; Dunbar *et al*., 2001; Marsh, 1999). For example, the TRF-107bp was assigned to two different archaeal orders *Methanobacteriales (*hydrogenotrophic methanogens) and *Methanosarcinales* (acetolastic methanogens) (Alsouleman *et al*., 2016). However, after data analysis, the TRFLP results were sufficient for comparative analyses as they were able to tracking of the microbial community dynamics and follow the main changes in the microbiome structure in response to different PM levels addition.

Due to the high community complexity and broad range of the involved metabolic interactions in anaerobic digestion system (e.g. Nettmann *et al*., 2010; Wang *et al*., 2010; Carballa *et al*., 2011; Regueiro *et al*., 2012; Fotidis *et al*., 2014; Cabezas *et al*., 2015; Theuerl *et al*., 2015; Toumi *et al*., 2015), especially in the anaerobic digestion of process-risk feedstocks (nitrogen-rich manure), an expanding knowledge of the microbial community structure and dynamic variations correlated with physico-chemical process parameters is of high importance. Therefore, and due to the results of

this study, the application of the TRFLP technique as pre-screening analysis could be followed by a next generation sequencing technique to have a deeper and more accurate insight of the occurring microbial community in term of structure and functionality. The development of next generation sequencing (NGS) techniques enables us to study the complex microbial community structure from broader and deeper perspectives The application of these high-throughput sequencing technologies to 16S rRNA gene increased the resolution of the studying microbial communities in full-scale anaerobic digesters.

Different advantages of NGS over the Sanger sequencing techniques were characterized, which could be summarized as follows: (1) in vitro construction of the sequencing library, (2) in vitro clonal amplification of DNA fragments and (3) the amplified DNA templates are sequenced simultaneously in a massively parallel fashion without the requirement for a physical separation step.

On the other side, the time-consuming and complex nature of these high-throughput techniques is a potential bottleneck for full-scale anaerobic digestion application, The major disadvantage of these techniques are related to (1) the resulted short reads, (2) the relative higher error rate in addition to (3) the complexity and computationally demanding nature of required data analysis.

The first step in these technologies is the PCR of the desired gene, therefore specific primers for this technology are used (Cardenas and Tiedje, 2008; Wang and Qian, 2009). This step is followed by high-throughput sequencing of the resulting amplicons libraries by one of the four available NGS platforms. The choosing of the platforms depends mainly on the need of higher coverage or the need of higher sequences length (Shokralla *et al*., 2012).

These platform are: Roche 454 Pyrosequencing Genome Sequencer (Roche Diagnostics Corp. Branford, CT, USA), MiSeq and HiSeq 2000 (Illumina Inc. San Diego, CA, USA), AB SOLiD System (Life Technologies Corp. Carlsbad, CA, USA) and Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA) (Shokralla *et al*., 2012).

Each of the previous mentioned platforms has advantages and disadvantages. The major advantages of the 454 Pyrosequencing platforms are the relative long read length obtained and its relatively short run time. This made 454 Pyrosequencing platforms the most commonly used NGS platform for the analysis of environmental DNA for ecological applications. On the other side, the main drawbacks of this platform are the

homopolymer errors which lead to an overestimation of the number of rare phylotypes and the generated short reads which limited the taxonomic assignment of these sequences to the genus level.

The Ion PGM platforms present cheap alternative platform with relative long reads up to 200bp but with lower coverage than 454-pyrosequencing.

The main advantage of both Illumina and SOLiD systems compared to the two previous mentioned platform 454 pyrosequencing and Ion PGM is the high output per run (Cao *et al*., 2017; Levy and Myers, 2016; Cabezas *et al*., 2015; Buermans and Dunnen, 2014; Scholz *et al*., 2012)

5.2 Performance of the anaerobic digestion process during increasing amounts of poultry manure

5.2.1 The performance of the control reactor

Two level of disturbance were distinguished in the study; disturbance on the reactor performance level and disturbance on the microbial level. During the whole experimental period, the control reactor (CR) - which was feed with cattle slurry as sole feedstock (OLR of 3.0 g_{VS} L^{-1} d^{-1}) - showed no significant changes neither in the produced biogas (R_2 = 0.24 and $p < 0.001$; R_2 = 0.27; $p < 3.7 \times 10^{-25}$) in mesophilic and thermophilic condition respectively, nor in the investigated chemical parameters over the entire experimental period (Alsouleman *et al*., 2016; Alsouleman, 2019).

The higher biogas production in the thermophilic condition (392 ± 59 L_N kg $_{VS}^{-1}$ with CH_4 content of 60 ± 1%) comparing with the mesophilic one (376 ± 72 L_N kg $_{VS}^{-1}$ with CH_4 content of 62 ± 2%) agreed with the fact that the thermophilic AD has a higher metabolic rate and is hence expected to improve the overall process efficiency (Ahring, 2003; Wilson *et al*., 2008; Abbassi-Guendouz *et al*., 2013; Shi *et al*., 2013).

Several studies have shown that improvements in performance in thermophilic digestion comparing with mesophilic ones are mainly due to an increase in hydrolysis coefficient. (Song *et al*., 2004: Kim *et al*., 2006; Ge *et al*., 2011). The hydrolysis coefficient determines the speed of degradation, rather than to an increase in the fraction of degradable material which could explain the higher biogas yield in thermophilic condition with a lower methane content.

Also, the content of NH_4^+-N and NH_3 of the control reactors in both mesophilic and thermophilic conditions were very similar. The ammonium nitrogen NH_4^+-N content

ranged between 1.8 ± 0.2 g kg_{FM}^{-1} in mesophilic condition and 1.9 ± 0.3 g kg_{FM}^{-1} in thermophilic condition, while the calculated NH_3 value ranged between 0.07 ± 0.02 g kg_{FM}^{-1} and 0.08 ± 0.04 g kg_{FM}^{-1} respectively during the whole period.

As already known, the pH values in highly buffered systems like the systems in this study due the high level of alkalinity arising from the degraded proteinaceous wastes can be very stable (Gerardi, 2003). The pH value in the control reactor at both temperature regimes (mesophilic and thermophilic) varied between 7.3 and 7.8. Therefore, the biogas yield, the methane content in the biogas, and the VFA contents were considered the reliable parameters for process monitoring in terms of process indicators for (in-) stability (Murto *et al*., 2004; Westerholm *et al*., 2011). The VFA content was monitored during the whole experimental period. The VFA value in thermophilic control reactor reached to 1.5 g L^{-1} which is higher comparing with its value in mesophilic condition (not exceeding 0.5g L^{-1}). The higher VFA content in the thermophilic condition agreed with the fact that the thermophilic AD is characterized with reduced process stability (Gallert *et al*., 1998; Kim *et al*., 2006). However, the two previous values of VFA still reflected a stable anaerobic digestion process (Weiland, 2008; Laaber, 2011; Drosg, 2013; LfL, 2013).

5.2.2 The performance of the experimental reactor at low PM level (25% PM addition)

The first experimental phase (EP1) was initiated by applying a low PM level (25% PM based on VS), the higher temperature led to higher biogas yield of 403 ± 74 L_N kg $_{VS}^{-1}$ with CH_4 content of 58% ± 1% in thermophilic AD comparing with 341 ± 61 L_N kg $_{VS}^{-1}$ with CH_4 content of 62% ± 1% in mesophilic AD (Ahring, 2003; Wilson *et al*., 2008; Abbassi-Guendouz *et al*., 2013; Shi *et al*., 2013). As expected, the change of feedstock composition resulted in an increase in the NH_4^+-N content from 1.6 ± 0.1 g kg_{FM}^{-1} to 3.9 g kg_{FM}^{-1} (corresponding NH_3 content of 0.06 ± 0.01 g kg_{FM}^{-1} to 0.3 g kg_{FM}^{-1}) in mesophilic AD and from 1.9 g kg_{FM}^{-1} to 3 g kg_{FM}^{-1} (corresponding NH_3 content of 0.25 g kg_{FM}^{-1} to 0.4 g kg_{FM}^{-1}) in thermophilic AD. These values were still lower than the published thresholds for process inhibition at both mesophilic (Schnürer and Nordberg, 2008; Drosg, 2013) and thermophilic conditions (Angelidaki and Ahring, 1993; Niu *et al*., 2013; Lv *et al*., 2014).

However, the previous abiotic changes had no significant effect on the overall process performance in this experimental phase in both mesophilic and thermophilic condition

indicated by an almost stable biogas yield (R^2=0.3 x 10^{-3}; p=0.845) (R^2 = 0.31; p = 8.8 x 10^{-16}), respectively. Also, the VFA content in first experimental phase in both cases were rather constant.

It could be concluded that, with low PM level, the reactor performance was functionally stable as no significant effects (in terms of disturbance) on the reactor performance in both cases were recorded (Alsouleman *et al*., 2016; Alsouleman, 2019). The overall reactor performance was efficient and the total biogas yield and methane content in this experimental phase were comparable with those of the control reactors.

So, it is recommended to apply the same mixture on full-scale biogas plants in both cases. The thermophilic anaerobic digestion offers different advantage such as higher metabolic rates (Ahring, 2003; Wilson *et al*., 2008; Abbassi-Guendouz *et al*., 2013; Shi *et al*., 2013) and higher rates of destruction of pathogens due to the higher sanitization effect (Zábranská *et al*., 2002; Sahlström, 2003; Bagge *et al*., 2005; Dang *et al*., 2013). These aspects are very preferable especially when AD of animal wastes as treatments technology will be applied. On the other hand, the reduced stability of the thermophilic process compared to the mesophilic process, the higher CH_4 content of the biogas in mesophilic condition comparing with thermophilic one in addition to the high energy input for heating process in thermophilic condition (Gallert and Winter, 1997) should be considered in full-scale application.

5.2.3 **The performance of the experimental reactor at medium PM level (50% PM addition)**

In order to elucidate how much PM-derived NH_4^+-N can be tolerated by a mesophilic and thermophilic anaerobic microbiome, the amount of PM was doubled to 50% in the second experimental phase (EP2) denominated as medium PM level. The performance of the reactors in both cases as a response to this increasing was absolutely different.

In mesophilic AD, a serious process imbalance occurred shortly after doubling the amount of PM (after 20 days from starting the EP2). The EP2 started with 4.2 g kg_{FM}^{-1} of NH_4^+-N and a corresponding NH_3 content of 0.17 g kg_{FM}^{-1}. The mentioned values are similar to reported inhibition levels of the AD in mesophilic condition, especially an inhibition of the acetoclastic methanogenesis (Schnürer and Nordberg, 2008; Drosg, 2013). This serious disturbance or imbalance in the mesophilic anaerobic digestion process occurred with NH_4^+-N content of 5.9 g kg_{FM}^{-1} respectively a NH_3 content of

0.5g kg_{FM}^{-1}. This disturbance was indicated by a VFA accumulation of almost 10 g L^{-1} in combination with a strong reduction in biogas yield and methane content.

After a certain time with continuous addition of 50% PM, in this case approximately two weeks, the system started to recover again indicating an adaptation of the microbial community to the given environmental conditions. Despite the continuous increase in the NH_4^+-N content, a decrease in VFA content and subsequently an increase in biogas yield and methane content were observed which rose to similar values as before and almost stabilized until the end of this experimental phase (Alsouleman *et al*., 2016). These results demonstrated the ability of the mesophilic system to recover again. Hence, it can be assumed, that an adaption or acclimation of the involved microorganism took place. The comparison of the archaeal and bacterial community before and after this disturbance phase revealed fundamental changes which can completely explain the recovery of reactor performance after the disturbance.

While in the thermophilic condition, as was expected the acclimated microbial community to the abiotic parameters in the first experimental phase (NH_4^+-N content of 3 g kg_{FM}^{-1}) was distinctly negatively influenced by starting the EP2. The irreversible inhibition of the biogas production in this phase started with NH_4^+-N content of 4 g kg_{FM}^{-1} even this value is lower than the previous recorded inhibition values in thermophilic conditions (Angelidaki and Ahring 1993; Niu *et al*. 2013; Lv *et al*. 2014). As illustrated previously (Alsouleman, 2019), the reason for the reactor performance deterioration in thermophilic AD in this experimental phase is not related to just increasing in the NH_4^+-N content but rather to a multiplicity of the prevalent environmental factors, i.e. the increase in the VS content in combination with an increase of the salt content (quantified as by the electrical conductivity) which reached the reported threshold value of 30 mS cm^{-1} (Chen *et al*., 2008; De Vrieze *et al*., 2017). Therefore, the monitoring and controlling of the prevalent operational parameters is very necessary to evaluate accurately the direct effect of the poultry-manure-derived increase in NH_4^+-N and NH_3 content on the reactor performance.

The findings in this experimental phase revealed the efficiency of the long term mesophilic anaerobic digestion of the mixture of 50% PM and 50% CS (vol/vol based on volatile substances). The acclimated microbial community to the increasing content of NH_4^+-N induced by the medium PM level addition was able to tolerate the NH_4^+-N content as high as 8 g kg_{FM}^{-1}. So that in order to configure the biogas process with the same mixture as efficient as possible on full-scale plants, it has to be used with cautions

and especially with respect to the demands of the microbiome. In contrast, the thermophilic system was not able to resist the prevalent environmental conditions arising from the 50% PM addition (vol/vol based on volatile substances) and a complete failure occurred in this phase, even it tried to adapt for a short time which was expressed as called short-termed stable biogas yield phase (Alsouleman, 2019).

5.2.4 The performance of the experimental reactor at high PM level (75% PM addition)

A further increase of PM (high PM level) in mesophilic condition caused a continuous increase in the NH_4^+-N content which reached its highest value of 9.6 g kgFM^{-1} at the day 416 and stagnated at this level until the end of EP3. This mentioned value seemed to be not more handled any more by the occurring microbiome and an accumulation of 30.4 g L^{-1} of the VFA content was recorded. Also, the NH_3 content showed an unsteady behavior which could be related to a higher buffer capacity within the system. As a consequence, the biogas yield and methane content were negatively influenced resulting in a complete process failure and no recover in the reactor performance was recorded anymore. The fourth (and last) experimental phase (EP4) was started at day 479 by applying 100 % poultry manure. This phase lasted only for 22 days due to the fact that the supplied feedstock could not be any more converted to an efficient amount of methane. At the end of EP4 the system was characterized by a NH_4^+-N content of 11.7 g kg FM^{-1}, a VFA concentration of 44 g L^{-1} and a related NH_3 concentration of 0.7 g kg FM $^{-1}$. Consequently, the reactor feeding was stopped although the prevalent chemical parameters were analyzed for further 33 days whereby no significant changes were recorded.

While in thermophilic condition, after the complete reactor failure and the non-recovered inhibition in the EP2, no further increase in the PM was applied. Although the reactor feeding was stopped, the prevalent chemical parameters (VFA, conductivity, TS, NH_4^+-N, pH, NH_3) were analyzed for further 30 days whereby no significant changes were recorded.

5.3 The response of the microbiome to increased amounts of poultry manure

5.3.1 Composition of the microbial community in the control reactor

To figure out and follow the dynamics and response of the microbial community to the disturbances arising from the different added PM levels, the microbial community composition of the control reactor was considered as reference in this study. The control reactor showed a stable AD process over the entire experimental phase in both mesophilic and thermophilic conditions, although the microbial community structure in mesophilic condition differed evidently from that in thermophilic condition.

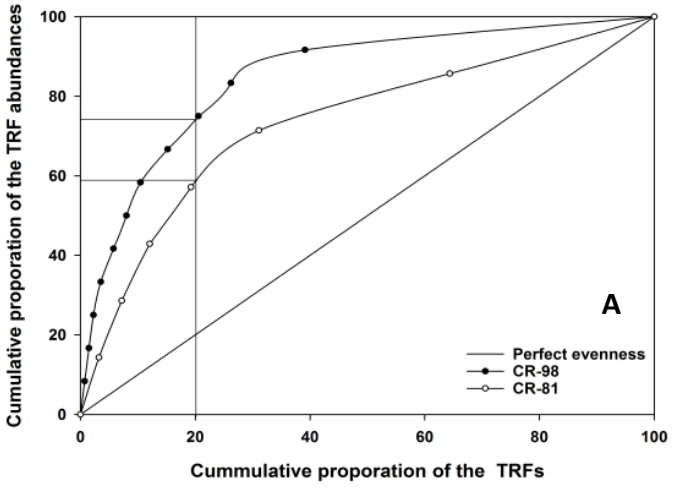
The bacterial community structure in mesophilic control reactor were characterized mainly by members from the phylum *Bacteroidetes* followed by members from the phylum *Firmicutes* and the WWE1 candidate division (depending on the relative abundance of the particular phylum to the whole bacterial community) while the archaeal community was dominated mainly by members from the genus *Methanosaeta*. So that, the assumed predominant pathway of methane formation in the mesophilic control reactor was the acetolastic pathway which in turn indicated a good performing reactor system as was previously reported by Regueiro *et al*. (2012). However, the results also indicated that a certain proportion of the produced biogas yield derived from the hydrogenotrophic pathway due to the presence of members belonging to the genus *Methanobrevibacter* (order *Methanobacteriales*) respectively the genus *Methanoculleus* (order *Methanomicrobiales*).

In thermophilic AD, the microbial community structure was characterized mainly by members from the phylum *Firmicutes Clostridiales, Bacillales*, at the bacterial level in combination with archaeal genera *Methanosarcina*, *Methanobrevibacter*, *Methanothermobacter* and *Methanoculleus*. This microbial structure under thermophilic conditions was in accordance with previous published results and supports the assumption of more or less stable microbial biocenoses and metabolic activity comparing with the mesophilic one. The previous mentioned microbial structure enabled to perform the hydrogenotrophic pathway of methane formation as pre-dominant pathway under thermophilic conditions which is in accordance with previous published results (Rademacher *et al*., 2012; Niu *et al*., 2013; De Vrieze *et al*., 2015; Pap *et al*., 2015; Campanaro *et al*., 2016).

To get information about the evenness and organisation of the microbial community, the Pareto-Lorenz evenness curves **Fig. 4** and the derived Gini coefficients (Alsouleman *et*

al., 2016; Alsouleman, 2019) were defined to facilitate the visualization of the evenness of the microbial communities. The Pareto-Lorenz curve distribution patterns of archaeal and bacterial TRFLP profiles were plotted based on the numbers of TRFs and their relative abundances. While a derived Gini Coefficient was calculated as the normalised area between the given Lorenz curve and the perfect evenness line.

Depending on the Pareto principle, the 20% of the bacterial TRFs (species) in both, mesophilic and thermophilic conditions, in this experimental phase accounted for approximately 41-43% of the whole bacterial relative abundance. This in turn reflected a balanced respectively well-established community (Verstraete *et al*., 2007; Marzorati *et al*., 2008; Carballa *et al*., 2011; Theuerl *et al*., 2015). While the 20% of the archaeal TRFs corresponded with 69% of the whole archaeal relative abundance in mesophilic AD and 47% in thermophilic AD. This in turn indicated that the thermophilic archaeal community was more even than the archaeal community under the mesophilic condition.



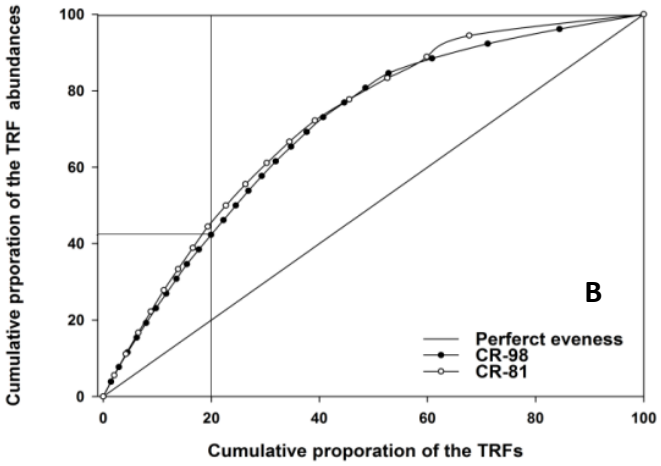


Figure 4: Pareto-Lorenz distribution curves based on 16S rRNA gene T-RFLP patterns of the archaeal (A) and bacterial (B) communities of the control reactor. The curve with filled circles represents the mesophilic condition and with empty circle represents thermophilic condition. The vertical line at the 0.2 *x* axis level is plotted to estimate the Pareto values as indicated by horizontal line. The 45° diagonal (perfect evenness) represents the perfect evenness of a community.

5.3.2 Microbial dynamics at low PM level (25% PM addition)

It is known from previous studies that the used feedstocks for AD affect the microbial community structure independent from general process conditions (Zhang *et al*., 2014; De Vrieze *et al*., 2017). Hence, the microbial community structures in both mesophilic and thermophilic AD were subjected to alteration by 25% PM addition due to changes in the nutrient availability as well as the prevalent abiotic parameters. These changes in the feedstock supply and in the nutrient availability had no significant effects on the reactor performance as was shown previously (Alsouleman *et al*., 2016: Fig. 1; Alsouleman,2019: Fig. 1).

The results showed that the bacterial community in the first experimental phase was affected clearly in both conditions. The calculated pairwise distance between the bacterial communities of the control reactor and the communities in the first experimental phase showed a change in the bacterial community composition up to 28% at mesophilic conditions **Tab. 2** and up to 17% at thermophilic conditions (Alsouleman, 2019).

The Shannon and the Richness indices for microbial diversity under thermophilic condition were considerably higher for bacterial than for archaeal communities in both control and experimental reactors in this experimental phase (Alsouleman, 2019).

Under mesophilic condition, the decreased abundance or even disappearance of some bacterial TRFs assigned either to the WWE1 candidate division or to the phylum *Bacteroidetes* by the 25% PM addition, reflected their sensitivity to changing reactor conditions. These bacterial TRFs might be possible indicator-organisms for a good reactor performance as their disappearance forecasts a subsequent process disturbance.

At the archaeal level in both mesophilic and thermophilic AD, the dominance of the obligate or facultative acetoclastic methanogens decreased clearly in combination with an increase in the predominance of hydrogenotrophic methanogens as was illustrated previously in detail (Alsouleman *et al*., 2016; Alsouleman, 2019). These results are in accordance with De Vrieze *et al*. (2015) and Fotidis *et al*. (2014) who reported that the obligate or facultative acetoclastic methanogens dominate the archaeal community at medium NH_4^+-N, VFA and/or salt concentrations. Furthermore, high concentrations of the mentioned parameters are positively correlated with a predominance of obligate hydrogenotrophic methanogens.

Under thermophilic condition, the clear decrease in the relative abundance of the TRFs assigned to the order *Methanoculleus* was recorded. These archaeal TRFs could be also potential indicator-organisms for the anaerobic digestion process disturbances.

Hydrogenotrophic methanogens utilize (CO_2) and molecular hydrogen (H_2) for methane production. Thus, the acetate produced by fermentative *Bacteria* has to be converted via the acetate oxidation pathway performed by syntrophic *Bacteria* (Dolfing, 2014; Westerholm *et al*., 2016). Thus, it can be assumed in this phase the significant contribution of the syntrophic acetate-oxidizing SAO bacteria. This fact could explain the previous recorded changes within the bacterial community composition of the EP1 and suggested also a strong dependence between the bacteria and archaea members.

Tab. 2: Similarity matrix in comparison of the bacterial communities over the mesophilic trial period. The calculated pairwise similarity considered both changes in the number and the relative abundance of each detected TRF within and between two samples

AD experimental phases	Experimental phase 1 (EP1)		Experimental phase 2 (EP2)						Experimental phase 3 (EP3)				Experimental phase 4 (EP4)	
	25% PM + 75% CS a) (vol/vol VS)		50% PM +50% CS (vol/vol VS)						75% PM +25% CS (vol/vol VS)				100 % CS (vol/vol VS)	
Sample	ER-98	ER-137	ER-155	ER-185	ER-207	ER-230	ER-274	ER-305	ER-319	ER-337	ER-372	ER-479	ER-490	ER-514
CR-98	78	72	37	48	31	66	23	59	60	30	46	34	7	10
ER-98		96	57	75	58	91	41	84	84	49	74	56	19	28
ER-137			76	86	72	89	70	88	87	50	69	55	18	23
ER-155				88	80	57	28	69	66	39	37	36	13	9
ER-185					92	78	42	89	86	54	62	49	20	21
ER-207						68	45	81	78	55	58	46	19	3
ER-230							57	91	94	65	79	67	31	30
ER-274								66	65	89	66	80	66	21
ER-305														
ER-319									97	72	79	67	37	32
ER-337										74	79	69	39	33
ER-372											72	75	65	29
ER-479												72	48	40
ER-490													75	30
ER-514														100

CS: Cattle Slurry; PM: Poultry manure; VS: Volatile substances

The previously mentioned changes in the microbial community are in agreement with the fact that the stable performance of the anaerobic digestion process indicates usually steady-state production and consumption of metabolites along the phases of this process. In contrast, a population shift at one of these process phases would likely require a concrete change in the remaining populations to maintain the stable state (Fernändenz *et al*., 1999).

As was known, the organization of the microbial community is the result of the action of the most fitting microorganisms to the prevalent environmental which in turn become dominant within the microbial structure (Marzorati *et al*., 2008; Wittebolle *et al*., 2009). Thus, the Lorenz curve is based on the assumption, that the distribution of species within a microbial community relates to the capacity of these species to compensate the disturbances and to conserve functionality even under perturbed conditions. The higher the Gini coefficient, the more uneven is the microbial community (Marzorati *et al*., 2008; Wittebolle *et al*., 2009; Theuerl *et al*., 2015).

As illustrated in **Fig. 5** and depending on the Pareto principle, in this experimental phase, 20% of the bacterial TRFs (assumed indicating species) in both mesophilic and thermophilic bacterial communities accounted for approximately 35 to 45% of the whole bacterial relative abundance. This in turn means that the most fitting species are dominant and present in high number species. Thus, the well-organized microbial community may explain its ability to deal with the environmental disturbances (new

feedstock and nutrient availability; low PM level) and save the process functionality as no significant effects on the reactor performance were recorded as was shown previously (Alsouleman *et al*., 2016: Fig. 1; Alsouleman, 2019: Fig. 1).

As was shown in Fig. 4, the thermophilic archaeal community organization was more even than that in mesophilic condition. This could be due to the predominance of the robust hydrogenotrophic methanogens which are considered the most fitting methanogens to the prevalent environmental condition in EP1 in the thermophilic condition. This archaeal community organization was able to deal with changing of the environmental condition (PM addition) and save its functionality.

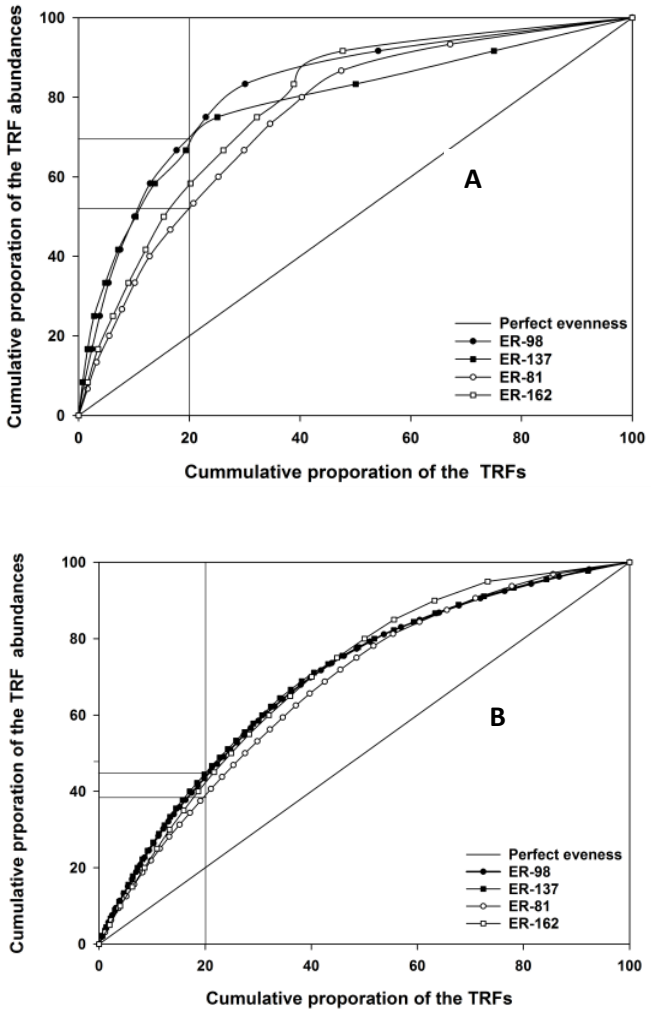


Figure 5: Pareto-Lorenz distribution curves based on 16S rRNA gene T-RFLP patterns of the archaeal (A) and bacterial (B) communities in the first experimental phase. The curve with filled circles represents the mesophilic condition and with empty circle represents thermophilic condition. The vertical line at the 0.2 *x* axis level is plotted to estimate the Pareto values as indicated by horizontal line. The 45° diagonal (perfect evenness) represents the perfect evenness of a community.

5.3.3 Microbial dynamics at medium PM level (50% PM addition)

In the second experimental phase with continuous addition of medium PM level, the TRFLP and the consequent identification of representatives for most abundant taxa, showed a significant difference in the microbial community structure in mesophilic and thermophilic conditions comparing with that under low PM level.

In the mesophilic AD experiment, at the beginning of the second experimental phase, an increase in the relative abundance of members from the order *Bacteroidetes* - which are reported as acid producers (Hahnke *et al*., 2016) - was recoded as a response to the change in the feedstock supply. These finding were supported by the applied indicator species analysis (ISA) as the highest significant indicator values for the TRFs related to this family ($IV \geq 70$ with $p \leq 0.05$) were recorded at the beginning of EP2 **Tab. 3**. Thus, this increased abundance may explain the subsequent VFA accumulation as several members from the order *Bacteroidales* have been reported as acid producers with acetic acid and propionic acid as main end products (Chen & Dong, 2005; Grabowski *et al*., 2005). Moreover, at the archaeal level, the *Bacteroidales*-dominated AD microbiomes were also dominated by the archaeal family *Methanosaetaceae* (De Vrieze *et al*., 2015; Alsouleman *et al*., 2016). This family is well-known to be significantly negative correlated with both increasing the VFA and NH_4^+-N content. Afterwards, a reorganisation of the bacterial community occurred as the *Bacteroidetes*-dominated microbiome was gradually replaced by members of the order *Clostridiales* (phylum *Firmicutes*). At this point it is questionable whether the VFAs produced by *Bacteriodales* led to self-inhibition or whether the increasing NH_4^+-N content suppressed their growth.

In addition to that, the continuous increasing in the NH_4^+-N content arising from the continuous addition of 50% PM may explain the subsequent VFA accumulation and as a consequence led to a replacement of the acetoclastic pathway of methane formation by hydrogenotrophic pathway on the archaeal level. The new microbial community structure is in accordance with the results of De Vrieze *et al*. (2015) and Fotidis *et al*. (2014). The subsequent change in the archaeal community structure would be likely a response to the change at the bacterial level which in turn formed a new microbial community able to maintain stable reactor performance. Fernandenz *et al*. (1999) proved also that the flexible community structure of their reactor characterising by

sequential replacement of microbiome's members led also to a stable reactor performance.

It could be assumed here, that the multiple populations in the mesophilic anaerobic digestion permit the replacement of negatively impacted population (*Bacteroidales*, acetoclastic pathway) by others (*Clostridiales*, hydrogenotrophic pathway) which were able to maintain the stability of the reactor performance.

The Gini Coefficient values derived from the Pareto-Lorenz patterns were between 0.37 and 0.45 for the bacterial communities and between 0.44 and 0.66 for the archaeal ones. These values indicated also well-established communities with intermediate evenness. This microbial community consisted not only of the generalists (such as TRF-152, TRF-181 in the bacterial community) that are mostly defined by their predominant occurrence but also specialists (such as TRF-65, TRF-169 in the bacterial community) which are able to compensate the environmental disturbances.

Concluding the addition of medium level of PM in mesophilic condition resulted in a new microbial community structure (*Clostridiales-Methanobacteriaceae*-dominated microbiome) which was functional redundant compared with the former one as the overall process rates were similar after the disturbance phase. It could be assumed that the microbial community in this experimental phase was extremely dynamic community due to its ability to maintain a functionality stable reactor performance. Here, the anaerobic microbial community enabled to adapt to changing environmental conditions by a natural-regulated and highly efficient microbial diversity management system.

Tab. 3: Indicator species analysis (ISA) for the given process conditions in the first two mesophilic experimental phases (EP 1 and EP 2) as the results revealed the most important shifts in the microbiome. Given are the values for the nitrogen as well the acid related parameter (left side) in combination with the detected indicator TRFs and their phylogenetic assignment (right side). ISA produces indicator values (IV ranging from 0 to 100, absent to exclusively present) for each TRF in defined groups of a given environment. Only IV with p > 0.05 is shown

Experimental phase	Sample name	Corresponding environmental categories [g kg_{FM}^{-1}]										TRF [bp]	Indicator species analyses with $p < 0.05$ [-]										Phyogenetic assignment of the detected TRFs [sorted by phylum,order, family]
		NH_4^+-N	NH_3	VFA	AA	PA	nBA	iBA	nVA	iVA	CA		IV NH_4^+-N	IV NH_3	IV VFA	IV AA	IV PA	IV nBA	IV iBA	IV nVA	IV iVA	IV CA	
EP 1	ER-day98	3,6	175	0,1	0,1	0,00	0,00	0,00	0,00	0,00	0,00	83	47	35	35	46	40	70	75	56	70	56	*Actinobacteria, Actinomycetales, Sanguibacteraceae*
EP 1	ER-day137	3,5	145	0,1	0,1	0,02	0,00	0,00	0,00	0,00	0,00	92	43	31	31	41	37	66	58	ns	66	ns	*Bacteroidetes, Bacteroidales, Porphyromonadaceae*
EP 2	ER-day155	3,9	177	0,1	0,1	0,00	0,00	0,00	0,00	0,00	0,00	93	43	29	29	38	39	76	61	61	76	61	*Bacteroidetes, Bacteroidales, Porphyromonadaceae*
												112	37	71	71	71	31	ns	35	ns	ns	ns	unknown *Bacteria*
												186	44	28	28	37	38	62	55	71	62	71	*Bacteroidetes, Bacteroidales*
EP 2	ER-day185	5,0	297	4,1	3,7	0,41	0,02	0,04	0,00	0,04	0,00	180	60	100	100	100	100	50	100	ns	50	ns	unknown *Bacteria*
EP 2	ER-day207	5,5	310	8,8	7,5	0,96	0,22	0,30	0,02	0,28	0,02	65	66	27	27	27	27	ns	ns	71	ns	71	*Firmicutes, Clostridiales*
												143	ns	37	37	41	40	57	51	80	57	80	*Firmicutes, Clostridiales, Peptococcaceae*
												239	72	51	51	51	51	52	68	88	52	88	unknown *Bacteria*
												544	ns	36	36	40	40	52	46	80	52	80	unknown *Bacteria*
EP 2	ER-day230	5,8	499	2,1	1,9	0,18	0,02	0,02	0,00	0,04	0,00	100	33	100	100	100	100	50	50	ns	50	ns	Bacteroidetes, Bacteroidales
EP 2	ER-day230	5,8	499	2,1	1,9	0,18	0,02	0,02	0,00	0,04	0,00	97	67	50	50	50	50	67	100	ns	67	ns	Bacteroidetes, Bacteroidales
EP 2	ER-day274	5,9	533	2,0	1,7	0,29	0,03	0,02	0,00	0,02	0,00												
EP 2	ER-day274	5,9	533	2,0	1,7	0,29	0,03	0,02	0,00	0,02	0,00	216	33	100	100	100	100	100	50	ns	100	ns	*Firmicutes*
												296	33	100	100	100	100	100	50	ns	100	ns	*Firmicutes, Clostridiales, Lachnospiraceae*
												481	ns	70	70	76	70	79	37	ns	79	ns	unknown *Bacteria*

EP = experimental phase, ER = experimental reactor, NH4+-N = ammonium nitrogen, NH3 = free ammonia nitrogen, VFA = volatile fatty acids, AA = acetic acid, PA = propionic acid, nBA = n-tutyric acid, iBA = Iso-butyric acid, nVA = n-vareic acid, iVA = Iso-valeric acid, CA = capronic acid, TRF = terminal restriction fragment, bp = base pair, IV = indicator value

While in thermophilic condition, the anaerobic microbiome adapted to thermophilic condition is much more sensitive for process disturbances arising from 50% PM which resulted in a complete process failure.

The Shannon's and Richness indices for the bacterial and archaeal communities were lower in this experimental phase comparing with those of control reactor and of the first experimental phase.

The Shannon's index of the sample ER-250 for the bacterial and archaeal communities under thermophilic condition reflected the lowest diversity of the microbial community during the entire experimental phase (Alsouleman, 2019).

The NMS analysis revealed that, a multiplicity of the prevalent environmental factors arising from medium PM level addition affected negatively the both archaeal and bacterial microbial community which caused later deterioration in thermophilic reactor performance.

The general trend of the archaeal community structure was towards the obligate hydrogenotrophic pathway. A clear increase in the relative abundance of TRF-336 assigned to the genus *Methanobrevibacter* was recorded and formed 68% of the entire archaeal community structure. Whereby a clear decrease in the relative abundance of TRF-627 assigned to the genus *Methanosarcina* completely. (Alsouleman, 2019).

This obligate hydrogenotrophic methanogens is well known to be the more stable/robust metabolic pathway considering the risk of ammonia toxicity (Chen *et al*., 2008; Demirel and Scherer, 2008; Fotidis *et al*., 2014). During the last years a lot is known about the inhibition thresholds of the NH_4^+-N respectively the NH_3 concentration, especially for the obligate and facultative acetolastic methanogens (e.g. De Vrieze *et al*., 2012; Niu *et al*., 2013; Niu *et al*., 2014) but on the other hand less information are available about the threshold values of the NH_4^+-N respectively the NH_3 concentration for the obligate hydrogenotrophic methanogens in thermophilic condition. So that and regarding the presented results, a threshold could be proposed at $\geq$ 4 g kg_{FM}^{-1} for NH_4^+-N corresponding to $\geq$ 0.5 g kg_{FM}^{-1} for NH_3 at 55°C (Alsouleman, 2019).

The NMS results showed that the multiplicity of the prevalent environmental factors arising from the 50% addition of PM caused inhibition of methanogenic activity which in turn forced the bacterial community to restructure (inhibition of the acid converting bacteria). A clear decrease in the relative abundance of TRF-94 assigned to the genus

Bacillales, and in the relative abundance of TRF-75 and TRF-216 assigned to the genus *Lactobacillales* (Alsouleman, 2019).

This suggested a strong dependence between *Bacteria* and *Archaea* members in the microbial community. It could be concluded here that the acclimated thermophilic microbial community failed to tolerate a medium PM level and a complete deterioration in the process occurred. Even though during the inhibition period, the microbial community was only able to compensate the prevalent operational parameter arising from medium PM level addition for a short time (second phase showing short-termed stable biogas yield) (Alsouleman, 2019).

Even the Pareto-Lorenz curves **Fig. 6** and the derived Gini coefficient values in this experimental phase (Alsouleman, 2019) reflected a well-balanced community composition which is assumed to be robust against the prevalent environmental factors but as mentioned previously the acclimated thermophilic microbial community failed to tolerate the multiplicity prevalent environmental factors arising from a medium PM level.

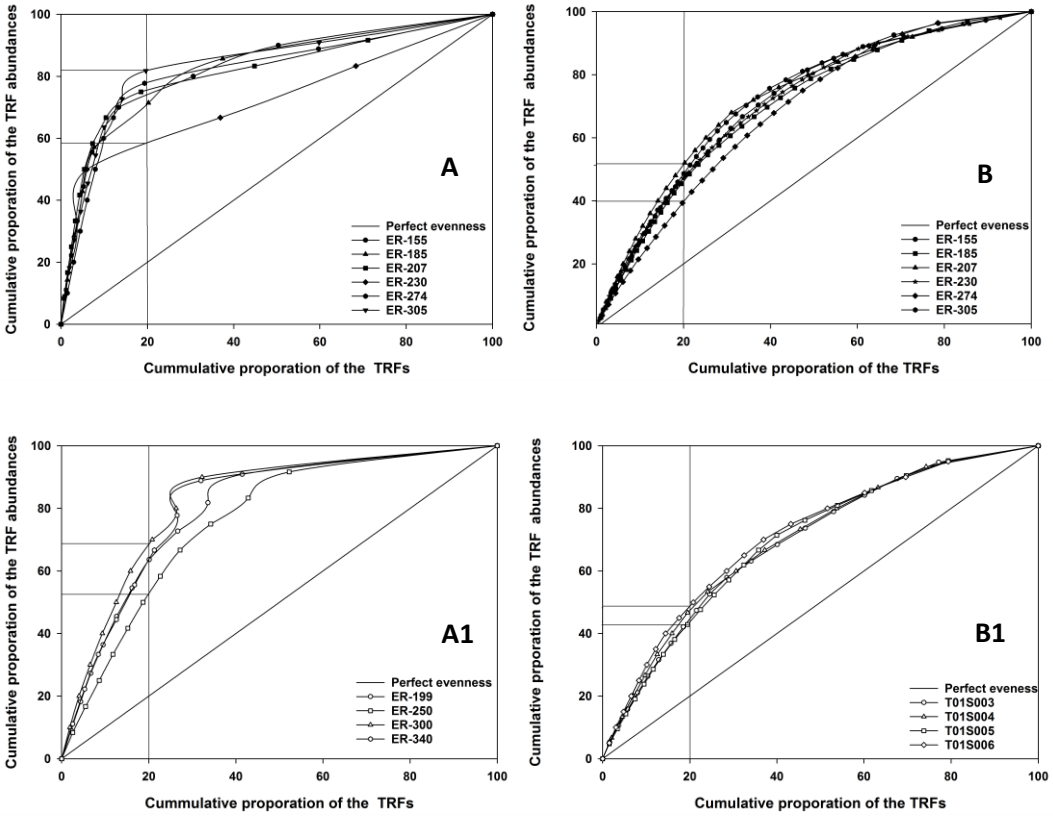


Figure 6: Pareto-Lorenz distribution curves based on 16S rRNA gene T-RFLP patterns of the bacterial communities in mesophilic condition (B); the archaeal communities in mesophilic condition (A); the bacterial communities in thermophilic condition (B1); the archaeal communities in mesophilic condition (**A1**) in the second experimental phase. The curve with filled circles represents the mesophilic condition and with empty circle represents thermophilic condition. The vertical line at the 0.2 *x* axis level is plotted to estimate the Pareto values as indicated by horizontal line. The 45° diagonal (perfect evenness) represents the perfect evenness of a community.

5.3.4 Microbial dynamics at high PM level (75% PM addition)

A further increase of PM (75% in EP3, respectively 100% in EP4) was not able to be tolerating anymore and a complete failure in the mesophilic reactor performance occurred.

According to the ongoing increase of the NH_4^+-N content during EP3, the trend of EP3 microbial community was moving on towards *a Clostridiales-Methanobacteriaceae*-dominated microbiome. Depending on the serious decrease in the biogas yield and methane content in this experimental phase, it could be assumed that the methanogenic activity was inhibited. A clear decrease in the relative abundance of the order *Methanobacteriales* (symbolized by TRF-107) and the genus *Methanoculleus* (symbolized by TRF-429bp) were recorded. Hence, it could be concluded that this inhibition of the methanogenic activity led to an inhibition in the activity of the acid converting bacteria which grows in syntrophic association with methanogens.

In this phase, the hydrolytic and acidogenic bacterial community members of the microbiome still converted the supplied feedstock/substrates into acids as identified by the continuous increase in the VFA content but the successive steps of conversion were completely inhibited. The occurring microbial community in this experimental phase could not compensate the induced process disturbance (the methanogenic activity was seriously inhibited), and a complete process failure occurred as a significant decrease in the biogas yield and methane content was recorded (Alsouleman *et al*., 2016).

In thermophilic condition, no more microbiological samples were analysed in this phase as a complete reactor failure was already detected from the previous phase.

6 Conclusions and Outlook

The findings of this study contribute to the basic understanding of the response of the microbial community in terms of restructuring and reorganization under increasing PM levels.

The results of this study proved that the CSTR was suitable to digest efficiently until the medium PM level on the lab-scale. However, further improvement of the reactor system is required to run the anaerobic digestion process of nitrogen rich substrate on full-scale efficiently; such as optimizing the mixing mechanism and separate the anaerobic digestion process of nitrogen-rich substance into two phases.

The finding of the microbial community analysis proved the validity of the TRFLP technique as pre-screening analysis which enables to follow the main microbial community dynamics due to the different added PM levels. More investigations are required to study deeply the structure and functionality of the involved microbial community as complex interacting microbial network.

The application of the next generation sequencing techniques could be applied in future to determine the different functional redundant microbial assemblage which can ensure a stable or resilient reactor performance.

With low and medium PM amounts, the acclimated occurring microbial community was able to adapt (in terms of restructuring and reorganization) to the new environmental conditions arising from changes in the feedstock supply and in the nutrient availability in mesophilic condition. The functional redundancy was the major microbial strategy in mesophilic condition ensuring an ongoing and stable biogas production as the compositional shifts did not affect significantly the reactor performance.

Hence it could be concluded, that under mesophilic condition the anaerobic digestion of low (25%PM+75% CS) and medium (50%PM+50% CS) PM levels (vol/vol based on volatile substances) could be applied efficiently on a full scale. Thereafter with increasing the PM amount, the microbial shifts under loss of functionality are likely to occur.

In contrast, under thermophilic condition, the acclimated occurring microbial community was able to adapt only to the low PM level. The multiplicity of prevalent operational factors arising from the application of medium level of PM was the reason for the reactor performance deterioration. Thus, the succession of the microbial community structure was unsuited to overcome the process disturbance. Therefore, deep investigations of the interaction or synergy effects of different operational factors on the microbial community are required. It could be concluded here that, the anaerobic digestion of low PM level could be applied without disturbances on a full scale, but with increasing the PM amount an irreversible inhibition are likely to take place.

Regarding the presented results, the threshold values of the NH_4^+-N respectively the NH_3 concentration for the obligate hydrogenotrophic methanogens in thermophilic condition could be proposed at ≥ 4 g kg_{FM}^{-1} for NH_4^+-N corresponding to ≥ 0.5 g kg_{FM}^{-1} for NH_3 at 55°C.

Also, the medium community organisation values (Gini coefficients) found in this study reflected a well-established microbial community in mesophilic with low and medium PM level and with low PM level in thermophilic condition. These microbial communities were robust and able to compensate the applied environmental disturbances and maintain the stability of the reactor performance.

These parameters were not be able to reflect the accurate and actual state of the microbial community in the thermophilic condition with medium PM level. Therefore, further research is required to optimize and determine the optimal value of these parameters independent of the applied molecular techniques.

Hence, in future the comprehensive identifying of the biogas process-relevant microorganisms especially operated with process-risk feedstocks like nitrogen-rich substances could be used as validation standards or as indicators for process emerging disturbances. Also, the co-occurrence network analyses, which provide a comprehensive picture of the interactions within the microbial community in a specific condition , explain how the disturbances (different added PM levels) affects firstly this interactions which in turn alter the function of the ecosystem (anaerobic digestion performance) and also give the opportunity to define the keystone species (a species who has a large impact and a great role in relation to its relative abundance in a specific ecosystem), should be applied to achieve highly efficient anaerobic digestion process .

Hence, the results of this study present a basis for more researches on the applicability of use nitrogen-rich manure for anaerobic digestion as alternative treatment technology for animal waste management on full-scale biogas reactor and as a bioenergy resource.

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DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen,

.....

KHULUD ALSOULEMAN

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen,

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KHULUD ALSOULEMAN

8 Appendix

A1: The relative distribution of the detected bacterial and archaeal terminal restrictions fragments (TRFs) in mesophilic experiment highlighted by colors according to an increasing abundance and their phylogenetic assignment

Domain	TRF [bp]	Sampling points & experimental phases															Phyogenetic assignment of the detected TRFs
		starter community	EP1		EP2						EP3				EP4		
			ER-98	ER-137	ER-155	ER-185	ER-207	ER-230	ER-274	ER-305	ER-319	ER-337	ER-372	ER-479	ER-490	ER-514	
		G_B = 0.41	G_B = 0.39	G_B = 0.40	G_B = 0.44	G_B = 0.41	G_B = 0.45	G_B = 0.43	G_B = 0.37	G_B = 0.45	G_B = 0.44	G_B = 0.44	G_B = 0.33	G_B = 0.39	G_B = 0.39	G_B = 0.38	
		G_A = 0.69	G_A = 0.64	G_A = 0.57	G_A = 0.65	G_A = 0.64	G_A = 0.63	G_A = 0.44	G_A = 0.64	G_A = 0.68	G_A = 0.62	G_A = 0.60	G_A = 0.49	G_A = 0.61	G_A = 0.56	G_A = 0.56	[sorted by phylum, class, order, family]
Bacteria	65	0	0	0	0	4	8	7	8	5	9	12	5	5	5	0	*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	83	3	3	4	4	0	0	0	0	0	0	4	0	0	0	0	*Firmicutes, Clostridia, Clostridiales*
	86	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*Actinobacteria, Actinobacteria, Actinomycetales, Sanguibacteraceae, Sanguibacter*
	92	15	5	13	27	17	18	7	0	9	9	3	0	2	0	0	*Bacteroidetes*
	93	3	4	4	4	3	0	0	0	0	0	0	0	0	0	0	*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	97	3	0	2	3	0	0	5	6	4	5	3	0	8	11	0	*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	100	0	0	0	0	0	0	7	7	3	3	4	0	5	0	0	*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	108	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*Bacteroidetes*
	112	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	n.d.
	132	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	*Firmicutes*
	143	0	2	0	0	3	2	3	0	0	0	0	0	0	2	0	*Firmicutes, Clostridia, Clostridiales, Peptococcaceae*
	152	5	4	4	3	3	6	5	3	5	5	5	0	0	0	0	*Firmicutes, Erysipelotrichia, Erysipelotrichales, Erysipelotrichaceae, Turicibacter*
	155	0	0	0	0	0	0	0	0	0	0	0	0	0	2	7	*Firmicutes, Bacilli, Bacillales, Staphylococcaceae*
	161	10	6	3	0	0	0	0	0	0	0	0	0	0	0	0	unknown *Bacteria*
	166	0	0	0	0	0	0	0	0	0	0	2	0	0	0	4	*Firmicutes, Bacilli, Bacillales*
	169	0	4	4	7	6	7	7	21	10	9	23	11	16	17	5	*Firmicutes, Clostridia, Clostridiales*
	180	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	*Firmicutes, Bacilli, Lactobacillales, Enterococcaceae*
	181	3	5	6	7	9	12	7	3	10	9	7	3	0	2	3	unknown *Bacteria*
	186	10	8	8	10	5	0	0	0	4	2	0	0	0	0	0	*Firmicutes, Clostridia, Clostridiales*
	192	8	0	0	3	0	0	3	0	0	0	0	0	0	0	0	*Firmicutes, Bacilli*
	194	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*Bacteroidetes, Bacteroidia, Bacteroidales*
	206	0	2	0	0	0	0	2	3	0	0	0	2	0	0	0	*Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae*
	213	0	3	2	0	0	0	0	0	2	2	0	2	0	2	9	*Firmicutes, Clostridia, Clostridiales*
	217	2	3	3	0	0	0	0	6	3	2	3	0	2	0	0	n.d.
	222	0	4	5	4	5	9	2	4	6	0	0	7	4	0	0	*Firmicutes, Erysipelotrichia, Erysipelotrichales,Erysipelotrichaceae,Turicibacter*
	225	0	0	0	0	0	0	0	0	0	0	6	6	0	2	0	*Proteobacteria , Gammaproteobacteria,Pseudomonadales, Pseudomonadaceae, Pseudomonas*
	228	4	5	3	0	0	0	0	2	0	0	0	2	5	6	0	*Proteobacteria , Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Ignatzschineria*
	233	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	*Firmicutes*
	236	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	239	0	0	0	0	0	3	0	3	0	0	0	3	0	0	0	*Firmicutes, Clostridia, Clostridiales, Syntrophomonadaceae*
	241	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	n.d.

A1 (continue)

Domain	TRF [bp]	Sampling points & experimental phases														Phyogenetic assignment of the detected TRFs
		starter community	EP1		EP2						EP3				EP4		
			ER-98	ER-137	ER-155	ER-185	ER-207	ER-230	ER-274	ER-305	ER-319	ER-337	ER-372	ER-479	ER-490	ER-514	
		G_B = 0.41	G_B = 0.39	G_B = 0.40	G_B = 0.44	G_B = 0.41	G_B = 0.45	G_B = 0.43	G_B = 0.37	G_B = 0.45	G_B = 0.44	G_B = 0.44	G_B = 0.33	G_B = 0.39	G_B = 0.39	G_B = 0.38	
		G_A = 0.69	G_A = 0.64	G_A = 0.57	G_A = 0.65	G_A = 0.64	G_A = 0.63	G_A = 0.44	G_A = 0.64	G_A = 0.68	G_A = 0.62	G_A = 0.60	G_A = 0.49	G_A = 0.61	G_A = 0.56	G_A = 0.56	[sorted by phylum, class, order, family]
Bacteria	290	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	*Synergistetes, Synergistia, Synergistales, Synergistaceae, Thermovirga*
	296	0	0	0	0	0	0	0	2	0	0	4	4	3	4	0	*Firmicutes ,Clostridia, Clostridiales, Ruminococcaceae* *Firmicutes, Clostridia, Clostridiales, Clostridiales_Incertae Sedis XI, Anaerococcus*
	297	0	0	0	0	0	0	0	0	0	0	0	0	5	8	0	*Firmicutes, Clostridia, Clostridiales, Lachnospiraceae* *Firmicutes,Clostridia, Clostridiales*
	298	0	0	0	3	3	3	3	6	5	5	8	6	4	8	13	*Firmicutes,Clostridia, Clostridiales*
	300	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	317	0	0	0	0	0	0	0	0	0	0	0	4	4	9	9	*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	368	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	n.d.
	373	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	n.d.
	378	0	0	0	0	0	0	0	0	0	0	0	2	0	0	3	n.d.
	393	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	n.d.
	453	0	0	0	2	0	2	0	2	0	0	0	0	0	0	0	n.d.
	481	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	n.d.
	487	0	0	0	0	0	0	0	2	0	0	0	3	0	0	0	*Firmicutes, Clostridia, Clostridiales, Clostridiales_Incertae Sedis XIII*
	489	0	0	0	0	0	0	0	0	0	0	0	0	0	2	9	n.d.
	491	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	n.d.
	537	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	n.d.
	543	0	0	0	0	2	0	0	0	0	0	0	0	3	4	9	n.d.
	544	0	2	2	3	5	7	0	0	0	2	0	4	3	0	0	n.d.
	553	0	0	0	0	0	0	0	2	0	2	0	2	3	0	0	*Firmicutes, Bacilli,Lactobacillales,Carnobacteriaceae* *Firmicutes, Bacilli, Lactobacillales, Streptococcaceae*
	558	0	0	0	0	0	0	0	0	0	0	0	4	2	4	4	*Firmicutes, Bacilli, Lactobacillales*
	564	0	0	0	2	6	5	0	3	8	6	0	3	0	0	0	*Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae*
	566	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3	*Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae*
	571	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	n.d.
Archaea	74	5	4	3	3	3	0	0	0	0	0	0	0	0	0	0	*Euryarchaeota, Methanomicrobia, Methanosarcinales*
	83																*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanosarcinaceae*
	107	63	49	25	49	63	26	31	6	6	5	3	0	0	0	0	*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanosaetaceae, Methanosaeta* *Euryarchaeota, Methanobacteria, Methanobacteriales*
	174	0	0	0	0	0	0	0	0	0	0	0	0	5	3	3	n.d.
	318	0	0	2	0	0	3	0	0	0	2	0	2	3	25	9	unknown *Archaea*
	319	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanobacterium*
	340	19	34	56	37	30	63	63	81	81	74	83	90	84	70	88	*Euryarchaeota, Methanobacteria, Methanobacteriale, Methanobacteriaceae*
	346	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae,Methanobrevibacter*
	429	7	5	6	3	0	0	3	7	6	10	10	6	0	0	0	*Euryarchaeota, Methanomicrobia, Methanomicrobiales, Methanomicrobiaceae, Methanoculleus*
	470	0	2	3	0	0	0	0	0	0	3	1	0	0	0	0	*Euryarchaeota, Methanomicrobia, Methanosarcinales*
	627	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	n.d.

A 2: Indicator species analysis (ISA) for the given process conditions in the two thermophilic experimental phases (EP 1 and EP 2). Given are the values for the nitrogen as well the acid related parameter (left side) in combination with the detected indicator TRFs and their phylogenetic assignment (right side). ISA produces indicator values (IV ranging from 0 to 100, absent to exclusively present) for each TRF in defined groups of a given environment. Only IV with p > 0.05 is shown

Sample name	Corresponding environmental categories										TRF	Indicator species analyses with $p < 0.05$										Phyogenetic assignment of the detected TRFs
	NH_4^+-N	NH_3	VFA	AA	PA	nBA	iBA	nVA	iVA	CA	[bp]	IV NH_4^+-N	IV NH_3	IV VFA	IV AA	IV PA	IV nBA	IV iBA	IV nVA	IV iVA	IV CA	[sorted by phylum,order, family]
	[mg kg_{FM}^{-1}]											[-]										
ER-day81	3000,0	341	210,0	210,0	66,00	4,00	7,00	12,00	12,00	0,00	94	69,1	54,6	54,6	54,6	54,6	54,6	54,6	54,6	54,6	69,1	*Firmicutes, Bacilli, Bacillales (Bacteroidetes)*
											294	75,3	38,2	38,2	38,2	38,2	38,2	38,2	38,2	38,2	75,3	*Firmicutes, Clostridia, Clostridiales*
											502	100	56,1	56,1	56,1	56,1	56,1	56,1	56,1	56,1	100	*nd*
											159	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Clostridia, Thermoanaerobacterales*
											288	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Clostridia, Clostridiales*
											294	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Clostridia, Clostridiales*
											376	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Clostridia, Halanaerobiales*
											453	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Clostridia, Clostridiales*
ER-day162	3000,0	267	83,0	83,0	25,00	0,00	0,00	0,00	0,00	0,00	288	50,7	40	40	40	40	40	40	40	40	50,7	*Firmicutes, Clostridiales, Clostridiales*
											228	62,5	39,4	39,4	39,4	39,4	39,4	39,4	39,4	39,4	62,5	*Bacteria*
											367	100	52,7	52,7	52,7	52,7	52,7	52,7	52,7	52,7	100	*Firmicutes, Clostridia, Halanaerobiales*
ER-day199	4000,0	407	254,0	254,0	94,00	5,00	10,00	5,00	28,00	5,00	141	61,5	44,4	44,4	44,4	44,4	44,4	44,4	44,4	44,4	61,5	*Firmicutes, Clostridia (Actinobacteria, Actinobacteria, Actinomycetales)*
ER-day250	5000,0	602	1309,0	1309,0	241,00	53,00	65,00	25,00	104,00	104,00	213	66,3	55,9	55,9	55,9	55,9	55,9	55,9	55,9	55,9	66,3	*Proteobacteria,Pseudomonadales (Xanthomonadales)*
ER-day300	6000,0	337	1461,0	1461,0	235,00	84,00	86,00	19,00	127,00	6,00	150	50	100	100	100	100	100	100	100	100	50	*Firmicutes, Bacilli, Bacillales (Firmicutes, Clostridia)*
											571	50	100	100	100	100	100	100	100	100	50	*nd*
											391	55,2	43,7	43,7	43,7	43,7	43,7	43,7	43,7	43,7	55,2	*Firmicutes,Lactobacillales, Lactobacillaceae , Lactobacillus*
											558	100	60,5	60,5	60,5	60,5	60,5	60,5	60,5	60,5	100	*Firmicutes, Bacili, Lactobacillales*
ER-day340	6000,0	651	3805,0	3805,0	303,00	114,00	103,00	10,00	167,00	6,00	466	50	100	100	100	100	100	100	100	100	50	*nd*