“All you have to decide, is what to do with the time that is given to you.”

Gandalf The Grey, in: J.R.R. Tolkien, ‘The Shadows of the Past’, The Fellowship of the Ring (London: George Allen & Unwin, 1954), p. 50.

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Methanosaeta vs. *Methanosarcina* in anaerobic digestion: the quest for enhanced biogas production

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Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Titel van het doctoraat in het Nederlands: *Methanosaeta* vs. *Methanosarcina* in anaerobe vergisting: de queeste voor een verbeterde biogas productie.

Cover image: Gustav Klimt (1862-1918), *The Swamp*, 1900, oil on canvas, 80 x 80 cm, private collection.

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A/B	Adsorptions-Belebungsverfahren
AD	Anaerobic Digestion
AnMBR	Anaerobic Membrane Bioreactor
ARDRA	Amplified Ribosomal DNA Restriction Analysis
AQDS	Anthraquinone-2,6-disulfonate
BES	Bioelectrochemical System(s)
BOD	Biological Oxygen Demand
CAS	Conventional Activated Sludge
CHP	Combined Heat and Power unit
Co	Community Organization
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DIET	Direct Interspecies Electron Transfer
DNA	Deoxyribonucleic Acid
dV	Cell potential
Dy	Community Dynamics
E_{an}	Anode potential
E_{cat}	Cathode potential
FA	Free Ammonia
FISH	Fluorescent In-situ Hybridization
FW	Fresh Weight
HM	Hydrogenotrophic Methanogenesis
HRT	Hydraulic Retention Time
J	Current density
KjN	Kjeldahl Nitrogen

KW	Kitchen Waste
LCFA	Long Chain Fatty Acids
MLVSS	Mixed Liquor Volatile Suspended Solids
MRM	Microbial Resource Management
OCP	Open Circuit Potential
OFMSW	Organic Fraction Municipal Solid Waste
OHPA	Obligate Hydrogen Producing Acetogens
OLAND	Oxygen-limited Autotrophic Nitrification/Denitrification
OTU	Operational Taxonomic Unit
PSD	Particle Size Distribution
qPCR	quantitative Polymerase Chain Reaction
rRNA	ribosomal Ribonucleic Acid
Rr	Range-weighted Richness
SAB	Syntrophic Acetogenic Bacteria
SAO	Syntrophic Acetate Oxidizing Bacteria
SEM	Scanning Electron Microscopy
SHE	Standard Hydrogen Electrode
SMA	Specific Methanogenic Activity
SRB	Sulphate Reducing Bacteria
SRT	Sludge Retention Time
STP	Standard Temperature and Pressure, 101325 Pa and 273.15 K
TAN	Total Ammonia Nitrogen
TMP	Trans Membrane Pressure
TPAD	Temperature-Phased Anaerobic Digestion
TS	Total Solids
TSS	Total Suspended solids

UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids
WHO	World Health Organization
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant

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CHAPTER 1: INTRODUCTION

This chapter has been partially redrafted after:

> De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W. 2012. *Methanosarcina*: The rediscovered methanogen for heavy duty biomethanation. Bioresource Technology, 112, 1-9.

1. The water-energy nexus

In our present western society the primary necessities of life, i.e. clean drinking water and food, shelter and clothing are widely available. However, maintaining our current way of life will lead to severe challenges for all of mankind in the near future. The increasing living standards in the developing countries, together with the growing world population are putting an increasing pressure on our natural resources, of which clean drinking water can be considered one of the most vital ones (Verstraete et al., 2009; Corcoran et al., 2010; WHO, 2012). In 2010, 89% of the world population, which accounts for in total 6.1 billion people, had access to improved drinking water sources, however, still leaving 11% (780 million people) with unimproved drinking water sources (WHO, 2012). The increasing demand, not only for direct potable purpose (which requires an average of 2-4 litres per person per day), but also for the production of food, as it takes 2000-5000 litre of water to produce the food for one person on a daily basis, combined with injudicious management, leads to further depletion of the existing fresh water sources (Jury & Vaux, 2007; Corcoran et al., 2010; Oelkers et al., 2011). This will result in a predicted water shortage for up to 7 billion people by the year 2050 (Verstraete et al., 2009). Consequently, enhanced monitoring and management of the existing fresh water sources, together with alternative technologies, will be of vital importance to maintain, and even improve drinking water provision to the world population (Jackson et al., 2001; Corcoran et al., 2010).

The increasing energy consumption is one of the other main problems the world is facing today. In 2010 the world energy production was estimated at 532 exajoules (10^{18} joules) or 12,717 Mtoe (IEA, 2012). A projected increase in the world energy production of 56% between 2010 and 2040 will lead to an increased consumption of fossil fuel (up to 80% of total energy consumption) and CO_2 emissions (IEA, 2013). Despite the fact that the production of renewable energy is increasing with 2.5 % per year, this accounts in total for only 10 % of the world's primary energy consumption or 53 exajoules (IEA, 2012; IEA, 2013). There is however a potential to cover 25 % of the world's energy needs by 2035 (25% or 156 exajoules) (IEA, 2012). Biofuels, such as wood, straw, charcoal, ethanol derived from maize, and methane-rich biogas show a great potential to limit global warming, create jobs in rural areas and improve energy security (IEA, 2011). Biomass currently only supplies 2 % of the world's electricity needs by the production of biogas, together with a combined heat and power (CHP) system. Despite the planning of so-called 'energy atolls' and the near-future application of wind turbines, able to operate at low wind velocities, wind and solar energy, at

present, only allow intermittent energy generation. This is in contrast to biogas production that can continuously provide electricity production in renewable energy schemes, and, therefore, still has a high growth potential (Kopetz, 2013; REN21, 2013).

Unfortunately, the abovementioned drinking water demands go hand in hand with a growing energy consumption. Increasing water demands and a growing awareness of the necessity of environmental protection in the last decades resulted in the conventional activated sludge system (CAS) that, at present, is the key biological system for industrial and domestic wastewater treatment (Verstraete et al., 2009; Verstraete & Vlaeminck, 2011). Notwithstanding, energy requirements for this wastewater treatment system are still far too high to apply this system on a global scale. Indeed, an estimated overall electricity consumption of 33 kWh (kWh_{el}) IE^{-1} $year^{-1}$ can be put forward, mainly due to aeration (up to 50 %) and pumping requirements (Wett et al., 2007; Verstraete & Vlaeminck, 2011). Energy recuperation by means of anaerobic digestion is one of the possible measures to counteract this energy gap, but this only results in maximum 20% energy recuperation, due to the low biodegradability (30-50%) of the waste activated sludge (WAS), if implemented at all (Ekama et al., 2007; Verstraete & Vlaeminck, 2011). The implementation of the so-called 'ZeroWasteWater' concept, in which the CAS will be replaced by the far more energy and cost efficient A/B (Adsorptions-Belebungsverfahren) process, would provide a suitable alternative, thus allowing not only water, but also nutrient and energy recovery (Boehnke et al., 1997; Wett et al., 2007; Verstraete & Vlaeminck, 2011). In this concept anaerobic digestion would take up a crucial role in the simultaneous recovery of energy, processing of the highly biodegradable sludge from the A-stage (A-sludge) and nutrient recuperation (Zeeman et al., 2008; Verstraete & Vlaeminck, 2011; De Vrieze et al., 2013a).

2. Anaerobic digestion as key technology in the future bio-based economy

Anaerobic digestion (AD) can be considered one of the first microbial technologies that allows energy recovery from low-value organic by-products. This technology has been applied at full-scale for several decades and it has the potential to become a key technology for renewable energy production (Mata-Alvarez et al., 2000; Angenent et al., 2004a; Verstraete et al., 2005; Holm-Nielsen et al., 2009; Appels et al., 2011; Tyagi & Lo, 2013). A variety of complex organic waste streams or by-products, such as waste activated sludge,

manure, slaughterhouse waste, the organic fraction of municipal solid waste (OFMSW), energy crops, algae biomass, molasses and vinasses, and different types of wastewater can be converted to biogas by means of AD (Debazua et al., 1991; Gunaseelan, 1997; Appels et al., 2008; Labatut et al., 2011; Zamalloa et al., 2011; Raposo et al., 2012; Zhang et al., 2014b). During the AD process, these substrates are gradually converted into the two main components of biogas, i.e. CH_4 (45-75%) and CO_2 (25-55%), by a diverse microbial consortium. Next to these two main components, traces of N_2, H_2, NH_3 and H_2S can also be produced, depending on the substrate and process conditions (Gerardi, 2003b; Tchobanoglous et al., 2003).

The main advantage of AD lies in its potential to not only treat and stabilize organic waste streams, but also recover energy from these streams in the form of the energy-rich CH_4 (Mata-Alvarez et al., 2000; Verstraete et al., 2005). In addition to the production of biogas, AD also has several other advantages compared to aerobic microbial technologies, such as a low cell yield, a high organic loading rate, limited nutrient demands, a minor environmental impact and low costs for operation and maintenance of the reactor system (Mata-Alvarez et al., 2000; Angenent et al., 2004a).

The production of biofuels and bio-based building blocks for the chemical industry, of which ethanol can be considered the most important one, is rapidly increasing (Sarkar et al., 2012). These biofuel production facilities or so-called 'bio-refineries' unfortunately also consume huge amounts of fresh water, leading to liquid waste streams that require adequate treatment before being discharged into the environment (Schornagel et al., 2012). For instance, the production of bio-ethanol by means of wet milling generates up to 20 litres of wastewater per litre of ethanol, containing high levels of chemical oxygen demand (COD) and biological demand (BOD), up to 100 and 50 g L^{-1}, respectively, at a low pH (4-4.5), which may result in serious environmental damage of the existing water bodies (Satyawali & Balakrishnan, 2008). To preserve the existing freshwater bodies, and to guaranty the success of these bio-refineries, validation of these side streams is necessary and perfectly fits within the framework of the 'zero waste bio-refinery' (Verstraete & Vlaeminck, 2011). In fact, AD can be considered the technique 'par excellence' to treat these waste streams, due to their high COD and BOD load. Assuming a conversion efficiency to methane of 50 %, the production of CH_4 from these wastewaters by means of AD could result in 0.35 m^3 of CH_4 per litre of ethanol produced. Taking an energy content of 10 kWh m^{-3} of CH_4 at a value of € 0.1 kWh^{-1}, this could result in € 0.35 added value per litre of bioethanol. As the market price of bio-ethanol itself ranges

between € 0.60 and 0.80 L^{-1}, the treatment of the wastewater could create an additional profit of roughly 50 %.

Hence, the central role of AD in our present and future economy cannot be ignored, as it plays a major role in two of the main processes crucial in ensuring the continuing existence of mankind. First, AD is and will continue to be the crucial process in industrial and household wastewater treatment, as it allows the recovery of energy, treatment of WAS and nutrient recovery, thus forming the basis of future energy-positive wastewater treatment. Second, AD may serve as the main process for energy recovery and wastewater treatment in the emerging bio-refineries, thus allowing a transition from a fossil fuel-based to a bio-based economy.

3. The anaerobic digestion process

3.1. The four stages in anaerobic digestion

Anaerobic digestion can be divided into four sequential stages, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis, during which complex organic substrates are gradually degraded to CH_4 and CO_2 by a specialized microbial consortium (Figure 1).

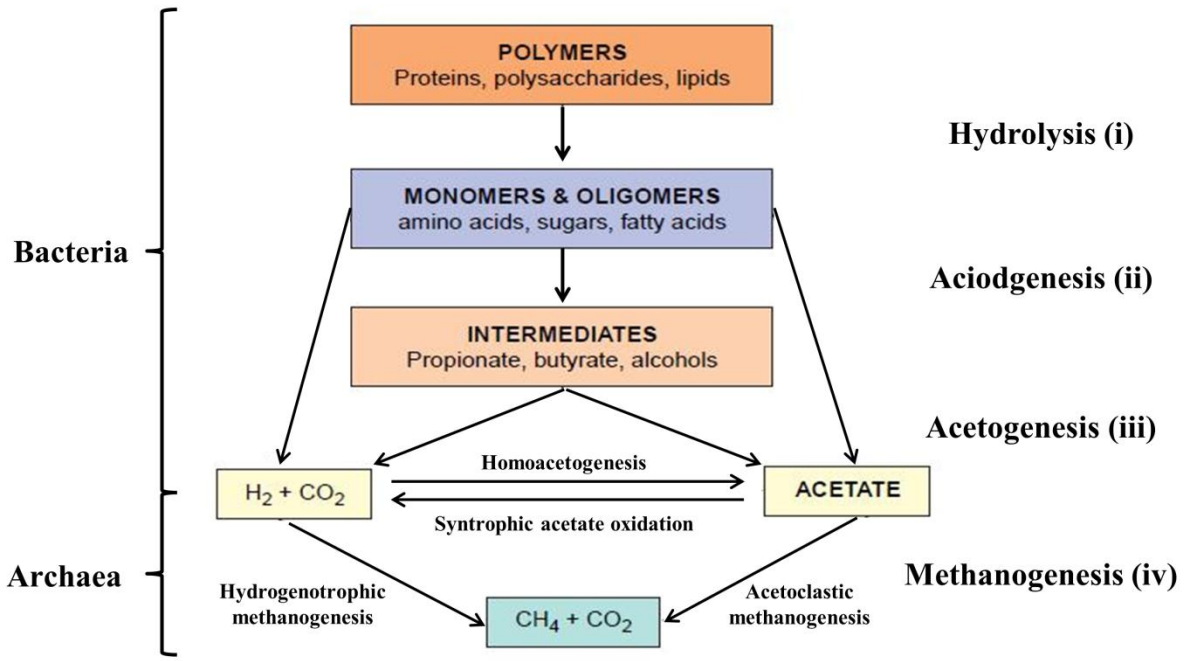

Figure 1.1 *Different stages of the anaerobic digestion process (adapted from Angenent et al. (2004a)).*

3.1.1. Hydrolysis

Hydrolysis is the first step in the AD process during which complex particulate organic substances, such as carbohydrates, lipids and proteins, are solubilized by means of exoenzymes, such as proteases, lipases, phosphatases, polysaccharases (e.g. amylases, cellulases), and esterases that are produced by hydrolytic bacteria (Gujer & Zehnder, 1983; Yang et al., 2010). These monomers mainly consist of amino acids, sugars, purines, pyrimidines and long-chain fatty acids. Micro-organisms that are involved in the hydrolysis step mainly belong to the Firmicutes, Bacteroidetes and Proteobacteria phyla (Cirne et al., 2007; Wang et al., 2010).

Hydrolysis is generally considered to be the rate-limiting step of the AD process, due to the complex nature of common substrates for AD, such as WAS, manure and other lignocellulose-rich substrates (Eastman & Ferguson, 1981; Vavilin et al., 1996; Appels et al., 2008). However, literature reports a wide range of hydrolysis rate constants, due to the difference in chemical composition of various substrates (Vavilin et al., 1996; Vavilin et al., 2008a; Rajagopal & Beline, 2011). The hydrolysis rate not only depends on the substrate composition, but also on the temperature, pH and sludge retention time (SRT) in the anaerobic digester (Eastman & Ferguson, 1981; Gujer & Zehnder, 1983; Miron et al., 2000; Batstone et al., 2009).

To improve the hydrolysis rate and efficiency, several pre-treatment methods have been developed. These treatments generally can be divided into four categories, i.e. physical, chemical, thermal and biological, also known as enzymatic, treatment methods (Monlau et al., 2013). However, to be economically feasible, only low energy intensive technologies are to be preferred as pre-treatment methods (Ma et al., 2011).

3.1.2. Acidogenesis

Acidogenesis or fermentation is the second step in the AD process that involves the conversion of the soluble monomers that are formed during hydrolysis to volatile fatty acids (butyrate, propionate and acetate), alcohols, CO_2, H_2, NH_3 and H_2S (Angenent et al., 2004a; Appels et al., 2008). Similar bacteria that are involved in hydrolysis are responsible for acidogenesis as well. The formation of volatile fatty acids (VFA) during the acidogenesis step may lead to a decrease in pH, hence a high buffering capacity and/or rapid further degradation of these VFA is required to maintain a constant pH (Gerardi, 2003e). Phase separation of the

AD process in two separate systems is sometimes applied to create favourable conditions for both acidogenic bacteria and methanogenic archaea (Shin et al., 2010).

3.1.3. Acetogenesis

Acetogenesis involves the conversion of the intermediary products, formed during the acidogenesis step, into acetate. The acetogenesis process involves several groups of micro-organisms, i.e. the syntrophic acetogenic bacteria (SAB), also called obligatory hydrogen-producing acetogenic bacteria (OHPA), homoacetogenic or syntrophic acetate oxidizing bacteria (SAO) and sulphate reducing bacteria (SRB) (Gerardi, 2003d; Angenent et al., 2004a).

Syntrophic acetogenic bacteria are able to convert VFA, such as butyrate and propionate to acetate and H_2. The H_2-concentration in the reactor suspension, however, plays an important role, as the acetogenic reactions are not thermodynamically favourable under standard conditions, with a $\Delta G^{0'}$ value of +48.3 kJ mole^{-1} for syntrophic butyrate oxidation (eq. 1.1) and +76.0 kJ mole^{-1} for syntrophic propionate oxidation (eq. 1.2) (Thauer et al., 1977; Schink, 1997; Kato & Watanabe, 2010; Muller et al., 2010; Schink & Stams, 2013):

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad (1.1)$$

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2 \quad (1.2)$$

These SAB therefore require the partnership of H_2-scavenging micro-organisms to maintain the metabolic activity that they could not achieve on their own, but find in the hydrogenotrophic methanogens. Especially for syntrophic propionate oxidation, a reduction of the H_2 partial pressure to a value < 10^{-4} atm is required to make the reaction energetically favourable (Schink, 1997; McInerney et al., 2008; Kato & Watanabe, 2010). Several groups of micro-organisms are involved in the syntrophic oxidation of propionate and butyrate. Most of these belong to the Syntrophobacterales order and the Peptococcaceae family (propionate) and Syntrophomonadaceae family (butyrate) (McInerney et al., 2008; Muller et al., 2010; Schink & Stams, 2013).

Syntrophic acetate oxidizing bacteria are able to oxidize acetate to H_2 and CO_2 (eq. 1.3), yet this reaction is, like syntrophic butyrate and propionate oxidation, thermodynamically highly unfavourable under standard conditions, with a $\Delta G^{0'}$ value of +104.6 kJ mole^{-1} (Thauer et al., 1977; Hattori, 2008; Schink & Stams, 2013).

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \quad (1.3)$$

As a result, syntrophic acetate oxidation can only take place at low values of H_2 partial pressure, i.e. between 2.6 and 74 Pa (Hattori, 2008). Only in this narrow interval of hydrogen gas concentration is the syntrophic acetate oxidation and the subsequent hydrogenotrophic methanogenesis (SAO-HM) coupling thermodynamically possible (Hattori, 2008). Hence, these acetate oxidizing bacteria also require H_2 scavenging partner organisms, which they find in hydrogenotrophic methanogens (Hattori, 2008; Nettmann et al., 2010). The SAO mainly belong to the bacterial orders Thermoanaerobacterales, Clostridiales and Thermotogales (Schnurer et al., 1996; Schnurer et al., 1997; Hattori et al., 2000; Balk et al., 2002; Hattori et al., 2005; Westerholm et al., 2010; Westerholm et al., 2011b).

Sulphate reducing bacteria are a last group of micro-organisms involved in the acetogenesis process. In general, SRB are able to use H_2, acetate, propionate and butyrate, as electron donor and sulphate as electron acceptor, therefore enabling them to directly influence acetogenesis (Barton, 1995; Huang et al., 2012; Zhang et al., 2013). From a thermodynamical point of view propionate is however preferred above acetate or other organic compounds as electron donor for sulphate reduction, with the exception of H_2, making SRB part of the acetogenic microbial community (Barton, 1995; Liamleam & Annachhatre, 2007; Chen et al., 2008; Zhang et al., 2013).

3.1.4. Methanogenesis

In the final step of the AD process methane is produced by means of two different pathways, i.e. acetoclastic methanogenesis, which involves the direct cleavage of acetate to methane and CO_2 (eq. 1.4), and hydrogenotrophic methanogenesis, during which CO_2 is reduced to methane by means of H_2 (eq. 1.5).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1.4)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (1.5)$$

Methanogenesis is carried out exclusively by archaea that belong to 5 different orders, i.e. the hydrogenotrophic orders Methanobacteriales, Methanomicrobiales, Methanococcales and Methanopyrales, and the acetoclastic order Methanosarcinales (Gerardi, 2003c; Liu & Whitman, 2008). The Methanococcales and Methanopyrales orders are usually absent in AD, because of their preference for extreme environments (Ollivier et al., 1998; Cavalier-Smith, 2002; Huber et al., 2002; Nercessian et al., 2003). The Methanosarcinales order contains the

strict acetoclastic Methanosaetaceae family and the mixotrophic Methanosarcinaceae family, of which the members are able to perform both acetoclastic and hydrogenotrophic methanogenesis, and are even able to use formate, methanol, methylamines, methylsulphide, dimethylsulphide and CO as substrate for methane production (Ferguson et al., 1996; Ferry, 1999; Rother & Metcalf, 2004; Liu & Whitman, 2008; Bizukojc et al., 2010; Ferry, 2011; Kumar et al., 2011).

However, one of the major drawbacks of AD is the sensitivity of the methanogenic consortium to different environmental factors. An abrupt change in pH, an increase in salt or organic matter concentration, an alteration of the organic loading rate (OLR) or the introduction of a toxic compound often causes system failure (Chen et al., 2008; Ma et al., 2009; Wijekoon et al., 2011). Overloading is a frequent problem in AD, since it leads to accumulation of fatty acids, as these are no longer efficiently removed by the methanogens. This is mainly due to their low growth rates, compared to the acidogenic and acetogenic bacteria, which causes the uncoupling of the acetogenic bacteria and the methanogens (Gujer & Zehnder, 1983). A well-balanced equilibrium between the different trophic levels is therefore of crucial importance to ensure a stable AD process with high methane production.

3.2. Operational parameters

Operational control in AD is crucial to maintain high methane production levels. Several parameters, of which sludge retention time (SRT), temperature, pH and the availability of growth factors are most important, can be monitored and, if necessary, adjusted during operation.

3.2.1. Sludge retention time (SRT)

The SRT can be considered an estimation of the average time that the biomass, which includes both substrate and micro-organisms involved in the AD process, remains in the digester. A SRT value of at least 10 to 12 days is recommended, yet, in most full-scale continuous stirred tank (CSTR) installations the SRT usually varies between 18 and 150 days, or even higher, depending on the reactor conditions**,** reactor temperature (mesophilic or thermophilic) and substrate composition (Gossett & Belser, 1982; Gerardi, 2003a; Appels et al., 2008).

Maintaining a sufficient high SRT value is necessary for two reasons. First, hydrolysis is considered the rate-limiting step, hence sufficient time is required for hydrolysis to take place. However, this mainly depends on the substrate composition itself (Miron et al., 2000; Batstone et al., 2009). Second, the SRT should be higher than the doubling time of the microbial component with the lowest growth rate. Methanogenic archaea in general have low growth rates, compared to the majority of the bacterial community (Gujer & Zehnder, 1983; Zhang & Noike, 1994; Gerardi, 2003a). Indeed, doubling times in the order of 4 to 6 days were deducted for methanogens (Gujer & Zehnder, 1983; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b). Although these value lie far below the minimum SRT value of 10 to 12 days, they are valid for optimal growth conditions, which are rarely present in AD. So, a safety margin of at least a factor 2 to 3 needs to be applied to avoid wash-out of the methanogens and subsequent process failure (Appels et al., 2008; Nges & Liu, 2010).

3.2.2. Temperature

Anaerobic digestion takes place at a wide temperature range, varying from psychrophilic digestion at 15°C to hyperthermophilic digestion at 70°C. Generally, AD takes place at mesophilic conditions, i.e. in the range of 30 to 40°C with an optimum of 35 to 37°C, or thermophilic conditions in the range of 50 to 60°C, with an optimum of 54°C (Van Lier, 1995; Gerardi, 2003f). In full-scale installations mainly mesophilic conditions are applied, yet both mesophilic and thermophilic digestion have their advantages and disadvantages.

Thermophilic AD results in higher microbial growth rates, degradation rates and conversion efficiencies, hence, lower SRT values can be applied, and higher biogas production rates are obtained, compared to mesophilic conditions (Veeken & Hamelers, 1999; Leven et al., 2007). However, this is not always the case, as thermophilic AD often shows process instability, which is reflected in higher residual VFA concentrations, compared to mesophilic digestion (Labatut et al., 2014).Thermophilic digestion also leads to a higher reduction of pathogens, such as *Salmonella* sp., *E. coli* and *Enterococcus* sp., which are present in the digestate (Leven et al., 2007; Kjerstadius et al., 2013). Mesophilic digestion requires less energy input for heating purposes, and is also less susceptible to failure, due to for example a lower free ammonia (FA) concentration during degradation of N-rich waste streams at mesophilic conditions compared to thermophilic conditions (Gallert & Winter, 1997; Leven et al., 2007; Labatut et al., 2014).

Apart from the different temperature ranges at which AD can take place, the tolerance of the microbial community to abrupt changes in temperature can be important in view of the overall stability of the AD process. The presence of heat shock genes and their products (heat shock proteins) in eukaryotic, bacterial and archaeal cells is often an indication of their tolerance against heat shocks, but also other forms of stress, e.g. high ammonium and salt concentrations (Macario et al., 1999; Zhang et al., 2006). In general, fluctuations in temperature should remain as low as possible, i.e. < 1°C per day for thermophilic AD and maximum 2-3°C per day for mesophilic AD (Gerardi, 2003f).

3.2.3. pH

The optimal pH level of AD lies between 6.8 and 7.5, which corresponds to the optimum range of most methanogens (Gujer & Zehnder, 1983; Appels et al., 2008). Indeed, most methanogens only perform well within this narrow pH range, although variation between 6.5 and 8.0 is possible, which is in contrast to the hydrolytic and acidogenic bacteria that only require a pH above 4.5 to 5.0 (Gerardi, 2003e).

A decrease in pH below the optimal range is often caused by overloading the AD reactor. An increase of the loading rate may lead to the accumulation of VFA, eliciting toxic effects and causing the pH to decrease to suboptimal conditions, which can cause a decrease in methanogenic activity (Gujer & Zehnder, 1983; Appels et al., 2008; Chen et al., 2008; Ma et al., 2009). Most methanogens are sensitive to a drop in pH with more than 0.5 units and/or an accumulation of fatty acids, especially acetate and propionate, to concentrations exceeding 3000 mg COD L^{-1} (Liu et al., 1985; Baloch et al., 2007). Therefore, increasing the organic loading rate (OLR) in AD should always be performed with great care and consideration. Autoregulation of the pH in AD can, however, take place due to two buffer systems, i.e. the (bi-)carbonate buffer (eq. 1.6) and the ammonia buffer (eq. 1.7), as both CO_2 and NH_3 are produced during the AD process (Gerardi, 2003e).

$$CO_2 + H_2O \leftrightarrow H_2CO_3^- \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^- \quad (1.6)$$

$$NH_3 + H^+ \leftrightarrow NH_4^+ \quad (1.7)$$

Supplementation of additional buffers to the digester, such as phosphate buffers, may be required in case of high residual VFA concentrations, due to high organic loading rates, or during feeding with substrates rich in carbohydrates, which have a low potential buffer capacity (Gerardi, 2003e).

3.2.4. Essential growth factors

Essential growth factors can be divided into macronutrients, i.e. C, N, P, K, Na, Ca, Mg and S, and micronutrients or trace elements, of which B, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se and W are the most important (Feng et al., 2010; Pobeheim et al., 2010; Demirel & Scherer, 2011; Schattauer et al., 2011). A wide optimal range of concentrations of these different growth factors have been reported in literature, and can also be found in full-scale digesters, sometimes spanning several orders of magnitude (Schattauer et al., 2011). These elements all play a crucial role in at least one of the metabolic pathways in AD, hence their presence, but especially their bioavailability, is crucial to maintain methane production. Nutrient limitation, due to mono-digestion of nutrient-poor substrates, or limitations in bioavailability, caused by precipitation or complexation, should be avoided (Gonzalez-Gil et al., 2003; Zandvoort et al., 2006; Aquino & Stuckey, 2007; Lebuhn et al., 2008; Vintiloiu et al., 2012; Hutnan et al., 2013).

The direct addition of trace elements to the digester may overcome these limitation, but also proliferates additional operational costs, especially since in some cases high amounts are required to reach acceptable bioavailability levels (Zandvoort et al., 2006). An inexpensive solution to nutrient limitation is co-digestion with nutrient-rich substrates. Co-digestion of manure or sewage sludge with other nutrient-poor substrates, such as energy crops and glycerol waste, increases nutrient availability in the digester, due to their high nutrient content (Park et al., 2006; Fang et al., 2011a; Razaviarani et al., 2013). Nevertheless, a well-informed decision on the co-substrate and blend ratio in terms of nutrient supplementation is important to ensure positive synergistic effects (Mata-Alvarez et al., 2011; Borowski & Weatherley, 2013).

3.3. Limiting and inhibiting factors

Anaerobic digestion is susceptible to different forms of disruption, because of its delicate balance between the microbial groups in the different stages, of which the methanogens are most vulnerable (Gujer & Zehnder, 1983; Sawayama et al., 2004; Chen et al., 2008). In view of this vulnerability, the four most common forms of stress in AD, i.e. organic overloading, ammonia toxicity, sulphate and sulphide inhibition, and high salt concentrations are discussed.

3.3.1. Organic overloading and increasing VFA

Anaerobic digesters are vulnerable to overloading, which can disrupt their operational stability (Gujer & Zehnder, 1983). A too fast increase of the loading rate leads to the accumulation of VFA, eliciting toxic effects and causing the pH to decrease to suboptimal conditions, subsequently causing a decrease in methanogenic activity (Gujer & Zehnder, 1983; Appels et al., 2008; Chen et al., 2008; Ma et al., 2009).

Organic overloading initially results in the accumulation of acetate and H_2, which cannot be converted to CH_4 fast enough by the slow-growing methanogens, and is thus followed by uncoupling of acetogenesis and methanogenesis (Gujer & Zehnder, 1983; Shin et al., 2011). Due to the accumulation of acetate and H_2, the syntrophic propionate and butyrate oxidation becomes thermodynamically unfavourable, which then in turn also accumulate, leading to a further decrease in pH, inhibition of methanogenesis and subsequent failure of the entire AD process (Thauer et al., 1977; Schink, 1997; Kato & Watanabe, 2010; Muller et al., 2010; Schink & Stams, 2013). Fast recovery after failure or even prevention of failure, by means of early-warning systems, is crucial from an economical point of view, and stresses the importance of a careful monitoring strategy of the AD process. A drastic decrease in OLR is most often the best treatment, yet unfavourable from a practical and economical point of view (Gallert & Winter, 2008; Retfalvi et al., 2011). Hence, several other monitoring and restoring methods have been developed in recent years.

Conventional monitoring of AD takes place by means of, if possible, on-line measurement of the classical parameters in AD, such as biogas production and composition, pH, VFA and buffer capacity or alkalinity (Bjornsson et al., 2000; Boe et al., 2010). Several on-line measurement technologies have been developed, such as headspace chromatography based VFA sensors, near infrared monitoring of VFA, dissolved H_2 concentration monitoring by means of a hydrogen-sensitive palladium–metal oxide semiconductor sensor in combination with a membrane for liquid-to-gas transfer, and even an electronic nose to detect overloading (Bjornsson et al., 2001; Holm-Nielsen et al., 2008; Holm-Nielsen & Esbensen, 2011; Boe & Angelidaki, 2012; Adam et al., 2013). Moreover, alternative early-warning indicators also have been applied, such as the VFA:Ca ratio (Kleybocker et al., 2012). The implementation of stabilization or remediation methods, such as the addition of CaO, creation of micro-aerobic conditions (stimulation of hydrolysis, acidogenesis and acetogenesis), stimulation of SRB or the introduction of composite ion exchangers has been successful as well (Mitra et al., 1998; Liamleam & Annachhatre, 2007; Kleyböcker et al., 2012; Ramos & Fdz-Polanco, 2013).

Clearly, the key issue to obtain stable AD with high CH_4 production values is finding an optimal balance between a high OLR and optimal conditions for the methanogenic community (Shin et al., 2011).

3.3.2. Ammonia and ammonium

During the AD process, the degradation of nitrogenous organic matter, mostly proteins, amino acids and urea causes the release of ammonia into the aqueous solution (Krylova et al., 1997; Sawayama et al., 2004; Chen et al., 2008). Total ammonia nitrogen (TAN) can be present in both the ammonium ion ($\mathbf{NH_4^+}$) and the free ammonia (NH_3) form (eq. 1.7) (Calli et al., 2005a; Chen et al., 2008; Schnurer & Nordberg, 2008). The exact ratio mainly depends on the pH and temperature in the reactor, with the free ammonia (FA) form being the most inhibiting to the microbial community. The amount of free ammonia released increases with rising pH and temperature, for the same level of TAN, according to eq. 1.8 (Anthonisen et al., 1976; Schnurer & Nordberg, 2008; El Hadj et al., 2009). Consequently, free ammonia (mg N L^{-1}) can be calculated based on the pH, temperature (T, °C) and TAN concentration:

$$NH_3 = \frac{TAN \cdot 10^{pH}}{e^{\frac{6344}{273+T}} + 10^{pH}} \quad (1.8)$$

In the majority of anaerobic digesters, the methanogens are most susceptible to high levels of TAN, exceeding 3000 to 4000 mg N L^{-1} (Krylova et al., 1997; Chen et al., 2008; Schnurer & Nordberg, 2008). Several strategies have been applied to avoid ammonia toxicity in AD. First, slow adaptation of the AD process to increasing TAN concentrations improves ammonia tolerance and has led to tolerance to TAN concentrations up to 7000 mg N L^{-1}, high above the TAN levels normally eliciting inhibition in AD (Hashimoto, 1986; Hansen et al., 1998; Calli et al., 2005b; Schnurer & Nordberg, 2008). However, operation at high TAN concentrations, and high free ammonia concentrations, depending on the reactor temperature and pH, requires the application of high SRT values, in the order of 30-60 days, and long adaptation periods to avoid washout of the methanogenic community (Schnurer & Nordberg, 2008; Garcia & Angenent, 2009). A second strategy involves direct removal of ammonia in AD by means of stripping or electrochemical recovery, thus avoiding toxic effects (Walker et al., 2011; Desloover et al., 2012; Zhang et al., 2012b; Serna-Maza et al., 2014). A third and last strategy is co-digestion of nitrogen-rich substrates, such as manure and slaughterhouse waste, with substrates with low nitrogen content, such as WAS (Borowski & Weatherley, 2013; Pitk et al., 2013).

3.3.3. Sulphate and sulphide

Anaerobic digestion of sulphate-rich waste streams results in the formation of sulphides, due to the activity of sulphate reducing bacteria (SRB). These SRB can reduce sulphate under anaerobic conditions while using H_2 and organic compounds, such as acetate, propionate and butyrate, as electron donors (Barton, 1995; Huang et al., 2012; Zhang et al., 2013). Overall methane production can, however, be (partially) inhibited by the activity of SRB, a process which can take place on three different levels.

Primary inhibition is caused by the competition for common substrates between acetogenic bacteria and methanogenic archaea (Karhadkar et al., 1987; Harada et al., 1994; Chen et al., 2008). Indeed, SRB are able to use both H_2 and acetate, the main precursors for CH_4 production, as electron acceptor (Koster et al., 1986; Hulshoff Pol et al., 1998; Chen et al., 2008). However, from a thermodynamical point of view propionate is preferred above acetate as electron donor for sulphate reduction, hence, both methanogenesis and acetogenesis are in competition with sulphate reduction (Barton, 1995; Liamleam & Annachhatre, 2007; Chen et al., 2008; Zhang et al., 2013).

Secondary inhibition is mainly caused by the toxicity of sulphide to methanogens that are most sensitive to elevated sulphide concentrations, in relation to the acidogenic and acetogenic bacteria (Hulshoff Pol et al., 1998; Chen et al., 2008). In general, the toxicity of sulphide is attributed to the free sulphide (H_2S) content, because of its ability to permeate freely through the cell membrane (Hulshoff Pol et al., 1998; Chen et al., 2008). The inhibiting effect of H_2S appears to be lower for granular sludge in comparison to suspended sludge at low and neutral pH (7.0-7.2), whereas similar inhibition levels are reported at higher pH values (7.8-8.0) (Visser et al., 1996; Hulshoff Pol et al., 1998). The higher tolerance of granular sludge to H_2S can be attributed to the pH gradient present in anaerobic granules (Koster et al., 1986; Lens et al., 1998). Sulphide inhibition of methanogens therefore depends on the characteristics of the sludge. In suspended sludge, inhibition levels are determined by the free sulphide concentration, whereas in granular sludge total sulphide ($TS = H_2S + HS^- + S^{2-}$) concentration determines the level of toxicity (Visser et al., 1996; Hulshoff Pol et al., 1998; Lens et al., 1998). Notwithstanding, literature reports a wide range of H_2S and TS concentrations causing 50% inhibition of methanogenesis, with values ranging between 20 and 1000 mg S L^{-1} for H_2S and 80-1250 mg S L^{-1} for TS (Isa et al., 1986b; Koster et al., 1986; Karhadkar et al., 1987; Visser et al., 1996; Hulshoff Pol et al., 1998; Lens et al., 1998).

A third and final form of inhibition of the AD process lies in the potential precipitation of trace elements, such as Fe, Co and Ni, due to the presence of sulphides, which subsequently results in a reduced bioavailability (Isa et al., 1986a; Gonzalez-Gil et al., 2003; Patidar & Tare, 2004; Zandvoort et al., 2006; Aquino & Stuckey, 2007; Jansen et al., 2007).

Generally, from an operational point of view, the degree to which biogas production is affected by these three inhibition processes, strongly depends on the COD/SO_4-S ratio (Gimenez et al., 2011). A COD/SO_4-S ratio > 10 will favour methanogenesis as the main process, while a COD/SO_4-S ratio < 1 will strongly emphasize sulphate reduction as the dominant process, with both processes taking place at COD/SO_4-S values between 1 and 10 (Isa et al., 1986b; Choi & Rim, 1991; Hulshoff Pol et al., 1998; O'Flaherty et al., 1998).

3.3.4. Salt

High salt levels in AD cause the bacterial and archaeal community to perish, because the elevated osmotic pressure may dehydrate the cell (Chen et al., 2008; Oh et al., 2008; Fang et al., 2011b). Especially AD of waste from the food processing industry encounters high salt concentrations in anaerobic digestion, i.e. concentrations of 4000 to 8000 mg Na^+ L^{-1} for pure food waste were measured (Feijoo et al., 1995; Omil et al., 1995; Omil et al., 1996; Chen et al., 2008; Oh et al., 2008). Salt stress can be attributed mostly to cations, of which sodium, calcium, potassium and magnesium are the most important (Omil et al., 1996; Appels et al., 2008; Chen et al., 2008). Methanogens are, like other archaea, negatively affected by high salt concentrations, whereas low concentrations are beneficial for growth, with reported values of 350 mg Na^+ L^{-1} to be optimal for methanogens, values between 3500 and 5500 mg Na^+ L^{-1} causing moderate, and values over 8000 mg Na^+ L^{-1} leading to severe impairment (Omil et al., 1996; Appels et al., 2008; Chen et al., 2008). Similar inhibiting concentrations are reported for potassium, with values of 400 mg K^+ L^{-1} being optimal for methanogens, and a concentration of 5.85 g K^+ L^{-1} causing 50% inhibition (Chen et al., 2008). However, a high range of both Na and K concentrations causing inhibition in AD are reported in literature, which depends on several factors, such as pH, temperature, TAN concentration, and the presence of other cations (Feijoo et al., 1995; Appels et al., 2008; Chen et al., 2008).

Similar to high TAN concentrations, the adaptation of the methanogenic community to high salt concentrations, by slowly increasing the salt content in the feed, is also possible, which results in a tolerance to salt shocks of up to 25 g Na^+ L^{-1} (de Baere et al., 1984; Lefebvre et

al., 2007). The adaptation of micro-organisms to high salt concentration mainly relies on their ability to produce or take up compatible solutes, e.g. osmoprotectants, to counteract the osmotic stress (Roessler & Muller, 2001; Empadinhas & da Costa, 2008). Indeed, the addition of osmoprotectants, such as glycine betaine, in AD can lead to a 2-fold increase in methane production at salt concentrations up to 14 g Na^+ L^{-1} (Oh et al., 2008). The adaptation to high salt concentrations in AD also can be increased by the introduction of an electric field, leading to COD removal efficiencies up to 93% at a salt concentration of 20 g Na^+ L^{-1} (Zhang et al., 2012a). A final strategy to decrease salt toxicity in AD involves the (in)direct extraction of salts from the digestate by means of an electrochemical cell (Desloover et al., 2012; Zhao et al., 2013).

3.4. Anaerobic digestion reactor technologies

Several AD reactor technologies that have been developed are established on full-scale level. The three most common reactor configurations, i.e. the continuous stirred tank reactor (CSTR), the upflow anaerobic sludge blanket (UASB) reactor and the anaerobic membrane bioreactor (AnMBR) are discussed.

3.4.1. Continuous stirred tank reactor (CSTR)

The CSTR configuration is the most basic and most common AD reactor system applied at full scale. In this system, the organic waste streams to be treated are directly and homogenously mixed with the digestate upon feeding. Hence, the retention time of the anaerobic biomass (SRT) and hydraulic retention time (HRT) are equal. The active anaerobic biomass in the digestate is kept in suspension by means of mechanical mixing, sludge recirculation or biogas recirculation (Karim et al., 2005; Kaparaju et al., 2008). In general, the applied mixing method is of little importance, with the exception of AD of waste streams with high solid content > 15%, in which biogas recirculation proves to be insufficient (Karim et al., 2005). However, it is clear that a certain degree of mixing is necessary to ensure optimal contact between the active anaerobic biomass and the substrate to be digested, thus increasing biogas production (Karim et al., 2005; Kaparaju et al., 2008).

In general, the CSTR configuration is used for waste streams that are rich in solids, such as waste activated sludge, manure, OFMSW and energy crops (Sundberg et al., 2013). The OLR in these systems is usually rather low, in comparison with other reactor configurations, with

values between 2.5 and 5.0 kg COD m^{-3} d^{-1}, although in some cases OLR values up to 10.0 kg COD m^{-3} d^{-1} are applied (Pycke et al., 2011; Sundberg et al., 2013).

3.4.2. Upflow anaerobic sludge blanket (UASB)

The development of the UASB technology dates back to the late seventies, and was initially designed for anaerobic treatment of liquid waste streams with high COD concentration (starting from 1.0 and even up to 200 g COD L^{-1}) and low solids content (Lettinga et al., 1980; Rajeshwari et al., 2000). Nowadays, a wide range of wastewater types, such as brewery, potato factory, paper mill, sugar based and dairy industry based wastewaters can be treated at full scale by means of this technology, allowing both wastewater treatment and energy recuperation (Lettinga, 1995; Leclerc et al., 2004; Pycke et al., 2011; Werner et al., 2011).

The key feature of this reactor technology is the presence of a sludge blanket at the bottom of the reactor that consists of microbial granules, in which the conversion of the organic waste to biogas takes place (Lettinga et al., 1980). The reactor is operated in upflow mode, typically at an upflow velocity of 1m h^{-1}. The combination of this upflow velocity and shear stress, due to biogas production, forces the anaerobic micro-organisms to form dense granules with good settling properties, to avoid wash-out (Hulshoff Pol et al., 2004; Wu et al., 2012). To obtain an efficient separation of the sludge, liquid and biogas phases a 3-phase separator device, which allows the biogas to separate from the liquid, and granules that were dragged upwards by rising biogas bubbles to settle again, is installed in the reactor (Figure 1.2) (Chong et al., 2012; Wu et al., 2012).

The phase separator divides the reactor into two zones, i.e. the settling zone, in which the sludge that was dragged up by the biogas settles, and the sludge zone, containing the concentrated sludge bed (Aiyuk et al., 2006). Due to the active retention of the sludge granules in the reactor, by means of the 3-phase separator, uncoupling of the HRT and SRT is possible, which allows operation at high SRT (approximately infinite) values while treating high wastewater flow rates at low HRT, as low as 2 hours (Lettinga & Hulshoff Pol, 1991; Van Lier et al., 2001; Leitao et al., 2005; Aiyuk et al., 2006). The combination of the presence of the 3-phase separator and the good settling properties of the sludge allows operation at a high sludge concentration (up to 40 kg VSS m^{-3} (volatile suspended solids)) and corresponding OLR (up to 15-20 kg COD m^{-3} d^{-1}) (Lettinga et al., 1980; Lettinga & Hulshoff Pol, 1991; Hulshoff Pol et al., 2004; Aiyuk et al., 2006).

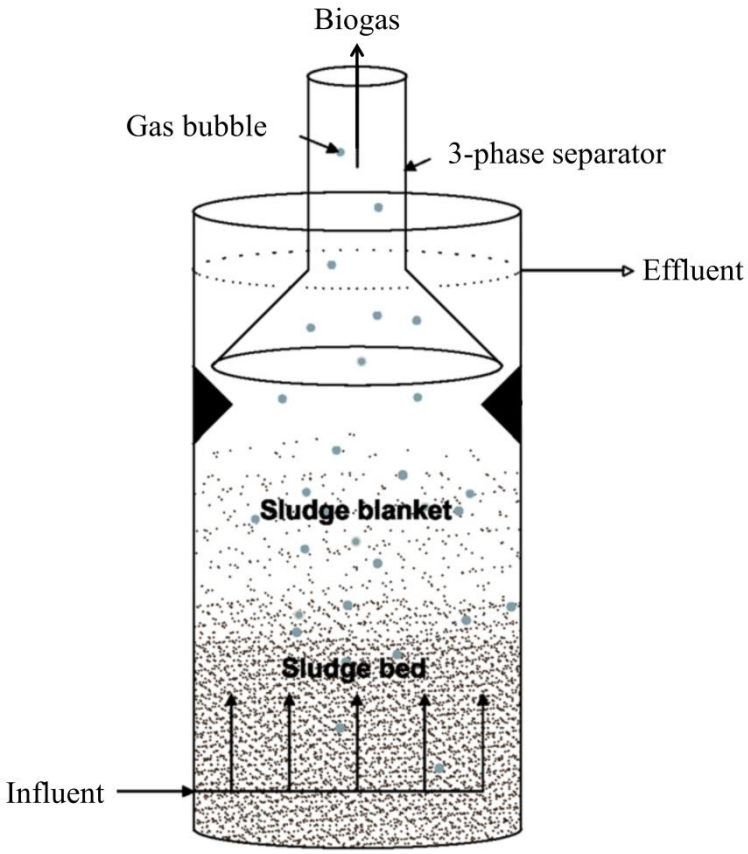

Figure 1.2 *Schematic representation of an UASB reactor, adapted from Chong et al. (2012).*

Efficient granulation and preservation of these granules is a crucial process in UASB reactors, to maintain efficient COD removal and biogas production. The granulation process can be divided in 2 subsequent phases. First, the formation of a precursor or nucleus takes place, followed by the actual growth of the granule, starting from the nucleus (Lettinga, 1988; Chen & Lun, 1993; Hulshoff Pol et al., 2004).

The first step is considered the most important one. *Methanosaeta* sp. play a crucial role, as they form small aggregates, induced by turbulence, due to their filamentous shape (Lettinga, 1988; Quarmby & Forster, 1995; Hulshoff Pol et al., 2004; Zheng et al., 2006). *Methanosarcina* sp. can however also attribute to nucleus formation, due to their ability to grow in clumps and to produce extracellular polymeric substances (EPS), onto which other micro-organisms, such as *Methanosaeta* sp., can attach (Chen & Lun, 1993; Hulshoff Pol et al., 2004). In the second phase the entrapment of other microbial species, e.g. bacteria that grow in syntrophy with methanogens, allows the formation of actual granules that, due to the shear forces, acquire a spherical shape (Lettinga, 1988; Chen & Lun, 1993; Hulshoff Pol et al., 2004). Several anaerobic granulation models have been developed, yet in general it is assumed that cell-to-cell communication or quorum sensing is most effective in developing

anaerobic granules, and organizing the structure of the granule-associated micro-organisms (Liu et al., 2003; Feng et al., 2014). This cell-to-cell mechanism supposedly allows the formation of a layered structure, often observed in anaerobic granules, with (acetoclastic) methanogens in the centre, (syntrophic) acetogenic bacteria in the middle and fermenting/hydrolysing bacteria in the outer layers (Figure 1.3) (Macleod et al., 1990; Liu et al., 2002; Hulshoff Pol et al., 2004; Abreu et al., 2007; Satoh et al., 2007). The structural organization of micro-organisms in these granules, hence, not only allows efficient electron and metabolite transfer between the different trophic levels, it also protects the vulnerable methanogens against different forms of stress, such as high VFA and sulphide concentrations, due to the presence of a pH gradient in these granules (Koster et al., 1986; Lens et al., 1998; Liu et al., 2002; Satoh et al., 2007).

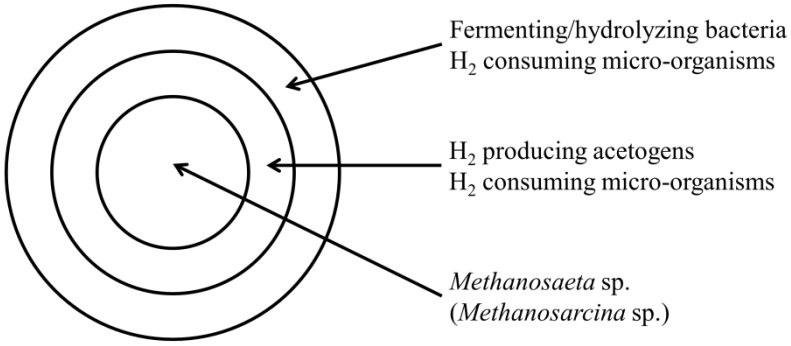

Figure 1.3 *Structural organization of anaerobic granules, adapted from Macleod et al. (1990) & Hulshoff Pol et al. (2004).*

3.4.3. Anaerobic membrane bioreactor (AnMBR)

Since the beginning of the nineties membrane systems have been successfully integrated in the aerobic wastewater treatment system for the treatment of both domestic and industrial wastewater (Liao et al., 2006b; Judd, 2008). Membrane systems have several advantages over conventional systems, such as improved effluent quality, low surface area requirements, operation at high biomass concentration, which allows high OLR values, complete biomass retention and low sludge production (Liao et al., 2006b; Judd, 2008; Meng et al., 2012; Lin et al., 2013). In recent years, increased research has been conducted to develop anaerobic membrane systems. However, full-scale applications remain limited, in contrast to CSTR and UASB reactor configurations, with the first full-scale application in the year 2000, yet increasing pilot and full-scale AnMBR applications are currently being installed all over the

world (Christian et al., 2010; Meng et al., 2012; Skouteris et al., 2012; Smith et al., 2012; Lin et al., 2013).

Two different AnMBR configurations can be distinguished, i.e. a side-stream system that consist of an external cross-flow membrane unit, and an internal submerged system (Figure 1.4). In the side-stream system the membrane is separated from the bioreactor and a pumping system is required to push the permeate (effluent) through the membrane. The cross-flow velocity of the permeate across the membrane is the main mechanism to avoid cake layer formation (Liao et al., 2006b; Smith et al., 2012). In the submerged configuration, the membrane can be immersed directly in the reactor or placed in an external chamber. In both systems a vacuum is applied to pull the effluent or permeate through the membrane, which is in contrast to the side-stream system. The application of a vacuum, in contrast to a pumping system, does not allow direct liquid velocity control, hence cake layer formation needs to be avoided by means of biogas scouring across the membrane surface (Cui & Wright, 1996; Liao et al., 2006b; Smith et al., 2012).

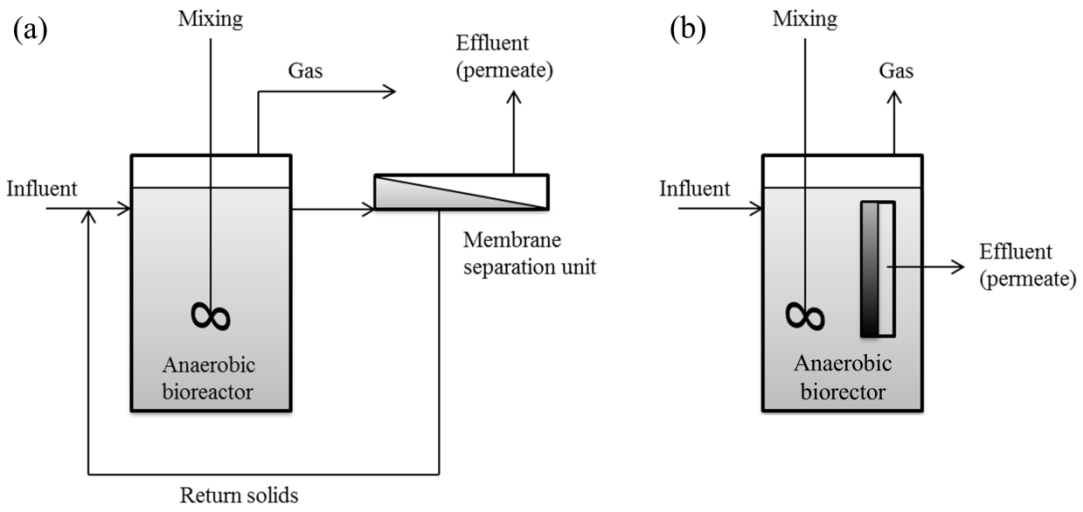

Figure 1.4 *Schematic representation of (a) a side-stream and (b) a submerged AnMBR system.*

In general, the side-stream configuration allows better fouling control, due to the direct steering of the cross-flow velocity, easier replacement of the membrane units and higher fluxes. However, the main disadvantages lies in the high energy consumption, up to 10 kWh m^{-3} effluent, when high fluxes are required (Le-Clech et al., 2006; Judd, 2008; Lin et al., 2013). Submerged systems generally require lower energy consumption and less intensive cleaning, yet the membrane units are more difficult to replace and slightly lower fluxes can be applied (Le-Clech et al., 2006; Meng et al., 2012; Lin et al., 2013).

The main potential of AnMBR systems lies in their ability to combine the advantages of UASB and CSTR systems. Indeed, AnMBR systems have several advantages over conventional anaerobic treatment technologies, such as high effluent quality, low surface requirements, total biomass retention, low start-up time, low sludge production, high SRT values, possibility of treatment of wastewater with high solids content, high biomass concentrations (up to 40 kg VSS m^{-3}), high flow rates, and high OLR (up to 25 kg COD m^{-3} d^{-1}) (Jeison & van Lier, 2008; Van Zyl et al., 2008; Skouteris et al., 2012; Stuckey, 2012; Zamalloa et al., 2012; Lin et al., 2013). The permeate or effluent flux through the membrane depends on several parameters, such as the membrane type, feed composition, biomass concentration, and the degree of biogas scouring (Field et al., 1995). To maintain a stable flux in the membrane reactor, the critical flux should be determined for each specific case (Field et al., 1995).

Apart from their advantages over other anaerobic technologies, AnMBR systems have two main disadvantages, i.e. the high initial cost of the membrane and fouling of the membrane during operation, although recent developments led to a decrease in cost and increased fouling resistance of the membranes (Choo et al., 2000; Le-Clech et al., 2006; Judd, 2008; Charfi et al., 2012; Lin et al., 2013).

4. *Methanosaeta* vs. *Methanosarcina* in anaerobic digestion

Acetoclastic methanogenesis is one of the two main pathways that allow the production of methane in AD. At present, only two methanogenic genera, i.e. *Methanosaeta* and *Methanosarcina*, able to perform acetoclastic methanogenesis, have been reported, as the former genus of *Methanothrix* was rejected and incorporated in the *Methanosaeta* genus (Boone, 1991; Gerardi, 2003c; Conklin et al., 2006; Tindall et al., 2008). The *Methanosaeta* and *Methanosarcina* genera, however, greatly differ in terms of morphology, physiology and metabolic potential. Hence, the specific role of these acetoclastic methanogens in AD is extensively discussed.

4.1. *Methanosarcina*: the robust methanogen

Methanosarcinaceae are metabolically and physiologically the most versatile methanogens, which can be related to the fact that they have, by far, the largest known archaeal genome (Galagan et al., 2002; Maeder et al., 2006).

In contrast to other methanogens that only possess a single pathway, *Methanosarcina* sp. are able to produce methane by means of four different pathways, i.e. CO_2 reduction with H_2, methyl reduction by means of H_2, acetoclastic cleavage of acetate to CO_2 and CH_4 and methylotrophic catabolism of C-1 compounds (Deppenmeier et al., 2002; Galagan et al., 2002; Welander & Metcalf, 2005; Maeder et al., 2006). This allows them to use a wide range of substrates for direct methane production, such as acetate, CO_2, formate, methanol, methylamines, methylsulphide, dimethylsulphide and CO (Ferguson et al., 1996; Ferry, 1999; Rother & Metcalf, 2004; Liu & Whitman, 2008; Bizukojc et al., 2010; Ferry, 2011; Kumar et al., 2011). Moreover, *Methanosarcina barkeri* has been shown to be able to use acetate not only as substrate for methane production, but also as substrate for syntrophic acetate oxidation in co-culture with the sulphate reducing *Desulfovibrio vulgaris* (Phelps et al., 1985). However, the ability of *Methanosarcina* to perform syntrophic acetate oxidation warrants further research, as this has not been demonstrated in other studies.

Hydrogen gas plays an important role in the production of methane by *Methanosarcina* sp., as it provides electrons for substrate reduction to methane in two of the four pathways. However, in most cases very little to no hydrogen gas is measured in the gas phase in the anaerobic digester, which may indicate that it is converted to methane immediately or that no hydrogen gas is produced at all (Sasaki et al., 2010b; Liu et al., 2011a; Liu et al., 2011b; Sasaki et al., 2011a). It was already demonstrated in a microbial electrolysis cell that an electric current can be used to reduce CO_2 to methane without the interference of hydrogen gas as electron carrier. This process therefore was called electromethanogenesis (Cheng et al., 2009). However, more in-depth research will be required to validate these results. Another study, using activated carbon to promote direct interspecies electron transfer (DIET), demonstrated that *Geobacter* species can form syntrophic associations with *Methanosarcina* sp. in the absence of H_2, hence via DIET (Lovley, 2011; Liu et al., 2012). However, up till now, DIET between *Geobacter* and *Methanosaeta* or *Methanosarcina* only has been shown with ethanol as substrate, thus, further research will be necessary to confirm whether this process actually takes place in AD. The addition of (semi)conductive iron oxides to enriched cultures of methanogenic communities in another study strongly accelerated methane-associated growth of *Geobacter*,

indicating electric syntrophy via (semi)conductive iron oxides between *Geobacter* and *Methanosarcina*, resulting in accelerated methanogenesis (Kato et al., 2012). Despite the fact that in this research enhanced methanogenesis was demonstrated by adding these minerals, both when using acetate and ethanol as substrate, syntrophic acetate oxidation could have taken place. Hence, these results, again, only could be confirmed when using ethanol as substrate. These findings suggest that DIET may alter the conventional concept of ATP generation, and can be an important mechanism for CO_2 and methyl reduction based methanogenesis by *Methanosarcina* sp. in AD (Morita et al., 2011; Liu et al., 2012). Nonetheless, these results are, up till now, only obtained with ethanol as substrate, therefore, further research will be required. As DIET may lead to a better conservation of energy, the direct transfer of electrons to *Methanosarcina* sp. might be the most efficient, and therefore the preferable way to produce methane for *Methanosarcina* sp. growing in syntrophy with other micro-organisms (Summers et al., 2010; Lovley, 2011; Morita et al., 2011).

Next to their extensive metabolic potentials, Methanosarcinaceae are unique compared to other methanogens in their ability to form complex multicellular structures (Galagan et al., 2002; Maeder et al., 2006). Depending on the growth phase and environmental conditions, *Methanosarcina* sp. can be present in AD as single cells or in multicellular clusters (Figure 1.5) (Macario et al., 1999; Galagan et al., 2002; Calli et al., 2005a; Maeder et al., 2006; Goberna et al., 2010). The formation of these clusters is often related to an adaptation response to stress, and the ability of *Methanosarcina* sp. to colonize different ecological niches (Galagan et al., 2002; Francoleon et al., 2009). The capability of *Methanosarcina* sp. to form clusters can be explained by their unique surface structure. The surface layers (S-layers) of most micro-organisms only contain a few abundant proteins, and are involved in surface recognition and cell adhesion (Mayerhofer et al., 1998; Sleytr & Beveridge, 1999; Francoleon et al., 2009). It was discovered that these S-layers in *Methanosarcina* sp. are associated with a few hundreds of proteins, thus expanding the possibilities for niche colonization and cell adhesion (Francoleon et al., 2009). This may be one of the explanations for the ability of *Methanosarcina* sp. to grow in cell clusters and to adhere onto a wide variation of surfaces.

In general, the growth rates of methanogenic archaea are quite low compared to the other micro-organisms present in AD (Gujer & Zehnder, 1983; Zhang & Noike, 1994; Gerardi, 2003a). Nonetheless, *Methanosarcina* sp. are characterized by a high μ_{max} of 0.60 d^{-1}, doubling times in the order of 1.0 to 1.2 days, and a half-saturation constant (K_s) of 200 to 280 mg COD L^{-1} for acetate**,** compared to other methanogens, especially *Methanosaeta* sp.

that, in general, show lower μ_{max} and K_s values and higher doubling times (Table 1.1) (Gujer & Zehnder, 1983; McMahon et al., 2004; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b; Bialek et al., 2011; Supaphol et al., 2011). The higher K_s value and thus lower affinity of *Methanosarcina* sp. for acetate can be attributed to their spherical form and thus higher volume-to-surface ratio and their growth in cell clusters. This may limit the intake of acetate in the cell (Calli et al., 2005a; Calli et al., 2005b; Conklin et al., 2006; Goberna et al., 2010).

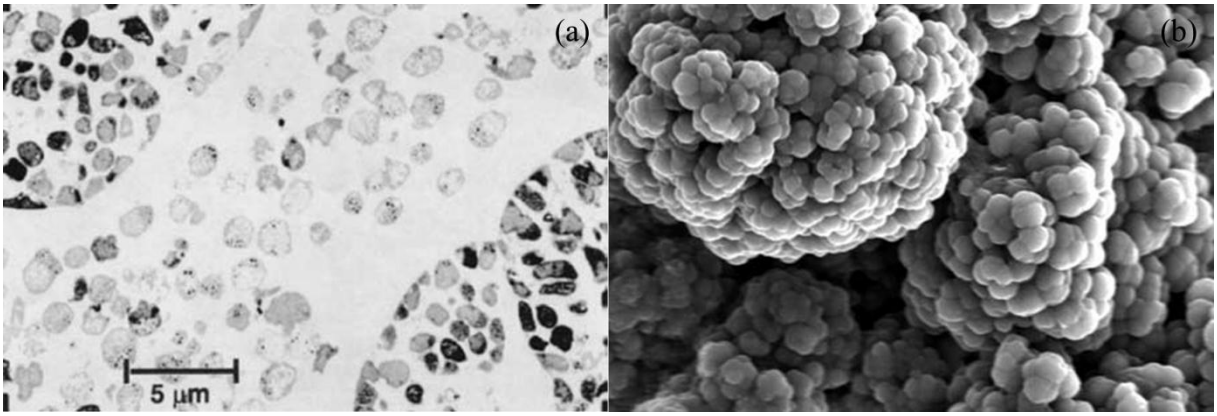

Figure 1.5 *Different morphological forms of Methanosarcina sp. A thin-section electron micrograph picture (a), visualizing both single cells (centre of micrograph) and multicellular clusters (top left, bottom right) in a pure culture medium and a SEM (scanning electron microscopy) image (b) showing the multicellular Methanosarcina cluster in a lab-scale AD reactor (Galagan et al., 2002; Conklin et al., 2006).*

Methanosarcinaceae can be found in a wide range of ecological niches, such as mesophilic and thermophilic digesters, waste activated sludge, garden soils, animal faeces, oil wells, acidic peatland soils, permafrost soils, highly polluted sediments of river estuaries, and even cold and/or deep anoxic sediments, which indicates their ability to survive under circumstances that are considered to be unsuitable for methanogenic growth (Simankova et al., 2001; Galagan et al., 2002; von Klein et al., 2002; Singh et al., 2005; Morozova & Wagner, 2007; Spanheimer & Muller, 2008; Saia et al., 2010; Xing et al., 2010; Steinberg & Regan, 2011). Consequently, *Methanosarcina* sp. are considered to be tolerant against different stressors (Table 1.1) (Calli et al., 2005a; Conklin et al., 2006; Thauer et al., 2008; Shin et al., 2011).

Table 1.1 *Characteristics of Methanosarcina and Methanosaeta*

Parameter	*Methanosaeta*	*Methanosarcina*	Reference
μ_{max} (d^{-1})	0.20	0.60	Gujer & Zehnder, 1983; Jetten et al., 1990; Jetten et al., 1992; Masse & Droste, 2000; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b; Tomei et al., 2009
K_s (mg COD L^{-1})	10 - 50	200 - 280	Gujer & Zehnder, 1983; Jetten et al., 1990; Jetten et al., 1992; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b; Tomei et al., 2009
NH_4^+ (mg N L^{-1})	< 3000	< 7000	Schnurer et al., 1999; Angenent et al., 2002; Calli et al., 2005a; Karakashev et al., 2006; Schnurer & Nordberg, 2008; Nettmann et al., 2010
Na^+ (mg L^{-1})	< 10000	< 18000	Rinzema et al., 1988; Spanheimer & Muller, 2008
pH-range	6.5 - 8.5	5 - 8	Liu et al., 1985; Ma et al., 2006; van Leerdam et al., 2008; Staley et al., 2011; Steinberg & Regan, 2011
pH-shock	< 0.5	0.8 – 1.0	Liu et al., 1985; Baloch et al., 2007
Temperature range (°C)	7 - 65	1 - 70	Simankova et al., 2001; Ma et al., 2006; Leven et al., 2007; Tang et al., 2008; Goberna et al., 2010; Xing et al., 2010; Siggins et al., 2011
Acetate concentration (mg L^{-1})	< 3000	< 15000	Liu et al., 1985; McMahon et al., 2004; Conklin et al., 2006; Yu et al., 2006; Baloch et al., 2007; Qu et al., 2009b; Hao et al., 2011; Staley et al., 2011

First, *Methanosarcina* sp. seem to be more tolerant to high TAN concentrations, up to 7000 mg N L^{-1}, compared to other methanogens, particularly *Methanosaeta* sp., which are no longer detected at TAN concentrations exceeding 3000 mg N L^{-1} (Schnurer et al., 1999; Calli et al., 2005a; Calli et al., 2005b; Karakashev et al., 2006; Schnurer & Nordberg, 2008; Goberna et al., 2010). The resistance of *Methanosarcina* sp. against high ammonium concentrations can be attributed to (1) their relative large cell size and spherical form, and (2) their ability to grow in clusters, in contrast to other methanogens (Calli et al., 2005a; Calli et al., 2005b; Goberna et al., 2010). The large cell size and spherical form of *Methanosarcina* corresponds to a higher volume-to-surface ratio. Combined with the formation of clusters, this leads to a much lower ammonia diffusion per unit of cell mass, compared to filamentous methanogens, and thus induces a higher tolerance to high concentrations of ammonia (Calli et al., 2005a; Calli et al., 2005b; Vavilin et al., 2008b; Goberna et al., 2010). As the ammonium concentration rises, single cells of *Methanosarcina* sp. can group together to form clusters thus lowering the ammonium toxicity. Reactor stability can therefore be correlated to the consistency of these clusters (Calli et al., 2005b). As long as the cluster formation is not disturbed by certain chemicals or high shear forces, the resistance to ammonium remains high.

Second, several *Methanosarcina* sp. demonstrate no significant decrease in methane production at pH values which deviate from the narrow optimal pH range in AD (van Leerdam et al., 2008; Steinberg & Regan, 2009; Staley et al., 2011; Steinberg & Regan, 2011). Initiation of methanogenesis has been reported in municipal solid waste at a concentration of total volatile fatty acids (VFA) exceeding 15000 mg COD L^{-1}, and a corresponding low pH that varied between 5.0 and 6.25 (Staley et al., 2011). *Methanosarcina barkeri* was detected as the sole methanogen responsible for the generation of methane (Staley et al., 2011). Actively growing *Methanosarcina* sp. have even been detected in acidic peatland soils at a pH lower than 5.0 (Steinberg & Regan, 2011). *Methanosarcina mazei* on the other hand has been detected in an anaerobic bioreactor degrading methanethiol at a pH of 8.3 (van Leerdam et al., 2008).

Third, several species of *Methanosarcina* are tolerant to high levels of salt, more specifically to sodium, to concentrations up to 18000 mg Na^+ L^{-1} (Morozova & Wagner, 2007; Spanheimer & Muller, 2008; Saia et al., 2010; Vyrides et al., 2010). Several *Methanosarcina* sp. strains show a good response to high salt concentrations, when transferred to a medium with high salinity, by changing their cell physiology, which includes both the accumulation of solutes (osmoprotectants**)**, export of Na^+ and uptake of K^+ (Roessler & Muller, 2001;

Empadinhas & da Costa, 2008). Hence, *Methanosarcina* sp. from different origins are able to grow at high salt concentrations, and can tolerate high salt shocks, because of a rapid physiological response in a matter of a few hours up to even a few minutes (Martin et al., 1999; Martin et al., 2000; Roessler & Muller, 2001; Spanheimer & Muller, 2008).

Finally, *Methanosarcina* sp. are also able to grow at a wide temperature range, from 1 to 70°C (Simankova et al., 2001; von Klein et al., 2002; Singh et al., 2005; Tang et al., 2008; Xing et al., 2010). Apart from this ability to grow at different temperature ranges, the tolerance of *Methanosarcina* to abrupt changes in temperature can also be important in view of its role as the robust methanogen in AD. The presence of heat shock genes and their products (heat shock proteins) in eukaryotic, bacterial and archaeal cells is most often an indication of their tolerance against heat shocks, but also other forms of stress, e.g. high ammonium and salt concentrations (Macario et al., 1999; Zhang et al., 2006). The first heat shock protein gene of the Hsp70 family observed in archaeal cells, was detected in several *Methanosarcina* sp., which may indicate that *Methanosarcina* is the heat shock tolerant methanogen in anaerobic digesters (Macario et al., 1991; Demacario & Macario, 1994; Bult et al., 1996; Macario et al., 1999; Zhang et al., 2006). Cold stress regulating genes, i.e. elongation factor 2 genes (*aef2*), are also present in the genome of *Methanosarcina thermophila* (Thomas & Cavicchioli, 1998). This indicates that *Methanosarcina* sp. can respond both to cooling and heating changes in AD.

4.2. *Methanosaeta*: the efficient methanogen

Methanosaetaceae are obligate acetoclastic methanogens and were, until recently, reported to exclusively use acetate as substrate for methane production (Raskin et al., 1994; Zhu et al., 2012). Genome analysis of different species, however, revealed a genome size of about half the size of *Methanosarcina* sp., yet remarkable differences were detected among the genomes of *Methanosaeta* sp. (Smith & Ingram-Smith, 2007; Barber et al., 2011; Zhu et al., 2012).

In-depth analysis of the genome of several *Methanosaeta* sp. revealed the presence of genes encoding enzymes for the CO_2 reduction pathway, which indicates that *Methanosaeta* sp. is more metabolically diverse than previously anticipated (Smith & Ingram-Smith, 2007; Zhu et al., 2012). The investigation of aggregates in UASB reactors divulged that two species present in these aggregates (a *Geobacter sp.* and *Methanosaeta consilii*) are apparently able to exchange electrons via DIET, without the presence of hydrogen gas as electron carrier**,**

however, only with ethanol as substrate (Morita et al., 2011). Transcriptomic, radiotracer, and genetic analysis confirmed that another *Methanosaeta* sp., *Methanosaeta harundinacea*, was accepting electrons via DIET for the reduction of CO_2 to methane, which proves that *Methanosaeta* sp. are actually able to use not only acetate, but also CO_2 for the production of methane (Rotaru et al., 2014). However, the discovery of this new pathway for methane production by *Methanosaeta* requires further research, as this pathway has, up till now, only been demonstrated using ethanol as substrate (Morita et al., 2011; Rotaru et al., 2014).

Methanosaetaceae can be present in different morphologies in AD, ranging from long filaments to short rods and small cocci, yet in most cases *Methanosaeta* sp. are filamentous archaea (Figure 1.6) (Janssen, 2003; Hulshoff Pol et al., 2004; Calli et al., 2005a; Rotaru et al., 2014).

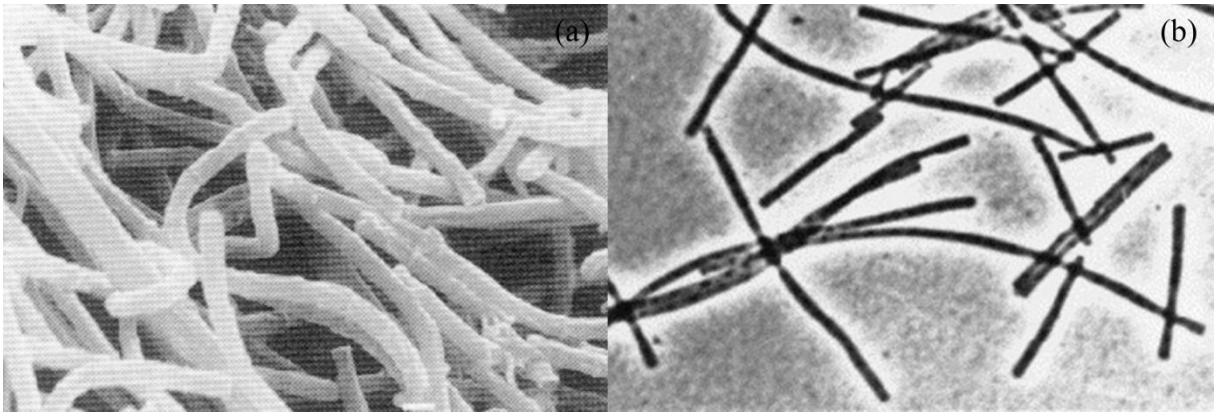

Figure 1.6 *Scanning electron microscopy (a) picture of Methanosaeta sp. in anaerobic granules and phase contrast photomicrograph (b) of a Methanosaeta sp. enrichment culture (Janssen, 2003; Hulshoff Pol et al., 2004).*

Due to their filamentous morphology, *Methanosaeta* sp. have two distinct advantages over other methanogens. First, their filamentous morphology allows the formation of 'spaghetti'-like structures that lead to the formation of anaerobic granules, making *Methanosaeta* sp. the key micro-organisms in anaerobic granulation (Lettinga, 1988; Quarmby & Forster, 1995; Diaz et al., 2003; Angenent et al., 2004b; Hulshoff Pol et al., 2004; Zheng et al., 2006). In some cases, however, a too high abundance of *Methanosaeta* sp. can lead to the formation of bulking granular sludge, thus implying the need to incorporate other non-filamentous methanogens in granular sludge as well (Diaz et al., 2003; Hulshoff Pol et al., 2004; Li et al., 2008). Second, the filamentous structure of *Methanosaeta* sp. correlates to a high surface-to-volume ratio, which allows them, together with their specific metabolic physiology, to grow at low acetate concentrations (Jetten et al., 1989; Jetten et al., 1992; Calli et al., 2005a).

Indeed, *Methanosaeta* sp. are characterized by a half-saturation constant (K_s) for acetate between 10 and 50 mg COD L^{-1}. In relation, *Methanosaeta* sp., however, have a low μ_{max} of 0.20 d^{-1} and doubling times in the order of 4 to 6 days at optimal conditions (Table 1.1), making them the slowest growing methanogens and therefore highly susceptible to wash-out (Gujer & Zehnder, 1983; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b).

Thanks to their high affinity for acetate, *Methanosaeta* sp. are able to grow in diverse ecological niches, such as rice paddy soils, aquifers contaminated with hydrocarbon- and chlorinated solvents, and boreal peatland ecosystems, niches that are characterized by low acetate concentrations (Jetten et al., 1992; Dojka et al., 1998; Grosskopf et al., 1998; Galand et al., 2005). *Methanosaeta* sp. are therefore, mainly due to their high affinity for acetate, considered as the predominant methane producers on earth (Smith & Ingram-Smith, 2007). Additionally, their important role in AD is uncontested, since they not only have a vital contribution to anaerobic granulation, they also allow efficient acetate conversion to methane, thus obtaining high COD removal rates. Hence, *Methanosaeta* sp. are responsible for at least part of the acetoclastic methanogenesis in almost every anaerobic digester, irrespective of the substrate and reactor configuration, operating at stable conditions, i.e. concentrations of TAN, Na^+ and acetate below the threshold values for *Methanosaeta* (Table 1.1) (Leclerc et al., 2004; Nelson et al., 2011; Sundberg et al., 2013; Williams et al., 2013).

The filamentous morphology of *Methanosaeta* sp. has, however, also a distinct **dis**advantage in terms of their overall stress tolerance. Because of the high surface-to-volume ratio, the diffusion of ammonia per unit of cell mass is much higher compared to other methanogens, thus inducing a lower tolerance to high TAN concentrations (Table 1.1) (Calli et al., 2005a; Calli et al., 2005b; Vavilin et al., 2008b; Goberna et al., 2010). Hence, in most AD systems, *Methanosaeta* sp. are no longer detected at TAN concentrations exceeding 3000 mg N L^{-1} (Schnurer et al., 1999; Karakashev et al., 2006; Schnurer & Nordberg, 2008). Moreover, high mixing intensities are also detrimental for *Methanosaeta* sp., since this leads to destruction of their filamentous structure (Hoffmann et al., 2008).

4.3. *Methanosarcina* and *Methanosaeta*: a perfect partnership?

Methanosarcina and *Methanosaeta* are, until present, the only known genera able to perform acetoclastic methanogenesis. However, despite their mutual preference for the same substrate, i.e. acetate, *Methanosaeta* and *Methanosarcina* are completely different in terms of overall

growth kinetics (Jetten et al., 1992; Conklin et al., 2006). *Methanosarcina* has a three times higher μ_{max}, a lower doubling rate, and a K_s value that is on average a factor 10 higher than *Methanosaeta* (Table 1.1). This has led to the overall conclusion that *Methanosarcina* is the prevailing acetoclastic methanogen at high acetate concentrations, while *Methanosaeta* becomes dominant at lower acetate concentrations (McMahon et al., 2001; McHugh et al., 2003; Conklin et al., 2006). *Methanosaeta sp.* in general dominate at acetate concentrations not exceeding 100 to 150 mg COD L^{-1}, whereas *Methanosarcina* becomes dominant at acetate concentrations above 250 to 500 mg COD L^{-1} (Conklin et al., 2006; Yu et al., 2006; Blume et al., 2010).

In AD, an increase in the concentration of acetate and other VFA is related to a decrease in performance and (partial) inhibition of methanogenesis (Chen et al., 2008). Indeed, increasing VFA concentrations indicate that *Methanosaeta* is apparently no longer able to maintain acetate at low concentrations, which can be attributed to two possible causes. First, overloading of the anaerobic digester results in increased acetate concentrations, because the slow growing *Methanosaeta* cannot follow the acidogenic and acetogenic bacteria that are producing the VFA (Gujer & Zehnder, 1983; Shin et al., 2011). Second, the threshold values of TAN, Na^+ (Table 1.1), other salts or even other potential toxicants for *Methanosaeta* are exceeded, which then results in partial or even total inhibition of the acetoclastic methanogenesis by *Methanosaeta* (McMahon et al., 2001; McMahon et al., 2004; Karakashev et al., 2005; Chen et al., 2008). In both cases, however, conditions become more favourable for *Methanosarcina*, compared to *Methanosaeta*, due to their higher tolerance to elevated TAN, Na^+ and acetate concentrations (Table 1.1) .

A shift from a *Methanosaeta* to a *Methanosarcina* dominated (acetoclastic) methanogenic community at increased organic loading rates and/or higher levels of common stressors, such as TAN or Na^+ and other salts, has been reported in several studies (McMahon et al., 2004; Conklin et al., 2006; Blume et al., 2010; Shin et al., 2010; Shin et al., 2011; Merlino et al., 2012; Merlino et al., 2013; Williams et al., 2013). However, in various other cases, a shift from a *Methanosaeta* dominated methanogenic community to a methanogenic community dominated by hydrogenotrophic methanogens was observed (Jang et al.; Delbes et al., 2001; Munk et al., 2010; Song et al., 2010; Hao et al., 2013). Hence, three potential pathway shifts can take place when *Methanosaeta* is no longer able to perform acetoclastic methanogenesis, due to changing conditions. First, a shift from a *Methanosaeta* to a *Methanosarcina* dominated acetoclastic methanogenesis can take place (Shigematsu et al., 2003; Conklin et

al., 2006; Vavilin et al., 2008b; Garcia et al., 2011; Lins et al., 2014). Second, a similar shift in the methanogenic community can occur, yet the main methanogenic pathway of *Methanosarcina* becomes hydrogenotrophic instead of acetoclastic methanogenesis (Karakashev et al., 2006; Hao et al., 2011; Karlsson et al., 2012; Westerholm et al., 2012b; Ho et al., 2013; Sun et al., 2014). Third, *Methanosaeta* can be replaced by strictly hydrogenotrophic methanogens, belonging to the Methanobacteriales and/or Methanomicrobiales order (Schnurer et al., 1999; Angenent et al., 2002; Hao et al., 2013; Lins et al., 2014). As all three pathway shifts have been observed and validated in AD, it remains unclear which specific transition to expect under specific conditions.

Notwithstanding, the crucial role of both *Methanosaeta* and *Methanosarcina* in AD is beyond a doubt. *Methanosaeta* clearly has a distinct advantage over *Methanosarcina* and other hydrogenotrophic methanogens at low acetate concentrations, whereas *Methanosarcina* can take over from *Methanosaeta* at changing conditions.

5. Objectives and outline of this research

Anaerobic digestion is a well-established technology that can be considered the first microbial technology that allows energy recovery from complex organic waste streams. As such, this technology has been applied at full-scale for several decades. However, despite its wide-spread application, this technology still poses several challenges, such as organic overloading, ammonia toxicity and salt toxicity. These problems can be related to the fact that, despite several attempts, the exact behaviour of the microbial community in AD is still unknown.

The main goal of this research was to evaluate the response of the microbial community in AD to changes in operational conditions, to allow better and more solid engineering, and to improve biogas production and process stability in AD. The main focus was aimed at the methanogenic community, as methanogenesis is generally considered as the weak link in the chain, because of the sensitivity of the methanogenic consortium to different environmental factors, although also bacterial community composition and organization were evaluated. Several strategies were applied to improve biogas production and process stability in AD, by (in)directly influencing the microbial community, which could be divided in two main categories. The first category involved the application of operational management strategies, including co-digestion (Chapter 2 and 3), feeding pattern variation (Chapter 4), and inoculum selection (Chapter 5), to improve biogas production and process stability. These operational

management strategies can be applied relatively easily in existing AD plants or AD plants in a start-up phase, as they only require a minor modification in the operational strategy and no profound technological modification of the plant. The second category of strategies covered the development of new technologies, i.e. the introduction of a cell potential by means of carbon felt electrodes (Chapter 6), and active biomass retention using AnMBR units, in which two different membrane fouling prevention strategies were applied (Chapter 7). These two strategies require a more thorough modification, to be applied in full-scale AD plants, yet, their positive impact on biogas production and process stability could prove to be substantial. In a last chapter (Chapter 8) the microbial community results of the lab-scale reactors were compared with full-scale plant samples to relate lab-scale results to actual full-scale plants. In a final general discussion (Chapter 9) the main results are discussed, after which future perspectives and research suggestions are proposed, followed by an overall conclusion.

Chapter 2 involved the co-digestion of A-sludge (highly biodegradable sludge from the A-stage of the Adsorptions-Belebungsverfahren) with kitchen waste. The high nutrient content of this Fe-rich A-sludge was anticipated to stabilize AD of kitchen waste. Different combinations of A-sludge and kitchen waste were evaluated at both mesophilic and thermophilic conditions.

The optimal combination of A-sludge and kitchen waste, as determined in Chapter 2, served as a basis for the co-digestion of A-sludge with kitchen waste or molasses in **Chapter 3**. In this chapter, the objective was to evaluate the exact mechanism of A-sludge as a stabilizing agent during anaerobic co-digestion with kitchen waste or molasses. In a first phase concentrated sterilized or active A-sludge was digested together with kitchen waste or molasses, whereas in a second phase diluted A-sludge was used. By means of these results, it was evaluated whether the main stabilizing effect of A-sludge in AD was through substrate dilution, micronutrient supplementation or additional biomass inoculation. The influence of the different combinations of A-sludge and kitchen waste or molasses on the bacterial and methanogenic community was investigated, and related to operational conditions.

The organic loading rate is considered one of the most important operational parameters that determine process stability in AD. In **Chapter 4**, a different feeding pattern was applied to increase operational stability in AD. A short-term stress test was carried out to evaluate a potential increase in stress tolerance, related to the feeding pattern. The effect of the feeding pattern on the evenness, dynamics and richness of the bacterial community, as well as the composition of the methanogenic community was investigated.

The microbial community in AD plays a crucial role in terms of stable methane production and stress tolerance. In **Chapter 5**, five different inocula were selected for the start-up and continuous operation of five lab-scale reactors. The ability of these inocula to achieve stable methane production, and tolerate increasing ammonium pulses was evaluated. Correlations were established between the microbial community and methane production, volatile fatty acids and ammonium concentration during stable and high ammonium concentration conditions. The role of the microbial community structure was investigated, and key microbial players for the stability and robustness of the digester were identified.

Bioelectrochemical systems can be used for numerous (lab-scale) applications, ranging from electrical power production to product formation, starting from complex (liquid) organic waste streams. In **Chapter 6**, a bioelectrochemical system was introduced in AD, to evaluate its stabilizing potential during molasses digestion. Lab-scale digesters were operated in the presence or absence of electrodes, in open (no applied potential) and closed circuit conditions. The (in)direct influence of the bioelectrochemical system on the microbial community was investigated.

The utilization of membrane technologies in wastewater treatment has increased in recent years, however, so far with limited applications in anaerobic wastewater treatment. In **Chapter 7**, two different anaerobic membrane bioreactor configurations with different fouling prevention strategies, one with biogas recirculation and one with a vibrating membrane, were used to digest concentrated and diluted molasses wastewater. The methanogenic community and its response to the different reactor configurations and wastewater composition were evaluated.

Finally, it was hypothesized that different operational parameters might lead to particular conformations of microbial communities AD. In **Chapter 8**, a total of 38 samples were collected from 29 stable full-scale AD plants, to obtain an overview of their microbial community. Correlations between operational parameters and the microbial community were determined, as well as between the different microbial groups. Potential clustering of the samples was investigated, and environmental and operational parameters driving the overall microbial community composition were identified.

In the final discussion, the results of the different chapters were combined, to evaluate the effect of the different management strategies, both on operational and microbial level. A comparison was made between the lab-scale microbial community results and the microbial community composition and organization in full-scale plants. These overall results were used

to enlighten future challenges and perspectives, and to draft an overall conclusion concerning the exact role of, in specific, *Methanosaeta* and *Methanosarcina*, and, in general, the microbial community in anaerobic digestion.

CHAPTER 2: HIGH-RATE IRON-RICH ACTIVATED SLUDGE AS STABILIZING AGENT FOR THE ANAEROBIC DIGESTION OF KITCHEN WASTE

This chapter has been redrafted after:

De Vrieze, J., De Lathouwer, L., Verstraete, W., Boon, N. 2013. High-rate iron-rich activated sludge as stabilizing agent for the anaerobic digestion of kitchen waste. Water Research, 47 (11), 3732-3741.

Abstract

Anaerobic digestion is a key technology in the bio-based economy that can be applied to convert a wide range of organic substrates into CH_4 and CO_2. Kitchen waste is a valuable substrate for anaerobic digestion, since it is an abundant source of organic matter. Yet, mono-digestion of kitchen waste often results in process failure. High-rate activated sludge or A-sludge is produced during the highly loaded first stage of the two-phase 'Adsorptions-Belebungsverfahren' or A/B activated sludge system for municipal wastewater treatment. In this specific case, the A-sludge was amended with $FeSO_4$ to enhance phosphorous removal and coagulation during the water treatment step. This study therefore evaluated whether this Fe-rich A-sludge could be used to obtain stable methanation and higher methane production values during co-digestion with kitchen waste. It was revealed that Fe-rich A-sludge can be a suitable co-substrate for kitchen waste; i.e. methane production rate values of 1.15 ± 0.22 and 1.12 ± 0.28 L L^{-1} d^{-1} were obtained during mesophilic and thermophilic co-digestion, respectively, of a feed-mixture consisting of 15% kitchen waste and 85% A-sludge. The thermophilic process led to higher residual volatile fatty acid concentrations, up to 2070 mg COD L^{-1}, and can therefore be considered less stable. Addition of micro- and macronutrients provided a more stable mono-digestion of kitchen waste, i.e. a methane production of 0.45 L L^{-1} d^{-1} was obtained in the micronutrient treatment compared to 0.30 L L^{-1} d^{-1} in the control treatment on day 61. Yet, methane production during mono-digestion of kitchen waste still decreased towards the end of the experiment, despite the addition of micronutrients. Methane production rates were related to the abundance of total archaea in the different reactors. This study showed that Fe-rich A-sludge and kitchen waste are suitable for co-digestion.

1. Introduction

Anaerobic digestion (AD) can be considered a key technology for bio-refinery side streams treatment in the future bio-based economy by converting low value organic by-products into biogas (Mata-Alvarez et al., 2000; Verstraete et al., 2005). The European Union demands that by the year 2020, 20% of European energy should be covered by renewable energy sources. The contribution of AD should be at least 25%; hence, further development of AD technology is crucial to meet the European standards (Holm-Nielsen et al. 2009). Anaerobic digestion offers numerous advantages over other processes for treating organic waste streams, such as the production of methane, a decrease and stabilization of organic waste, operation at a high organic loading rate (OLR), limited nutrient demands and low operational control and maintenance costs (Angenent et al., 2004a; Mata-Alvarez et al., 2011).

A wide range of organic substrates can be converted into CH_4 and CO_2 by means of AD. Kitchen waste (KW) is an interesting substrate for AD, since it is very abundant. Indeed, yearly 2.5 billion tonnes of KW are produced in Europe (Ma et al., 2011). The high energy content of 0.7 – 1.1 kWh kg^{-1} fresh weight (FW), high biodegradability (up to 90%) and high water content (70 – 80%) make KW a suitable candidate for AD (Banks et al., 2011; Ma et al., 2011). Nonetheless, the mono-digestion of KW can be problematic because of the high chemical oxygen demand (COD) and Kjeldahl Nitrogen (KjN) concentrations – up to 250 g COD L^{-1} and 35 g N L^{-1}, depending on the origin – which may lead to an unbalanced AD process ending up in acidification (Banks et al., 2011; Ma et al., 2011; Zhang et al., 2012c). Therefore, anaerobic mono-digestion of KW is mainly performed at low OLR (in the range of 1 – 3 g COD L^{-1} d^{-1}) to prevent the accumulation of volatile fatty acids (VFA) and subsequent process failure (Hecht & Griehl, 2009; Ma et al., 2011). Hence, co-digestion of KW with other organic waste streams may improve process stability. Several waste streams already have been successfully co-digested with KW to improve stability and biogas production, such as manure, paper waste and waste activated sludge (Kim et al., 2011; Zhang et al., 2012c). Another waste stream that might be suitable for co-digestion with KW is A-sludge.

A-sludge is generated in large quantities during the ‘Adsorptions-Belebungsverfahren’ or A/B process for municipal wastewater treatment (Boehnke et al., 1997). This technology is applied at full scale in the wastewater treatment plants of, among others, Strass, Austria and Nieuwveer, Breda, The Netherlands (Wett et al., 2007). The key element in this system is the highly loaded biological adsorption stage or A-stage, in which the organic carbon in the wastewater is converted into microbial sludge (Boehnke et al., 1997). The second stage, or B-

stage, consists of a nitrogen treatment step by means of conventional nitrification/denitrification or oxygen-limited autotrophic nitrification/denitrification (OLAND) (Verstraete & Vlaeminck, 2011). Anaerobic digestion of the energy-rich sludge of the A-stage, or A-sludge, greatly contributes to the lower energy requirements or even energy self-sufficiency of the A/B process (Wett et al., 2007; Verstraete & Vlaeminck, 2011). It is hypothesized that co-digestion of the A-sludge with other organic waste streams should greatly enhance biogas production (Verstraete & Vlaeminck, 2011). Co-digestion of A-sludge with KW may not only enhance biogas production, but also improve the stability of AD of KW, as A-sludge can provide the anaerobic digester with additional trace elements.

The main objective of this work was to evaluate whether co-digestion of A-sludge and KW could lead to (1) higher biogas production rates, (2) enhanced stability of KW digestion, under both mesophilic and thermophilic conditions and, (3) what the main mechanism was behind the potential stabilizing effect of the A-sludge. The Fe-rich A-sludge of a full scale treatment plant was used in this study. The high Fe-content of the A-sludge may attribute to an enhanced stability of the AD process, since Fe has an essential role in the metabolism of the micro-organisms, and can prevent sulphide inhibition (Zhang & Jahng, 2012). Co-digestion of KW with high-rate iron-rich activated sludge hence does not require the extra supplementation of Fe and allows for the re-use of the Fe that was dosed in the wastewater treatment plant for phosphorous removal.

2. Materials and methods

2.1 Substrates and sludge inoculum

Kitchen waste was obtained from the industrial kitchen of the Ghent University restaurant ‘De Brug’ (Ghent, Belgium). This KW consisted mostly of carbohydrates from bread, rice and potatoes, proteins and fats from cooked meat and fish, as well as cooked and non-cooked vegetables and fruits. The KW was thoroughly mixed in a kitchen blender and stored at 4°C. A-sludge was collected from the municipal WWTP (wastewater treatment plant) of Nieuwveer (Breda, the Netherlands). The characteristics of the A-sludge and KW are shown in Table 2.1. Note that in this plant $FeSO_4$ is added to the highly loaded A-stage, which results in a case-specific high Fe content of the sludge.

Mesophilic anaerobic sludge was collected from the sludge digester of the municipal WWTP of Ossemeersen, Ghent (Belgium). Thermophilic sludge originated from a thermophilic

anaerobic digester treating manure, KW, slaughterhouse waste and energy crops (Bio-gas Boeye, Beveren, Belgium).

Table 2.1 *Characteristics of the Fe-rich A-sludge and kitchen waste (KW) used to prepare the feeding of the reactors. All analyses were carried out in triplicate, except for the Fe, Cu, Mn, Zn, Ni, Mo, Co, Ca and Mg determination. The low COD/VS ratio for the KW is most likely related to an error in the COD analysis method.*

Parameter	Unit	Kitchen waste	A-sludge
Total COD	g kg^{-1} FW	260 ± 47	20.0 ± 7.3
Total solids	g kg^{-1} FW	255 ± 4	27.6 ± 0.4
Volatile solids	g kg^{-1} FW	240 ± 6	19.8 ± 0.4
Total ammonia nitrogen, TAN	mg N kg^{-1} FW	384 ± 4	313 ± 6
Kjeldahl nitrogen, KjN	mg N kg^{-1} FW	11930 ± 1060	1329 ± 27
Total phosphorus, TP	mg P kg^{-1} FW	710 ± 57	309 ± 17
COD:N ratio	-	21.8 ± 4.4	15.1 ± 5.5
COD:P ratio	-	366 ± 77	65 ± 24
TS:VS ratio	-	1.07 ± 0.03	1.40 ± 0.04
COD:VS ratio	-	1.08 ± 0.20	1.01 ± 0.37
Fe	mg kg^{-1} FW	9.52	1350
Cu	mg kg^{-1} FW	2.06	20.2
Mn	mg kg^{-1} FW	4.69	8.75
Zn	mg kg^{-1} FW	4.83	50.9
Ni	mg kg^{-1} FW	0.252	0.788
Mo	mg kg^{-1} FW	0.1	0.343
Co	mg kg^{-1} FW	0.012	0.259
Ca	mg kg^{-1} FW	308	887
Mg	mg kg^{-1} FW	218	96.7
Fe:P ratio	-	0.013 ± 0.001	4.37 ± 0.24

2.2. Experimental set-up and operation

2.2.1 Thermophilic and mesophilic co-digestion of kitchen waste and A-sludge

Five anaerobic lab-scale continuous stirred tank reactors (CSTR), each with a total volume of 1 L and a working volume of 800 mL (Figure 2.1), were operated for 91 days under thermophilic conditions (54 °C). The inoculum sludge was diluted with tap water until a volatile suspended solids (VSS) concentration of 10 g VSS L^{-1} was obtained. These five

reactors were given different combinations of A-sludge and KW, i.e. 100% A-sludge (A-Therm), 95% A-sludge and 5% KW (5-Therm), 90% A-sludge and 10% KW (10-Therm), 85% A-sludge and 15% KW (15-Therm) and 100% KW diluted with tap water (KW-Therm). The reactors were operated in a fed-batch mode, in which feeding took place three times a week. The experiment consisted of a start-up phase of 21 days, during which the organic loading rate (OLR) was gradually increased (Figure 2.2) and the HRT (hydraulic retention time)decreased from 80 to 40 days on day 14 and from 40 to 20 days on day 21. After day 21 the reactors were run for 70 days at a constant HRT of 20 days. The OLR, however, slightly changed through time (Figure 2.1), because on day 72 a new batch of A-sludge was used, and on day 86 a new batch of KW was used. No dilution with water was applied to maintain constant pH, and buffer and salt concentrations in the feed.

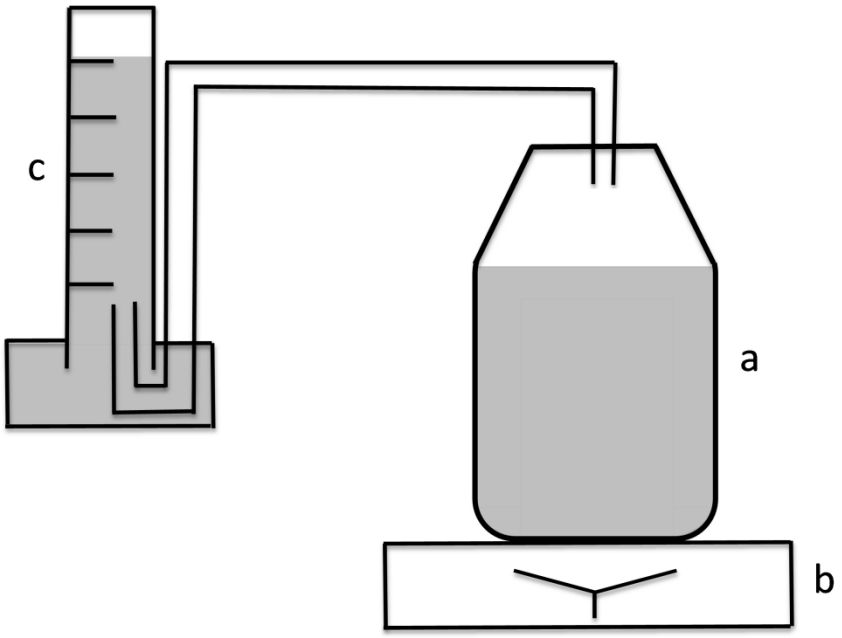

Figure 2.1 *Schematic overview of the reactor set-up for the mesophilic and thermophilic experiment**,** in which the reactor (a), magnetic stirring device (b) and gas collection device (c) are shown.*

The five mesophilic reactors were operated under the same conditions at a temperature of 34°C. They were given the same ratios of A-sludge and KW, i.e. 100% A-sludge (A-Mes), 95% A-sludge and 5% KW (5-Mes), 90% A-sludge and 10% KW (10-Mes), 85% A-sludge and 15% KW (15-Mes) and 100% KW diluted with tap water (KW-Mes). The operational parameters are presented in Table 2.2.

The pH of the reactors was monitored without adjustment three times a week. The biogas production and the percentage of methane in the biogas were also measured three times a

week and reported at STP (standard temperature and pressure) conditions. Effluent samples were taken three times a week for volatile fatty acids (VFA) analyses and once a week for total and volatile solids (TS and VS) and total ammonia nitrogen (TAN) analyses. A weekly biomass sample of 10 mL was taken to examine the microbial community as well. These samples were subsequently stored at -20 °C until DNA extraction was performed.

Table 2.2 *Operational parameters during the experimental period of the mesophilic and thermophilic co-digestion of kitchen waste (KW) and A-sludge, and the mesophilic mono-digestion of KW with additives. The organic loading rate is determined by the feed mixture.*

Parameter	Mesophilic co-digestion	Thermophilic co-digestion	Mesophilic with supplements
Substrate	A-sludge and/or KW	A-sludge and/or KW	KW
Duration (d)	70	70	63
Temperature (°C)	34	54	34
Organic loading rate, OLR (g COD L^{-1} d^{-1})	1.21 ± 0.07 to 3.13 ± 0.17	1.21 ± 0.07 to 3.13 ± 0.17	1.53 ± 0.12
Hydraulic retention time, HRT (d)	20	20	20

2.2.2. Mesophilic mono-digestion of kitchen waste with supplements

Five reactors were operated under similar conditions as in the mesophilic co-digestion of A-sludge and KW (Table 2.2). The five reactors were run for 77 days. The experiment consisted of a start-up phase of 14 days with a gradual increase of the OLR (Figure 2.2) and a decrease of the HRT from 80 to 20 days on day 14. From day 14 on the reactors were run for 63 days at a constant HRT of 20 days. All five reactors were fed KW, 10 times diluted with tap water at the same OLR, yet different supplements were added to the feed of the reactor. A control treatment was used with only KW as feed (KW-Control), which was similar to the KW-Mes reactor. A second reactor (KW-Macro) was given additional macro-nutrients in the following doses (in mg kg^{-1} FW): $CaCl_2.2H_2O$: 200, $MgCl_2.6H_2O$: 200, $Fe_2(SO_4)_3$: 100, NH_4Cl: 500. A third reactor (KW-Micro) was supplemented with additional micro-nutrients in the following amounts (in µg kg^{-1} FW): $NiCl_2.6H_2O$: 450, $MnCl_2.4H_2O$: 500, $FeSO_4.7H_2O$: 500, $ZnSO_4.7H_2O$: 100, H_3BO_3: 100, $Na_2MoO_4.2H_2O$: 50, $CoCl_2.6H_2O$: 50 and $CuSO_4.5H_2O$: 5. A fourth reactor (KW-Yeast) was given yeast extract as a supplement in a concentration of 200 mg kg^{-1} FW substrate. To a fifth reactor (KW-Methanostim), the commercial product

Methanostim Liquid (Avecom, Belgium) was given in a concentration of 1µL kg^{-1} FW substrate, as instructed by the manufacturer. This product consists of a solution of technical grade ferric chloride, cobalt chloride hexahydrate, yeast extract and citric acid solution, as described by Ma et al. (2009). The same set of parameters as in the mesophilic and thermophilic co-digestion of KW and A-sludge were monitored.

2.3. Microbial community analysis

Total DNA was extracted from the sludge samples by means of the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 200 mg of sample was taken for DNA extraction.The DNA concentration in the extracts was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands), by measuring the absorbance ratios at 260 nm and 280 nm.

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Analytical triplicates of a 10 to 100-fold dilution of the DNA-samples were analysed for total bacteria and total archaea. The general bacterial primers P338F (5'-ACTCCTACGGGAGGCAGCAG-3') and P518r (5'-ATTACCGCGGCTGCTGG-3'), as described by Ovreas et al. (1997), were used to quantify total bacteria. The primer sets used for total archaea (ARC) were previously described by Yu et al. (2005). A reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, WI, USA) containing 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water, 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure which consists of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C for total bacteria. The qPCR program for total archaea consisted of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. The qPCR data were represented as copies per gram of wet sludge.

2.4. Analytical techniques

TS, VS, TAN and total COD (COD_{tot}) were determined according to Standard Methods (Greenberg et al. 1992). VFA were extracted using diethyl ether and measured in a GC-2014

gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands), equipped with a DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector. A Compact GC (Global Analyser Solutions, Breda, the Netherlands), equipped with a Porabond precolumn and a Molsieve SA column was used to analyse biogas composition. Concentrations of CH_4, CO_2 and H_2 were determined with a thermal conductivity detector that has a lower detection limit of 1 ppmv for each gas component. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium). The total Fe, Cu, Mn, Zn, Ca and Mg contents of the A-sludge and KW were analysed by means of an ICP-OES VISTA MPX (Varian, Munich, Germany), whereas the total Co, Ni and Mo contents were analysed using an ICP-MS Elan DRC-e (PerkinElmer, Waltham, MA, USA). The samples were destructed in a CEM Mars 5 Microwave Accelerated Reaction System (International Equipment Trading Ltd, Vernon Hills, IL, USA) prior to analysis.

3. Results

3.1. Reactor performance

3.1.1. Thermophilic co-digestion of kitchen waste and A-sludge

During the first 21 days of the experiment, the A-Therm, 5-Therm, 10-Therm, 15-Therm and KW-Therm were run at a lower OLR to obtain a stable start-up phase. However, all 5 reactors demonstrated an accumulation of total VFA during the start-up phase to a value of 700, 540, 860, 4480 and 2050 mg COD L^{-1} in the A-Therm, 5-Therm, 10-Therm, 15-Therm and KW-Therm, respectively, on day 12, indicating a difficult start-up. After 21 days the OLR was increased to a stable value, depending on the feed mixture, which was only slightly elevated throughout the experimental phase (Figure 2.2a).

The methane production during the experimental period was on average 0.37 ± 0.09, 0.63 ± 0.15, 0.80 ± 0.15, 1.12 ± 0.28 and 0.15 ± 0.17 L L^{-1} d^{-1} in the A-Therm, 5-Therm, 10-Therm, 15-Therm and KW-Therm, respectively (Figure 2.3a).

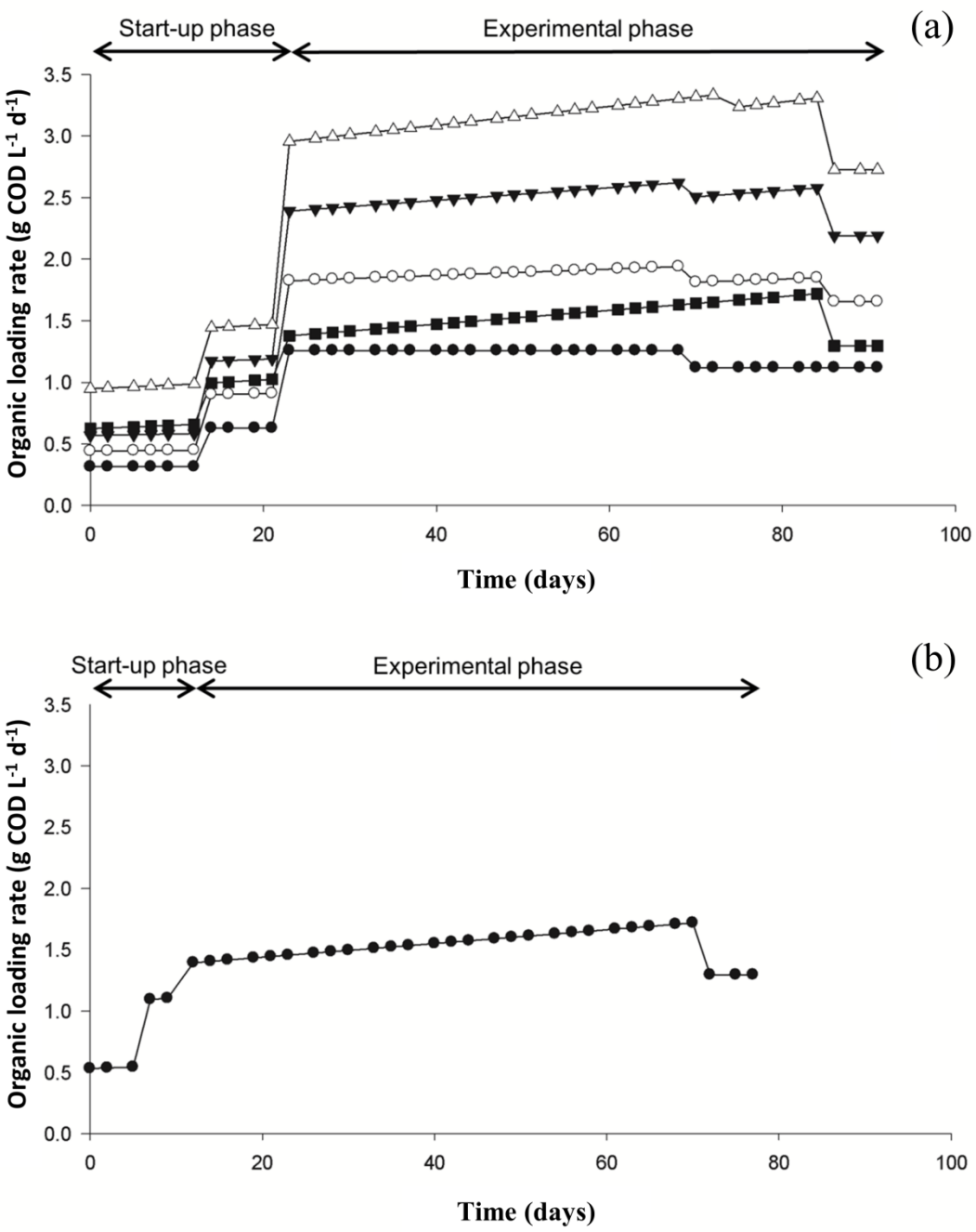

Figure 2.2 *Organic loading rate (OLR) of (a) the A-Therm and A-Mes (■), 5-Therm and 5-Mes (○), 10-Therm and 10-Mes (▼), 15-Therm and 15-Mes (Δ) and KW-Therm and KW-Mes (●) treatment and (b) the mesophilic mono-digestion of KW, enhanced with additives. The slight change in the OLR on day 72 was caused by the application of a new batch of A-sludge, whereas the change on day 86 was related to the usage of a new batch of kitchen waste.*

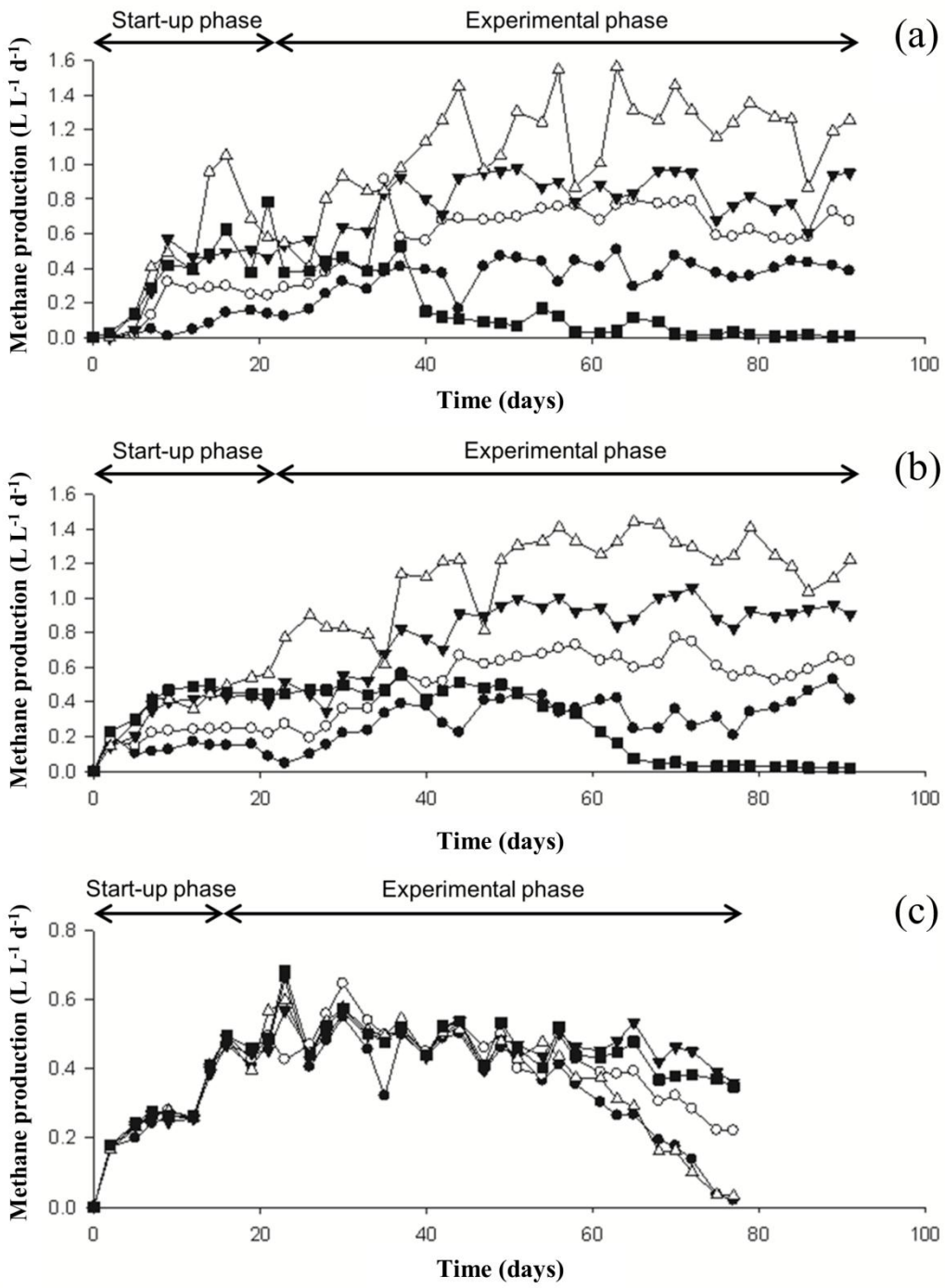

Figure 2.3 *Methane production of (a) the A-Therm (●), 5-Therm (○), 10-Therm (▼), 15-Therm (Δ) and KW-Therm (■) treatment in the thermophilic co-digestion of A-sludge and KW, (b) the A-Mes (●), 5-Mes (○), 10-Mes (▼), 15-Mes (Δ) and KW-Mes (■) treatment in the mesophilic co-digestion of A-sludge and KW and (c) the KW-Control (●), KW-Macro (○), KW-Micro (▼), KW-Yeast (Δ) and KW-Methanostim (■) treatment in the mesophilic mono-digestion of KW, enhanced with additives.*

The pH remained stable throughout the experimental phase in the A-Therm, 5-Therm, 10-Therm and 15-Therm, with average pH values of 7.38 ± 0.13, 7.35 ± 0.13, 7.37 ± 0.13 and 7.43 ± 0.13, respectively. Total VFA concentrations however demonstrate that there was a

slight level of overloading in the 15-Therm, with total VFA levels up to 2070 mg COD L^{-1} during the experimental phase (Figure 2.4a). The A-Therm, 5-Therm and 10-Therm only had limited accumulation of VFA with values never exceeding 470, 780 and 1150 mg COD L^{-1} respectively. The KW-Therm showed a faster decrease in methane production in comparison to the KW-Mes, with a decline from 0.46 L L^{-1} d^{-1} on day 30 to only 0.03 L L^{-1} d^{-1} on day 61, indicating a rapid acidification of the digester. These results are in correlation to the accumulation of VFA and decrease in pH, which reached a concentration of 8.0 g COD L^{-1} and a value of 4.39 on day 61, respectively.

The VS removal efficiencies in the A-Therm, 5-Therm, 10-Therm and 15-Therm had values of 50.4 ± 5.0, 67.7 ± 4.8, 72.6 ± 3.3 and 72.8 ± 2.9 % in the A-Therm, 5-Therm, 10-Therm and 15-Therm**,** respectively (Figure 2.5a). The removal efficiency of VS in the KW-Therm reached a value of 77.6 % on day 42, although at the end of the experiment, the removal efficiency declined to a value of 45.8 %.

3.1.2. Mesophilic co-digestion of kitchen waste and A-sludge

In comparison to the thermophilic co-digestion of KW and A-sludge, the mesophilic experiment also contained a start-up phase of 21 days, during which the A-Mes, 5-Mes, 10-Mes, 15-Mes and KW-Mes were operated at a lower OLR to obtain an effective start-up. A smooth start-up could be detected with a sudden increase in total VFA in the 15-Mes to a value of 3400 mg COD L^{-1} on day 16, which decreased back to levels below detection limit on day 26. After 21 days the OLR was applied in the exact same way as in the thermophilic test (Figure 2.2a). An average methane production of 0.32 ± 0.11, 0.56 ± 0.15, 0.83 ± 0.18, 1.15 ± 0.22 and 0.27 ± 0.21 L L^{-1} d^{-1} was detected in the A-Mes, 5-Mes, 10-Mes, 15-Mes and KW-Mes, respectively, during the experimental period. The methane production was highest in the 15-Mes reactor, followed by the 10-Mes, 5-Mes and A-Mes (Figure 2.3b). Apart from a small decrease during the start-up phase, the pH in the 15-Mes never dropped below 7.00, with an average pH-value of 7.27 ± 0.09 in the 15-Mes and 7.10 ± 0.10, 7.12 ± 0.12 and 7.19 ± 0.10 in the A-Mes, 5-Mes and 10-Mes, respectively. These results are also correlated to the total VFA concentrations in the reactor that never exceeded a value of 500 mg COD L^{-1} in the A-Mes, 5-Mes, 10-Mes and 15-Mes during the experimental phase (Figure 2.4b). However, the results are completely different for the KW-Mes reactor that showed a clear decrease in methane production from 0.56 L L^{-1} d^{-1} on day 37 to only 0.02 L L^{-1} d^{-1} at the end of the

experiment, despite the fact that it had a lower OLR than the 5-Mes, 10-Mes and 15-Mes, indicating severe process failure. These results are corroborated by the severe decrease in pH to a value of 4.57 and the accumulation of total VFA to a value of 11.4 g COD L^{-1} in the KW-Mes at the end of the experiment.

A similar stable VS removal efficiency was obtained in the 5-Mes, 10-Mes and 15-Mes, comparable to the thermophilic experiment, with values of 70.4 ± 4.3, 73.8 ± 3.4 and 73.1 ± 3.1 % respectively (Figure 2.5b). With a value of 54.9 ± 4.1 %, the VS removal efficiency in the A-Mes was substantially lower than in the 5-Mes, 10-Mes and 15-Mes reactors. In contrast, VS removal in the KW-Mes increased from 58.1 to 82.4 % on day 77, followed by a decrease to 76.6% at the end of the experiment.

3.1.3 Mesophilic digestion of kitchen waste with supplements

Mono-digestion of KW, supplemented with certain additives was performed for a period of 77 days, including a start-up period of 14 days, during which the OLR was gradually increased. A stable start-up was achieved, as total VFA accumulation did not exceed a value of 270 mg COD L^{-1} in the KW-Control, KW-Macro, KW-Micro, KW-Yeast and KW-Methanostim, and pH remained stable, albeit slightly lower than the optimum value of 7.2 (Chen et al. 2008, De Vrieze et al. 2012, Gujer and Zehnder 1983). A pH of 6.74, 6.77, 6.76, 6.77 and 6.77 was detected in the KW-Control, KW-Macro, KW-Micro, KW-Yeast and KW-Methanostim, respectively, on day 14. After 14 days the OLR increased to an equal stable value in all five reactors (Figure 2.2b). Methane production remained constant in all five reactors from day 14 until day 51 with average values of 0.46 ± 0.07, 0.49 ± 0.06, 0.48 ± 0.05, 0.49 ± 0.06 and 0.50 ± 0.06 L L^{-1} d^{-1} (Figure 2.3c) with stable pH values of 6.91 ± 0.11, 6.92 ± 0.10, 6.94 ± 0.10, 6.94 ± 0.11 and 6.95 ± 0.09 in the KW-Control, KW-Macro, KW-Micro, KW-Yeast and KW-Methanostim, respectively. Total VFA concentrations remained below 500 mg COD L^{-1} in all reactors (Figure 2.4c). After day 51, methane production rapidly decreased to a value of 0.02 and 0.03 L L^{-1} d^{-1} on day 77 in the KW-Control and KW-Yeast, respectively, which was in contrast to the KW-Macro, KW-Micro and KW-Methanostim, showing only a limited decrease to values of 0.22, 0.36 and 0.35 L^{-1} d^{-1}, respectively, on day 77 (Figure 2.3c). These results were confirmed by the differences in total VFA accumulation and pH, as pH values of 5.68, 6.50, 6.82, 6.00 and 6.85 and VFA concentrations of 6080, 3520, 680, 7490 and 2500 were measured in the KW-Control, KW-Macro, KW-Micro, KW-Yeast and KW-

Methanostim, respectively, on day 77. Methane production thus seemed to be maintained in the KW-Micro and KW-Methanostim, despite a certain degree of VFA accumulation. A 50% decrease in methane production was observed in the KW-Macro, with higher levels of VFA compared to the KW-Micro and KW-Methanostim, and methane production totally ceased in the KW-Control and KW-Yeast.

The VS removal efficiency results (Figure 2.5c) demonstrated that there was no remarkable difference between the different treatments, with average values of 77.7 ± 3.4, 78.5 ± 4.4, 79.5 ± 3.8, 77.7 ± 3.3 and 77.6 ± 4.4 % in the KW-Control, KW-Macro, KW-Micro, KW-Yeast and KW-Methanostim, respectively, during the experimental period. This is notwithstanding the different methane production values between the treatments at the end of the experiment.

3.2. Microbial community analysis

The microbial community in the different reactors, as well as in the A-sludge and KW, was analysed by means of real-time PCR. Total bacteria and total archaea were quantified. Selected samples were analysed at different time points during the test, more specifically the mesophilic and thermophilic inoculum samples on day 0, the reactor samples of the mesophilic and thermophilic co-digestion test on day 42, 63 and 84 and the reactor samples of the test with mono-digestion of KW, enhanced with additives on day 42 and 77.

The inoculum sludge samples showed similar values for total bacteria ($1.4 \times 10^{10} \pm 2.0 \times 10^{9}$, $1.8 \times 10^{10} \pm 9.9 \times 10^{8}$ and $6.9 \times 10^{9} \pm 9.0 \times 10^{8}$ copies g^{-1} wet sludge) and total archaea ($2.1 \times 10^{8} \pm 4.5 \times 10^{6}$, $2.1 \times 10^{8} \pm 8.7 \times 10^{6}$ and $1.4 \times 10^{8} \pm 1.3 \times 10^{7}$ copies g^{-1} wet sludge) in the mesophilic and thermophilic inoculum sludge for the co-digestion test and the mesophilic inoculum sludge for the KW mono-digestion test, respectively. Total bacteria reached an average value of $2.4 \times 10^{10} \pm 1.8 \times 10^{10}$ copies g^{-1} throughout the entire mesophilic and thermophilic co-digestion experiment, with the exception of the KW-Therm that showed a remarkable decrease to a value of $4.5 \times 10^{7} \pm 1.8 \times 10^{6}$ copies g^{-1} on day 84. Total archaea abundance was also almost similar to the inoculum sample throughout the entire mesophilic and thermophilic co-digestion experiment. Nonetheless, total archaea were slightly higher on day 84 in the 15-Mes and 15-Therm, compared to the A-Mes and A-Therm, with values of $3.0 \times 10^{9} \pm 1.2 \times 10^{8}$ and $5.6 \times 10^{9} \pm 1.0 \times 10^{9}$ copies g^{-1}, compared to $8.8 \times 10^{8} \pm 2.2 \times 10^{7}$ and $7.9 \times 10^{8} \pm 1.4 \times 10^{8}$ copies g^{-1}, respectively, although the differences were limited. Total

archaea showed a clear decrease towards the end of the experiment in the KW-Mes and KW-Therm, with values of 1.9 x 10^8 and 2.3 x 10^5 respectively on day 84, indicating a severe decrease of total archaea, especially in the KW-Therm. No remarkable differences in total archaea and total bacteria could be detected during the KW mono-digestion test. Total archaea showed a similar increase from 1.4 x 10^8 copies g^{-1} sludge in the inoculum sample to 9.1 x 10^8 ± 6.8 x 10^7 copies g^{-1} in all other samples on day 42 and 77, with the exception of the KW-Yeast sample on day 77 that reached a value of only 4.0 x 10^8 ± 4.4 x 10^7 copies g^{-1} for total archaea.

4. Discussion

Thermophilic AD of KW in combination with A-sludge resulted in stable methane production, although residual VFA concentrations pointed towards a certain instability in the process. Thermophilic mono-digestion of KW rapidly resulted in process failure. A similar pattern was observed during mesophilic digestion of KW and A-sludge, but the residual VFA concentrations were lower and mono-digestion of KW only took place after an initial lag period. Supplementation with additives during the mono-digestion of KW resulted in a more stable methane production when micro-nutrients were added, although these treatments showed gradual process inhibition towards the end of the experiment.

The thermophilic digestion process had a difficult start-up phase with elevated VFA concentrations and a decrease in pH, although all combinations of KW and A-sludge demonstrated stable methanation during the experimental phase. The residual VFA concentrations increased in case of a higher KW-content of the feed, with the highest value of 2070 mg COD L^{-1} observed in the 15-Therm, indicating overloading of the reactor. The high KjN content of the KW might be responsible for this, leading to a TAN concentration of 1560 mg N L^{-1} and a corresponding free ammonia (FA) concentration of 183 mg N L^{-1} at a pH of 7.54 causing a decrease in methanogenesis (Chen et al., 2008; De Vrieze et al., 2012). Mono-digestion of KW resulted in a severe drop in methane production on day 40 to a value of 0.15 L L^{-1} d^{-1}, with a corresponding total VFA concentration of 4450 mg L^{-1}, indicating severe process failure. This is similar to previous experiments in which KW was used as single substrate (Banks et al., 2011; Zhang & Jahng, 2012).

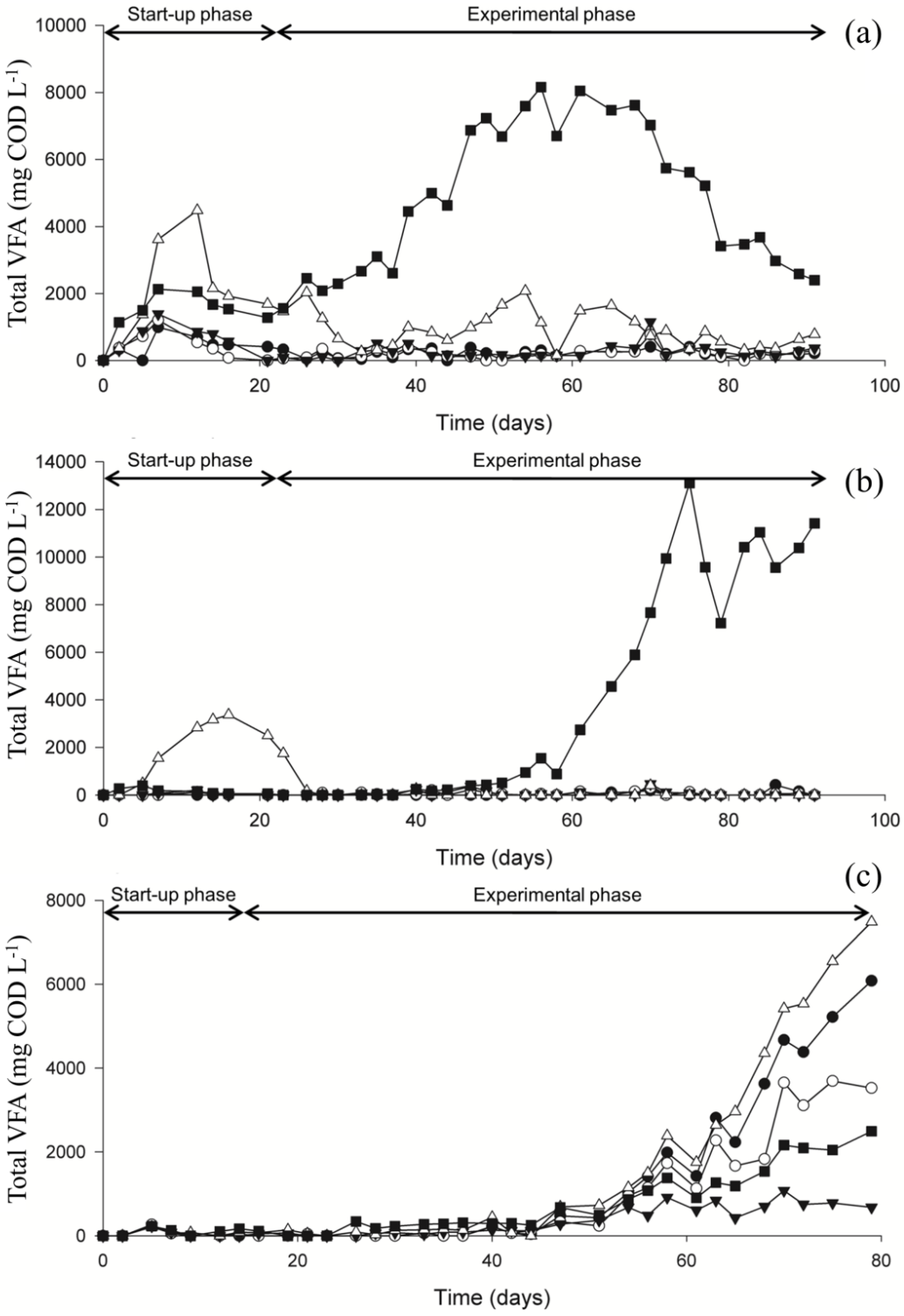

Figure 2.4 *Total VFA concentration (mg COD L^{-1}) of (a) the A-Therm (●), 5-Therm (○), 10-Therm (▼), 15-Therm (Δ) and KW-Therm (■) treatment in the thermophilic co-digestion of A-sludge and KW, (b) the A-Mes (●), 5-Mes (○), 10-Mes (▼), 15-Mes (Δ) and KW-Mes (■) treatment in the mesophilic co-digestion of A-sludge and KW and (c) the KW-Control (●), KW-Macro (○), KW-Micro (▼), KW-Yeast (Δ) and KW-Methanostim (■) treatment in the mesophilic mono-digestion of KW, enhanced with additives.*

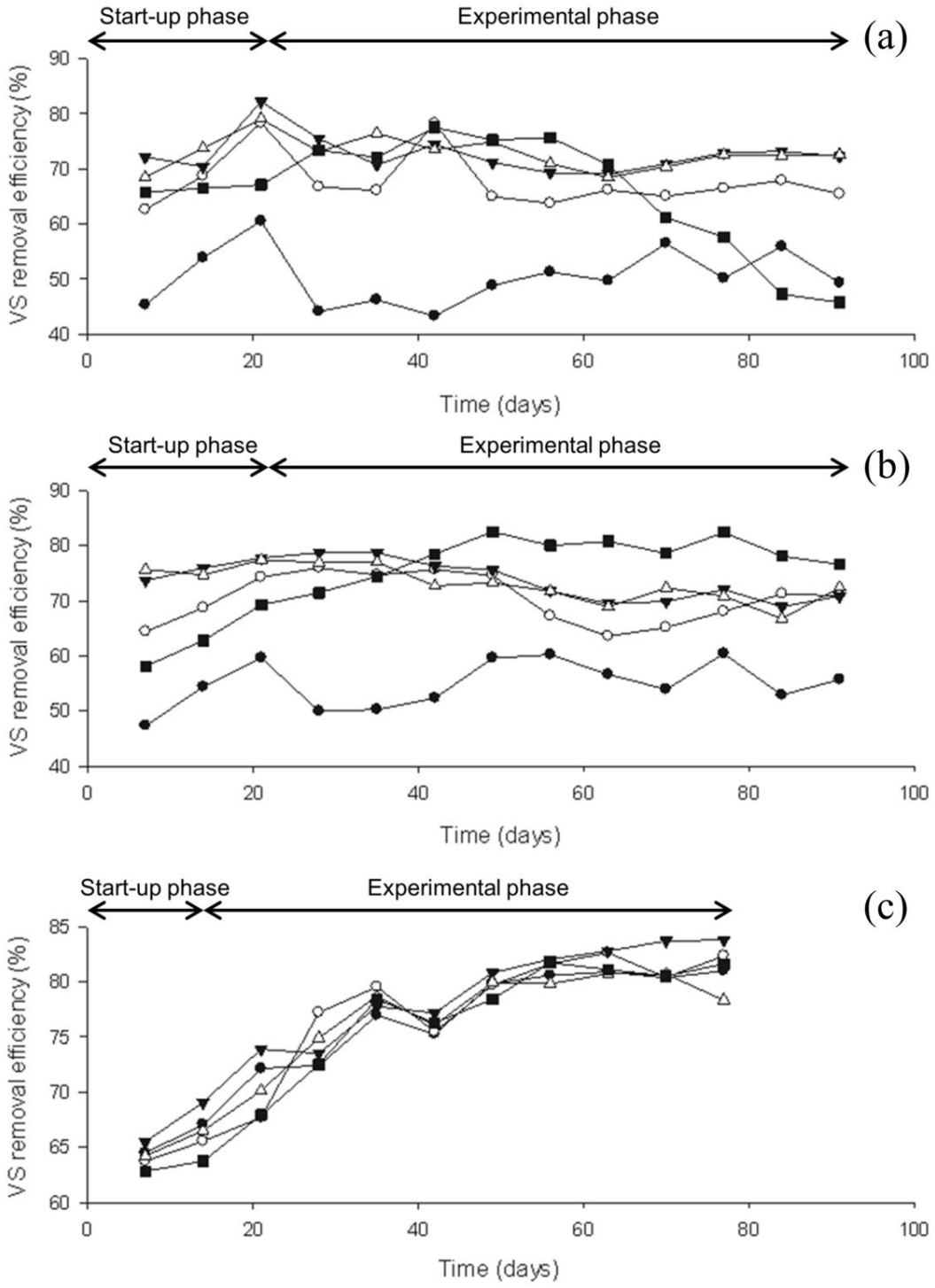

Figure 2.5 *VS removal efficiency (%) of (a) the A-Therm (●), 5-Therm (○), 10-Therm (▼), 15-Therm (Δ) and KW-Therm (■) treatment in the thermophilic co-digestion of A-sludge and KW, (b) the A-Mes (●), 5-Mes (○), 10-Mes (▼), 15-Mes (Δ) and KW-Mes (■) treatment in the mesophilic co-digestion of A-sludge and KW and (c) the KW-Control (●), KW-Macro (○), KW-Micro (▼), KW-Yeast (Δ) and KW-Methanostim (■) treatment in the mesophilic mono-digestion of KW, enhanced with additives.*

Mesophilic co-digestion of KW and A-sludge resulted in stable methane production. A smoother start-up was achieved and residual VFA levels remained below 500 mg COD L^{-1} during the entire experimental period, which is in contrast to the thermophilic co-digestion of KW and A-sludge, showing more VFA accumulation. Methane production results confirmed this, since average methane production was higher in the A-Therm and 5-Therm compared to the A-Mes and 5-Mes, respectively. When higher fractions of KW were added to the feed, the 10-Mes and 15-Mes achieved higher methane production values than the 10-Therm and 15-Therm, respectively. Mono-digestion of KW under mesophilic conditions maintained stable operation for about 20 days longer than under thermophilic conditions, although eventually methane production ceased and VFA accumulated, which was also observed in other studies (Hecht & Griehl, 2009; Carballa et al., 2011). Based on these results, it can be concluded that Fe-dosed A-sludge stabilizes KW digestion both in mesophilic and thermophilic conditions. VS removal results were similar in the 5-Therm, 10-Therm and 15-Therm compared to the 5-Mes, 10-Mes and 15-Mes, respectively. Yet, VS removal was on average higher in the A-Therm, compared to the A-Mes, with values of 50.4 and 54.9% respectively, which could be explained by the higher level of hydrolysis at thermophilic conditions (Lv et al., 2010). In contrast, VS removal in the KW-Therm decreased to a much lower value at the end of the experiment, compared to the KW-Mes, which relates to the faster process inhibition in the KW-Therm, compared to the KW-Mes. Although only partially confirmed by the VS removal results, the potential higher degree of hydrolysis that can be obtained at higher temperatures is neutralized by the higher build-up of fatty acids, which often occurs in thermophilic digestion (Lv et al., 2010). The higher stability of the mesophilic co-digestion process can be explained by the difference in TAN concentration, since higher TAN levels were detected at thermophilic conditions and the higher temperature and pH at thermophilic conditions led to a higher fraction of free ammonia, which is especially toxic to methanogens (Anthonisen et al., 1976; Chen et al., 2008; Schnurer & Nordberg, 2008; De Vrieze et al., 2012).

Both mesophilic and thermophilic experiments emphasized the stabilizing potential of Fe-rich A-sludge during KW digestion, which is in correlation to the stabilizing potential of sewage sludge and manure (Kim et al., 2011; Zhang et al., 2012c). The stabilizing influence of the Fe-rich A-sludge during KW digestion could be attributed to the presence of higher levels of macro- and micronutrients in the A-sludge, which are, with the exception of Mg, 2 to 140 times higher in our A-sludge compared to the KW (Table 2.1). This is in agreement with an earlier study that reported stable methanation of KW by adding Co, Fe, Mo and Ni to the

digester, of which Fe appeared to be the most important compound (Zhang & Jahng, 2012). Since our A-sludge contained around 140 times more Fe than KW, which can be explained by the fact that $FeSO_4$ is dosed in the A-stage of the municipal WWTP of Nieuwveer (Breda, the Netherlands), it is most likely the iron in the A-sludge that caused the stabilizing effect. Especially the Fe:P ratio, which is a factor 300 higher in our A-sludge compared to the KW, might be an explanation for the stabilizing effect of our A-sludge. Indeed, it was already demonstrated by Kleyböcker et al. (2012) that both phosphate and calcium played an important role in maintaining reactor stability, yet iron could replace the role of calcium in precipitating phosphate, thus forming iron phosphate precipitates to which VFA could be adsorbed. Further research should however be carried out to confirm this hypothesis. Overall, the role of Fe, either dosed to remove phosphorus or in this specific case to coagulate sludge, as a vital component in the AD process merits in-depth study. At present ranges of iron in waste activated sludge are very broad (2.9 to 80 mg/g TS) and do not relate to concomitant biogas production values in a coherent way (Park et al., 2006). The effect of several additives on the AD of KW was evaluated to estimate the role of A-sludge during KW digestion and to confirm that Fe is indeed the crucial limiting compound. The treatments to which macro- and micronutrients, both containing Fe, were added (KW-Macro, KW-Micro and KW-Methanostim) showed a more stable methanation process compared to the other two treatments. The addition of trace elements (KW-Micro and KW-Methanostim) was more effective compared to the addition of macronutrients (KW-Macro). However, all treatments expressed a decrease in methane production and an accumulation of VFA toward the end of the experiment, indicating that micronutrients could prevent immediate failure but not guarantee stable operation. This is in contrast to an other study in which Fe was considered essential to improve stability (Zhang & Jahng, 2012). The limited effect of the micronutrients could be attributed to the low amounts in which they were added. Together with the low contents in the KW feedstock itself, stable methanation could therefore only be supported in the beginning of the experiment, when sufficient levels of macro- and micronutrients were still available, provided by the inoculum sludge.

The microbial community was investigated by means of qPCR analysis on total archaea and total bacteria. Total archaea can be considered a valid estimation of total methanogens, because of the highly unfavourable conditions for non-methanogenic archaea in AD (Woese et al., 1990; Raskin et al., 1995). Total bacterial showed similar values in all reactors throughout the entire experiment, with the exception of the KW-Therm. The severe decline in

total bacteria in the KW-Therm on day 84 could be correlated to the high degree of process inhibition, as the pH decreased to a value of 4.03. Total archaea showed a certain degree of correlation to methane production, which is to be expected. The higher levels of total archaea in the 15-Mes and 15-Therm compared to the A-Mes and A-Therm can be directly correlated to the higher levels of methane production, which was on average three times higher in the 15-Mes and 15-Therm. Both the KW-Mes and KW-Therm showed a decrease in total archaea towards the end of the experiment, which can be directly correlated to the ceased methane production at the end of the experiment. The decrease was much more severe in the KW-Therm, indicating that methanogens were much more negatively affected in the KW-Therm, compared to the KW-Mes. The failing of the mono-digestion of KW was apparently caused by a decrease in the abundance of the methanogens. Indeed, methanogens are considered to be the most susceptible to inhibition or nutrient limitation, compared to the other micro-organisms in AD (Cresson et al., 2006; Chen et al., 2008; De Vrieze et al., 2012).

5. Conclusions

Mono-digestion of KW resulted in process failure and therefore it is recommended that KW be co-digested with other substrates. Co-digestion of KW with A-sludge led to stable methane production at mesophilic and thermophilic conditions, yet higher residual VFA concentrations during thermophilic digestion indicated a certain degree of instability. The addition of micro- and macronutrients resulted in a more stable mono-digestion of KW. This stabilizing effect can be potentially attributed to the high Fe content in the A-sludge compared to the KW. Total archaea positively correlated to methane production in the different reactors.

Acknowledgments

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CHAPTER 3: CO-DIGESTION OF MOLASSES OR KITCHEN WASTE WITH HIGH-RATE ACTIVATED SLUDGE RESULTS IN A DIVERSE MICROBIAL COMMUNITY WITH STABLE METHANE PRODUCTION.

This chapter has been redrafted after:

De Vrieze, J., Plovie, K., Verstraete, W., Boon, N. 2014. Co-digestion of molasses or kitchen waste with high-rate activated sludge results in a diverse microbial community with stable methane production. *Submitted to the Journal of Environmental Management.*

Abstract

Kitchen waste and molasses are organic waste streams with high organic content, and therefore, are interesting substrates for renewable energy production by means of anaerobic digestion. Both substrates, however, often cause inhibition of the anaerobic digestion process, when treated separately, hence, co-digestion with other substrates is required to ensure stable methane production. In this research, iron-rich A-sludge (sludge harvested from a high rate activated sludge system) was used to stabilize co-digestion with kitchen waste or molasses. Lab-scale digesters were fed with A-sludge and kitchen waste or molasses for a total period of 105 days. Increased methane production values revealed a stabilizing effect of concentrated A-sludge on kitchen waste digestion. Co-digestion of molasses with A-sludge also resulted in a higher methane production. Volumetric methane production rates up to 1.53 L L^{-1} d^{-1} for kitchen waste and 1.01 L L^{-1} d^{-1} for molasses were obtained by co-digestion with A-sludge. The stabilizing effect of A-sludge was attributed to its capacity to supplement various nutrients. Microbial community results demonstrated that both reactor conditions and substrate composition determined the nature of the bacterial community, although there was no direct influence of micro-organisms in the substrate itself, while the methanogenic community profile remained constant as long as optimal conditions were maintained.

1. Introduction

Anaerobic digestion (AD) can be considered a robust biochemical conversion process for the treatment of organic waste streams and the production of renewable energy (Appels et al., 2011). Full-scale applications are successfully installed all over the world, treating a wide variety of organic waste streams, with a yearly increase of 25% in the last years (Appels et al., 2011; Srirangan et al., 2012). Despite its successful application on an industrial scale, several operational parameters can affect proper performance, such as ammonia toxicity, overloading, salt stress, and deficiency of micronutrients, leading to acidification and subsequent failure of the process (Chen et al., 2008; Demirel & Scherer, 2011; De Vrieze et al., 2012). However, co-digestion can prevent process failure, as in-depth selection of suitable co-substrates with complementary characteristics can (1) favour positive interactions by the introduction of additional micronutrients, and (2) avoid inhibition by diluting concentrated waste streams, thus, increasing methane production (Mata-Alvarez et al., 2011; Bond et al., 2012). As such, numerous organic substrates already have been successfully co-digested on lab-scale and industrial scale (Mata-Alvarez et al., 2011; Balussou et al., 2012).

Sewage sludge is a very suitable substrate for co-digestion with other waste streams, such as the organic fraction of municipal solid waste, energy crops, manure and slaughterhouse waste (Balussou et al., 2012; Bond et al., 2012; Borowski & Weatherley, 2013; Pitk et al., 2013). A-sludge is high-rate activated sludge that is generated in the A-stage of the 'Adsorptions-Belebungsverfahren' or A/B process for municipal wastewater treatment (Boehnke et al., 1997). In this A-stage the organic carbon of the wastewater is converted into microbial biomass at very low sludge retention time values, as low as 2-3 days (Boehnke et al., 1997; Ge et al., 2013). It was demonstrated that this A-sludge had a high conversion efficiency to biogas (i.e. 85-90%) during AD (De Vrieze et al., 2013a; Ge et al., 2013). Hence, AD of A-sludge could lead to at least 30-50% energy savings and even energy self-sufficiency of the overall A/B process (Wett et al., 2007; Verstraete & Vlaeminck, 2011). Therefore, A-sludge can be suitable for co-digestion with other waste streams. Its high micronutrient content, low total nitrogen and salt concentrations, high biodegradability and intermediate organic matter content make A-sludge a substrate par excellence for co-digestion with substrates with excess of the latter (Borowski & Weatherley, 2013; Pitk et al., 2013).

Kitchen waste (KW) and molasses are widely available substrates**.** Indeed, a yearly world molasses production of 60 million tonnes is reached in recent years, and KW production in Europe alone is estimated at 2.5 billion tonnes per year (Chauhan et al., 2011; Ma et al., 2011;

Maung & Gustafson, 2011). Both substrates contain high levels of organic matter, which makes them suitable for biomethane production, however, their high Kjeldahl nitrogen and salt contents may cause failure of the AD process, when treated as single substrate (Satyawali & Balakrishnan, 2008; Banks et al., 2011; Ma et al., 2011). Molasses is an important sidestream product of the sugar production process that can be used as substrate for animal feed, but also as substrate for bio-ethanol production (Figure 3.1). In this Chapter, and also in Chapter 6 and 7 molasses was used as a model substrate of sidestream products from the sugar industry and bio-refinery. It was shown by De Vrieze et al. (2013a) that co-digestion of A-sludge and KW resulted in stable methane production, whereas digestion of only KW resulted in acidification of the digester and subsequent process failure. The exact role of A-sludge as stabilizing agent, however, still remains to be elucidated. First, A-sludge could dilute KW and molasses, thus avoiding organic overloading and/or inhibition of the methanogens through salt and ammonia stress (Satyawali & Balakrishnan, 2008; Banks et al., 2011; Ma et al., 2011). Second, sewage sludge in general, and A-sludge in particular, contains high levels of micronutrients, and therefore could serve as a stimulating agent in case of nutrient limitations (Zhang & Jahng, 2012; De Vrieze et al., 2013a). Third, A-sludge could act as a continuous inoculum, as it contains high concentrations of micro-organisms that may influence the microbial community in the anaerobic digester.

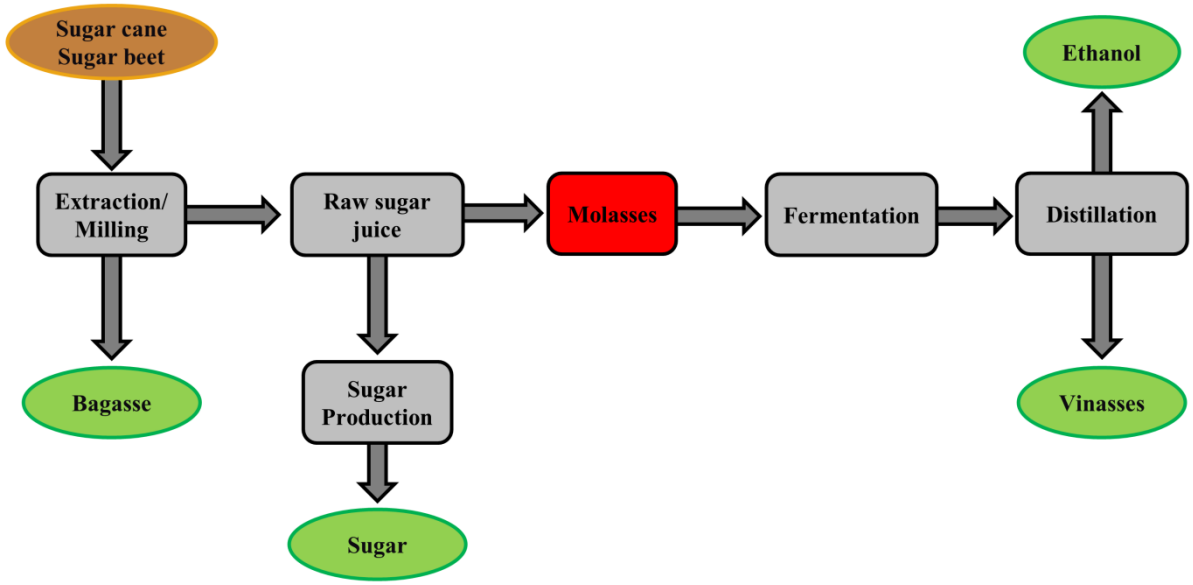

Figure 3.1 *Schematic overview of the central role of molasses in the sugar industry and bio-refinery (adapted from Di Nicola et al. (2011)).*

The objective of this research was therefore to determine the exact mechanism of A-sludge as stabilizing agent during co-digestion with KW or molasses in a continuous 105-days experiment. It was hypothesized that the main stabilizing mechanism of A-sludge was nutrient supplementation, rather than substrate dilution or additional biomass inoculation, because of its high nutrient content. This was evaluated by using concentrated sterilized or active A-sludge as co-substrate in a first phase, whereas in a second phase diluted A-sludge was used. The application of sterilized A-sludge, compared to active A-sludge, allows the investigation of the bioaugmentation potential of A-sludge, while the application of concentrated A-sludge, compared to diluted A-sludge, permits the evaluation of the nutrient supplementation hypothesis.

2. Materials and methods

2.1. Substrates and inoculum

The mesophilic anaerobic sludge that served as inoculum for the continuous digesters and the biochemical methane potential (BMP) test was obtained from the sludge digester of the municipal wastewater treatment plant of Dendermonde, Belgium. The sludge was diluted with tap water until a volatile suspended solids (VSS) concentration of 10 g L^{-1} was reached.

The A-sludge was obtained from the municipal wastewater treatment plant of Nieuwveer (Breda, the Netherlands), in which Fe was added to the A-stage, thus resulting in a case-specific high Fe content of the sludge. Two A-sludge batches that presented different compositions, i.e. concentrated A-sludge (Phase 1) and diluted A-sludge (Phase 2), were collected. The concentrated A-sludge was roughly two times more concentrated than the diluted sludge. The molasses, which originated from potato processing, were obtained from AVEVE, the Netherlands. The KW was obtained from the industrial kitchen of the Ghent University restaurant ‘De Brug’ (Gent, Belgium). This KW consisted of a various mixture of bread, rice, potatoes, (cooked) meat and fish, and cooked and non-cooked vegetables and fruits, and was thoroughly mixed with a kitchen blender. The A-sludge, molasses and KW were stored at 4 °C until use. The characteristics of the A-sludge (Phase 1 and 2), molasses and KW are shown in Table 3.1.

Table 3.1 *Characteristics of A-sludge1 (Phase 1), A-sludge2 (Phase 2), molasses and kitchen waste (KW) used to prepare the feeding of the reactors (n=3, except Fe and P). COD = chemical oxygen demand.*

Parameter	A-sludge1	A-sludge2	Kitchen waste	Molasses
Total COD (g L^{-1})	48.8 ± 12.0	22.6 ± 2.2	319 ± 33	452 ± 15
Total solids, TS (g L^{-1})	37.2 ± 0.3	19.9 ± 0.2	218 ± 2	615 ± 7
Volatile solids, VS (g L^{-1})	25.3 ± 0.4	13.3 ± 0.2	200 ± 2	450 ± 13
Total ammonia nitrogen, TAN (mg N L^{-1})	715 ± 20	n.d.	462 ± 4	1442 ± 11
Kjeldahl nitrogen, KjN (mg N L^{-1})	2866 ± 251	1895 ± 171	11303 ± 277	32400 ± 300
Total phosphorus, TP (mg P L^{-1})	941	436	575	6284
COD:N ratio	17.03 ± 4.44	11.93 ± 1.58	28.22 ± 3.00	13.95 ± 0.48
COD:P ratio	51.86 ± 12.75	51.83 ± 5.05	554.78 ± 57.39	71.93 ± 2.39
TS:VS ratio	1.47 ± 0.03	1.50 ± 0.03	1.09 ± 0.01	1.37 ± 0.04
COD:VS ratio	1.93 ± 0.48	1.70 ± 0.17	1.60 ± 0.17	1.00 ± 0.04
K (g L^{-1})	n.d.	n.d.	n.d.	81.4
Fe (mg L^{-1})	1538	733	12.6	63.6
Fe:P ratio	1.63	1.68	0.022	0.010

2.2. Experimental set-up and operation

2.2.1. Co-digestion experiment

Eight anaerobic glass lab-scale digesters with a total volume of 1 L and a working volume of 800 mL were applied as continuous stirred tank reactors (CSTR) and operated for 105 days at mesophilic conditions (34 ± 1 °C). The reactor itself (Figure 2.1) was a glass Schott Bottle (Duran Group GmbH, Mainz, Germany), closed with a rubber stopper trough which a plastic tube was inserted for biogas collection (a). Volumetric biogas production was collected in graduated columns for volumetric biogas production rate and composition determination (c). Mixing of the reactors was carried out using a magnetic stirrer (b).

The reactors were inoculated with anaerobic sludge at an initial concentration of 10 g VSS L^{-1} and feeding took place three times a week by replacing part of the sludge by fresh feed in a fed-batch mode. Each reactor was given a different feed, i.e. 100% A-sludge (Aact), 100% sterilized A-sludge (Aster), 85% A-sludge and 15% KW (Aact-KW), 85% sterilized A-sludge and 15% KW (Aster-KW), 90% A-sludge and 10% molasses (Aact-Mol), 90% sterilized A-sludge and 10% molasses (Aster-Mol), 15% KW and 85% tap water (KW) and 10% molasses and 90% tap water (Mol). The fractions of KW and molasses were chosen as to apply similar organic loading rates in the Aact-KW and Aster-KW, compared to the Aact-Mol and Aster-Mol, and to avoid overloading of the reactors. Sterilization of the A-sludge was carried out by autoclaving the sludge at 121 °C for 30 minutes.

The experiment consisted of a start-up phase of 28 days during which the organic loading rate (OLR) was gradually increased (Figure 3.2), and the sludge retention time (SRT) decreased from 80 to 20 days. Concentrated A-sludge (A-sludge1) was used during Phase 1 (day 28-62) and a new batch of more diluted A-sludge (no tap water addition) (A-sludge2) in Phase 2 (day 63-105), while the SRT was kept constant at 20 days, resulting in a higher OLR in Phase 1 than Phase 2 (Figure 3.2). No water was added to the A-sludge2, to maintain a similar pH, and buffer and salt concentration in A-sludge2 compared to A-sludge1.

Biogas production and composition, as well as the reactor pH (without adjustment) and volatile fatty acid (VFA) concentrations were analysed three times a week. Methane production values were reported at standard temperature and pressure (STP) conditions. Reactor samples were taken once a week for total and volatile solids (TS and VS) and total ammonia nitrogen (TAN) analysis. A biomass sample of 10 mLwas also collected on weekly basis, and stored at -20 °C for microbial community analysis.

2.2.2. Biochemical methane potential (BMP) test

A BMP test was carried out to estimate the anaerobic biodegradability of the A-sludge (sterilized and active A-sludge1 and 2), KW and molasses. The test was performed under mesophilic conditions (34 ± 1 °C) in triplicate in serum flasks with a total volume of 120 mL and a working volume of 80 mL. A control treatment that contained only inoculum was also performed in triplicate. The substrate to inoculum ratio was maintained at 0.5 g COD g^{-1} VSS. The required amounts of inoculum and substrate were added to the flask, after which they were connected to glass columns, in which biogas production was measured by means of water displacement. Biogas composition was evaluated at the end of the experiment, i.e. after 21 days. Methane yield was expressed as the volume of methane per gram of substrate VS, and COD yield as the fraction of substrate COD converted to methane. Both values were reported at STP conditions.

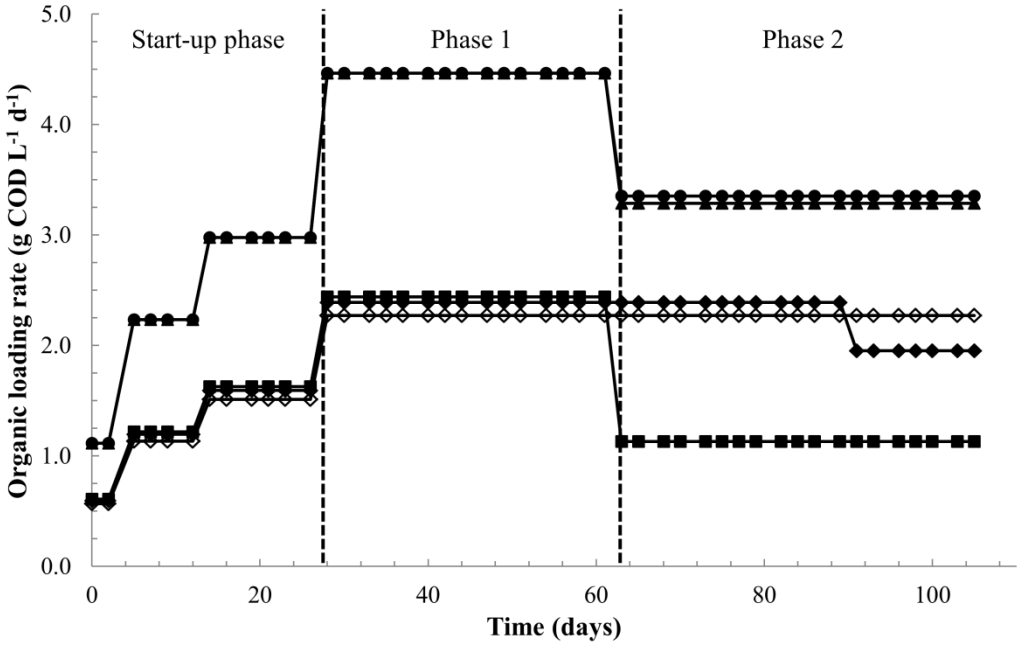

Figure 3.2 *Organic loading rate (OLR) of the 100% A-sludge (■), 85% A-sludge and 15% KW (●), 90% A-sludge and 10% molasses (▲), 15% KW and 85% tap water (♦) and 10% molasses and 90% tap water (◊) treatment. The OLR is the same for both the active and sterilized A-sludge treatment. In Phase 1 concentrated A-sludge (A-sludge1) was used, whereas in Phase 2 diluted A-sludge (A-sludge2) was used. The OLR was equal in the 85% A-sludge and 15% KW, and 90% A-sludge and 10% molasses treatment during the start-up phase and Phase 1, hence, the overlap of the symbols.*

2.2.3. Methanogenic activity test

A methanogenic activity test was set up to estimate the methanogenic potential of the A-sludge 1 and 2, both sterilized and active. This test was carried out in triplicate at 34 °C in sealed serum flasks with a total volume of 120 mL, in which 60 mL of the selected A-sludge was introduced. Biogas production was evaluated by means of gas pressure measurements, using a UMS-Tensiometer (Infield 7) device (UMS, Munchen, Germany), and reported at STP conditions. Biogas composition was measured after 1, 2, 4, 8, 14 and 21 days.

2.3. Microbial community analysis

Total DNA was extracted from the sludge samples by means of the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), following the manufacturer's instructions. Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 200 mg of sample was taken for DNA extraction.The DNA concentration in the extracts was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands), by measuring the absorbance ratios at 260 nm and 280 nm. The quality of the extracted DNA was evaluated on a 1% agarose gel.

Denaturing gradient gel electrophoresis (DGGE) was performed following the PCR protocol of Boon et al. (2002), using the total bacterial primers P338f-GC and P518r (Muyzer et al., 1993). The PCR was run with a 2720 thermal cycler (Applied Biosystems). The PCR product quality was verified on a 1% agarose gel. An INGENY phorU2X2 DGGE-system (Goes, the Netherlands) was subsequently used to prepare the 8% (w/v) polyacrylamide DGGE gel with a denaturing gradient ranging from 45% to 60%, consistent with the protocol of Boon et al. (2002). The obtained DGGE gel was processed using the Bionumerics software 5.1 (Applied Maths, Kortrijk, Belgium). Only bands with an intensity higher than 1% were considered. A matrix of similarities between the densiometric curves of the band patterns was calculated on the basis of the Pearson product-moment correlation coefficient.

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Analytical triplicates of a 10 to 100-fold dilution of the DNA-samples were analysed for the methanogenic populations Methanobacteriales, Methanomicrobiales, Methanosarcinaceae, and Methanosaetaceae. The primer sets used for the methanogenic populations Methanobacteriales (MBT), Methanomicrobiales (MMB),

Methanosarcinaceae (Msc), and Methanosaetaceae (Mst) were previously described by Yu et al. (2005). The reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, Wis) and consisted of 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure which consists of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C for all methanogenic populations. The qPCR data were represented as copies per gram of wet sludge.

2.4. Analytical techniques

Total and volatile suspended solids (TSS, VSS), TS and VS, TAN and chemical oxygen demand (COD) were determined according to Standard Methods. (Greenberg et al., 1992). Total P analysis was carried out by means of a Jenway 6400 spectrophotometer (Keison Products, Essex, UK), and the Fe content was analysed by means of an ICP-OES VISTA MPX (Varian, Munich, Germany). Biogas composition was analysed with a Compact GC (Global Analyser Solutions, Breda, the Netherlands), equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH_4, CO_2 and H_2 were determined using a thermal conductivity detector with a lower detection limit of 1 ppmv for each gas component. The volatile fatty acid (VFA) concentrations were measured by means of gas chromatography (GC-2014, Shimadzu®, The Netherlands) with a DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector (FID). Liquid samples were conditioned with sulphuric acid and sodium chloride, and 2-methyl hexanoic acid was used as internal standard for quantification of further extraction with diethyl ether. The prepared sample (1 µL) was injected at 200 ºC with a split ratio of 60 and a purge flow of 3 mL min^{-1}. The oven temperature increased by 6 ºC min^{-1} from 110 ºC to 165 ºC, where it was kept for 2 min. The FID had a temperature of 220 ºC. The carrier gas was nitrogen at a flow rate of 2.49 mL min^{-1}. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium).

3. Results

3.1. Co-digestion of KW and molasses with A-sludge

During the first 28 days of the experiment (start-up phase), methane production slowly increased in all treatments (Figure 3.3). The pH maintained values above 7.00, with the

exception of the KW treatment that demonstrated a pH value of 6.91 on day 23. Despite the stable pH, total VFA accumulated in several treatments during the start-up phase (Figure 3.4). Total VFA remained below 100 mg COD L^{-1} in the Aact, Aster, Aact-KW, Aster-KW, and KW during the first 28 days, whereas total VFA increased to 3.2, 2.1 and 0.9 g COD L^{-1} in the Aact-Mol, Aster-Mol and Mol treatments, respectively.

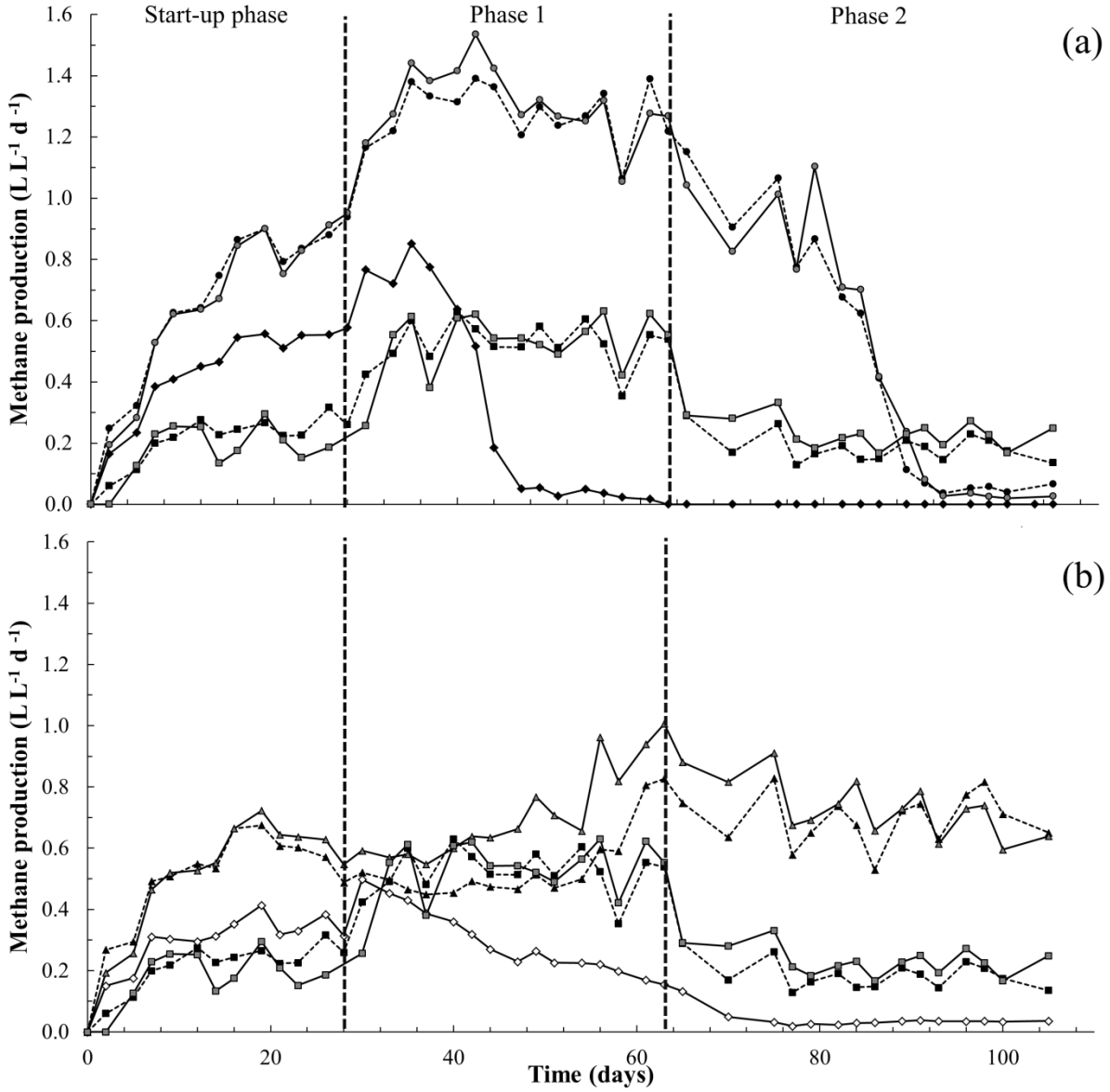

Figure 3.3 *Methane production of (a) the different combinations of kitchen waste, as well as the A-sludge control treatments, i.e. the 100% A-sludge (■), 100% sterilized A-sludge (■), 85% A-sludge and 15% KW (●), 85% sterilized A-sludge and 15% KW (●), and 15% KW and 85% tap water (♦) treatment, and (b) the different combinations of molasses, as well as the A-sludge control treatments, i.e. the 100% A-sludge (■), 100% sterilized A-sludge (■), 90% A-sludge and 10% molasses (▲), 90% sterilized A-sludge and 10% molasses (▲), and 10% molasses and 90% tap water (◊) treatment. The*

100% A-sludge (■) and 100% sterilized A-sludge (■) treatments were added to both figures for better comparison of the results.

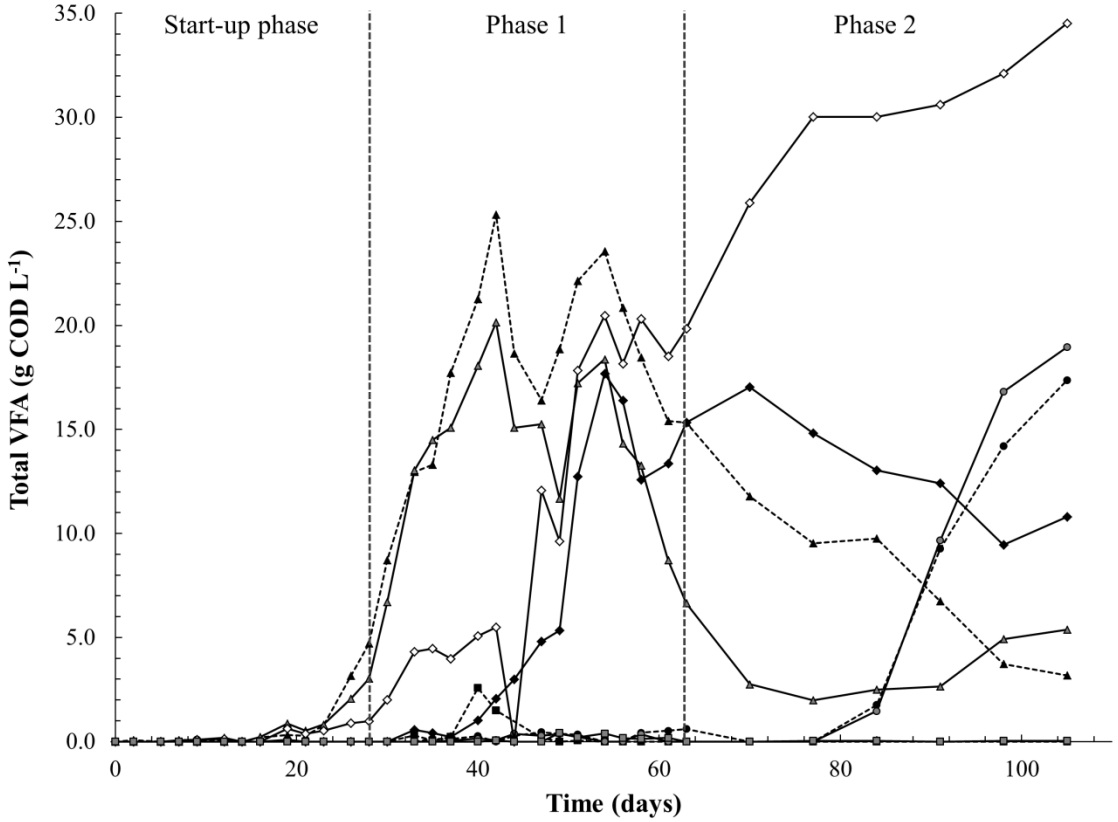

Figure 3.4 *Total volatile fatty acid (VFA) concentrations of the 100% A-sludge (■), 100% sterilized A-sludge (■), 85% A-sludge and 15% KW (●), 85% sterilized A-sludge and 15% KW (●), 15% KW and 85% tap water (♦), 90% A-sludge and 10% molasses (▲), 90% sterilized A-sludge and 10% molasses (▲), and 10% molasses and 90% tap water (◊) treatment.*

During Phase 1, co-digestion of concentrated A-sludge and KW resulted in stable methane production, with average values of 1.26 ± 0.12 L L^{-1} d^{-1} in Aact-KW and 1.29 ± 0.14 L L^{-1} d^{-1} in Aster-KW (Figure 3.3a). Total VFA remained below 600 mg COD L^{-1} in both treatments, in agreement with the stabilization of the pH within the optimal range (pH of 7.14 ± 0.07 and 7.14 ± 0.11 in Aact-KW and Aster-KW, respectively). Co-digestion of concentrated A-sludge and molasses reached only average methane production values of 0.54 ± 0.12 L L^{-1} d^{-1} in Aact-Mol and 0.70 ± 0.15 L L^{-1} d^{-1} in Aster-Mol (Phase 1, Figure 3.3b), despite the fact that the OLR was similar to the test with KW (Figure 3.2). Contrarily to co-digestion of A-sludge and KW, total VFA reached higher values of 25.3 g COD L^{-1} in the Aact-Mol and 20.1 g COD L^{-1} in the Aster-Mol (Figure 3.4). However, an average pH of 7.48 ± 0.13 and 7.61 ± 0.15 was maintained in Aact-Mol and Aster-Mol, respectively. Mono-digestion of both KW and

molasses failed when the OLR was fixed over 2 g COD $L^{-1}d^{-1}$ (methane production of 0.02 and 0.17 L L^{-1} d^{-1} and total VFA levels up to 13.4 and 18.5 g COD L^{-1} for the KW and Mol treatment, respectively, at the end of Phase 1). Meanwhile, digestion of both active and sterilized concentrated A-sludge remained constant during Phase 1 at average methane production values of 0.51 ± 0.10 L L^{-1} d^{-1} in Aact and 0.53 L L^{-1} d^{-1} in Aster. The pH remained above 7.00 in both treatments. The TAN concentration reached a maximum value of 2357 mg N L^{-1} in the Aact-Mol and 2127 mg N L^{-1} in the Aster-Mol at the end of Phase 1 (Figure 3.5), which corresponded to a free ammonia concentration of 98 and 133 mg N L^{-1}, respectively. Free ammonia concentrations remained below 11 mg N L^{-1} in the Aact-KW and Aster-KW.

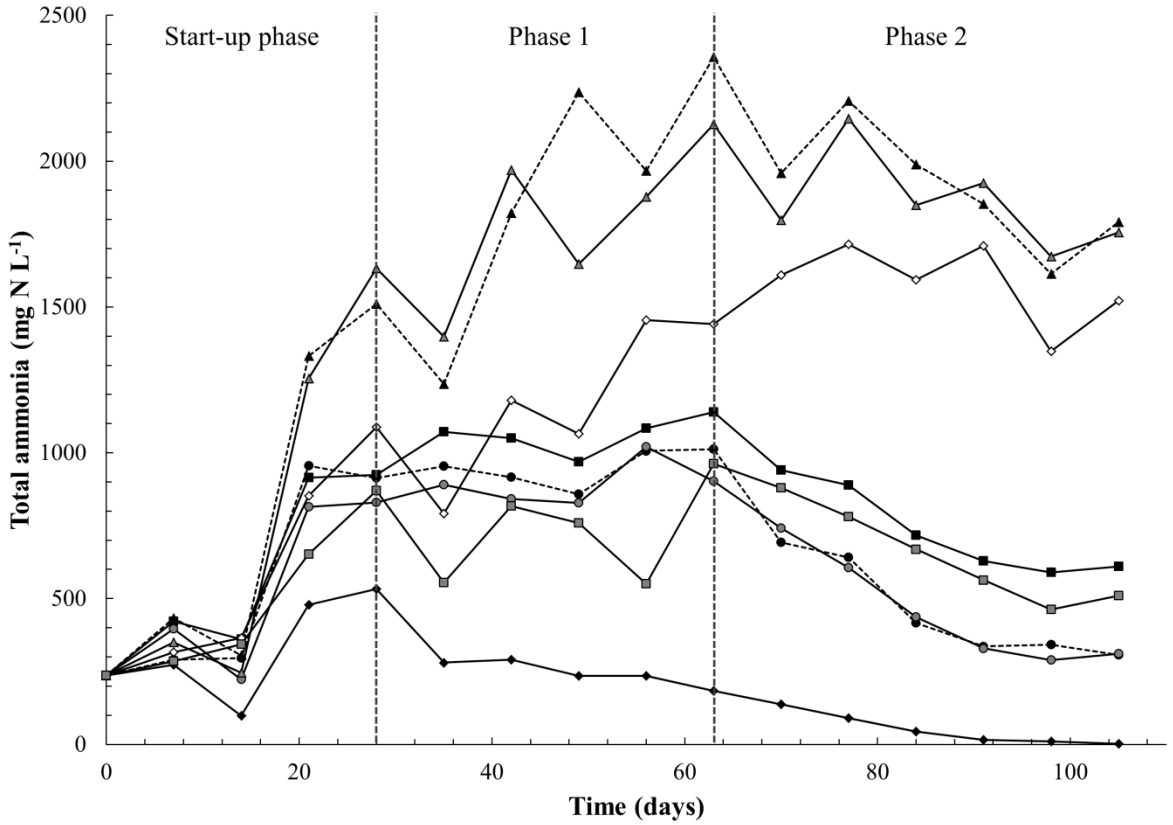

Figure 3.5 *Total ammonia concentrations (mg N L^{-1}) of the 100% A-sludge (■), 100% sterilized A-sludge (■), 85% A-sludge and 15% KW (●), 85% sterilized A-sludge and 15% KW (●), 15% KW and 85% tap water (♦), 90% A-sludge and 10% molasses (▲), 90% sterilized A-sludge and 10% molasses (▲), and 10% molasses and 90% tap water (◊) treatment.*

In Phase 2, diluted A-sludge was used for co-digestion with KW or molasses. The transition from concentrated to diluted A-sludge resulted in process failure for co-digestion of A-sludge with KW, while the performance of co-digestion of A-sludge with molasses even slightly increased (Figure 3.3). The decrease in methane production for KW co-digestion on day 91

(0.07 L L^{-1} d^{-1} in Aact-KW and 0.08 L L^{-1} d^{-1} in Aster-KW) was related to a lower pH (4.70 in Aact-KW and 4.95 in Aster-KW) and increased total VFA (9.3 g COD L^{-1} in Aact-KW and 9.7 g COD L^{-1} in Aster-KW). Methane production of molasses co-digestion was maintained at an average value of 0.70 ± 0.08 and 0.75 ± 0.11 L L^{-1} d^{-1} in Aact-Mol and Aster-Mol, respectively, in Phase 2 (Figure 3.3b). The pH remained constant at an average value of 7.86 ± 0.08 and 7.90 ± 0.15, and total VFA levels decreased to values of 3.2 and 5.4 g COD L^{-1} in Aact-Mol and Aster-Mol, respectively, at the end of Phase 2, which was 4-5 times lower compared to Phase 1 (Figure 3.4). A maximum TAN concentration of 2207 mg N L^{-1} was obtained in the Aact-Mol and 2145 mg N L^{-1} in the Aster-Mol in Phase 2 (Figure 3.5), which corresponded to a free ammonia concentration of 175 and 206 mg N L^{-1}, respectively.

3.2. Effect of A-sludge sterilization on methane production

A-sludge sterilization was carried out by autoclaving the sludge. Mono-digestion of active A-sludge (Aact) and sterilized A-sludge (Aster) resulted in both cases in stable methane production. Average methane production values of 0.51 ± 0.10 and 0.53 ± 0.10 L L^{-1} d^{-1} were obtained for Aact and Aster, respectively, during Phase 1 and 0.21 ± 0.10 and 0.25 ± 0.09 L L^{-1} d^{-1}, respectively, during Phase 2 (Figure 3.3). The decrease of methane production from concentrated to diluted A-sludge was in agreement with the reduced OLR and maximum methane potential, obtained from the BMP tests (Table 3.2). Methane production from the Aster was only slightly higher than the Aact. However, a punctual maximum total VFA concentration of 2.6 g COD L^{-1} was observed on day 40 for Aact, while this was only 410 mg COD L^{-1} in Aster (Figure 3.4).

Table 3.2 *Biochemical methane potential results of the A-sludge1 and 2 (active and sterilized), kitchen waste and molasses (n=3). All results are presented at STP conditions. Methane yield is expressed as the volume of methane per gram of substrate VS and COD yield as the fraction of substrate COD converted to methane.*

Substrate	Yield (mL CH_4 gVS^{-1})	COD Yield (%)
A-sludge1	306.9 ± 62.6	48.4 ± 9.9
A-sludge2	218.8 ± 71.5	34.5 ± 11.3
A-sludge1 – sterilized	412.5 ± 53.9	65.1 ± 8.5
A-sludge2 – sterilized	258.0 ± 61.1	40.7 ± 9.6
Molasses	275.3 ± 39.6	77.6 ± 11.2
Kitchen waste	262.4 ± 59.4	78.4 ± 9.0

Co-digestion of KW with A-sludge or sterilized A-sludge did not lead to a difference in methane production, with average values of 1.26 ± 0.12 and 1.29 ± 0.14 L L^{-1} d^{-1} for Aact-KW and Aster-KW, respectively, during Phase 1. A similar decrease in methane production was observed during Phase 2 (Figure 3.3a). Total VFA levels and pH values were also similar in Aact-KW and Aster-KW.

Co-digestion of molasses with sterilized A-sludge resulted in a slightly higher methane production, compared to co-digestion with active A-sludge during Phase 1, with methane production values of 0.54 ± 0.09 L L^{-1} d^{-1} for Aact-Mol and 0.70 ± 0.15 L L^{-1} d^{-1} for Aster-Mol. These results were in agreement with a lower pH in Aact-Mol (7.48 ± 0.13), compared to Aster-Mol (7.61 ± 0.15) and consequent lower total VFA concentrations in Aster, compared to Aact from day 37 to day 91 (Figure 3.4).

BMP test results confirmed the higher methane potential of the sterilized A-sludge compared to the active sludge, both for A-sludge 1 and 2 (Table 3.2). No indigenous methanogenic activity was observed in the sterilized sludge in the methanogenic activity tests, both in A-sludge1 and 2 after 21 days of operation. The active A-sludge showed indigenous methanogenic activity after 4 days of incubation at 34 ± 1 °C, with final values of 34.4 ± 1.3 and 48.8 ± 4.3 mL CH_4 g^{-1} VS, after 21 days, for A-sludge 1 and 2, respectively.

3.3. Microbial community analysis

3.3.1. Bacterial community clustering

Cluster analysis of the DGGE patterns was carried out by means of the Pearson correlation coefficient, to obtain on overview of bacterial community dynamics in the different treatments at different time points (Figure 3.6). First, the co-digestion treatments with sterilized and active sludge always clustered together, thus sterilization did not significantly affect the bacterial community. Second, both digesters treating only A-sludge (Aact and Aster) formed a single cluster, irrespective of the application of sterilization. Third, the application of molasses or KW as co-substrate for co-digestion with A-sludge does not seem to have a high differentiating impact on the total bacterial community. In fact, there appears to be a higher degree of clustering over time, and therefore also the degree of methane production and stability in the digester, than by co-substrate.

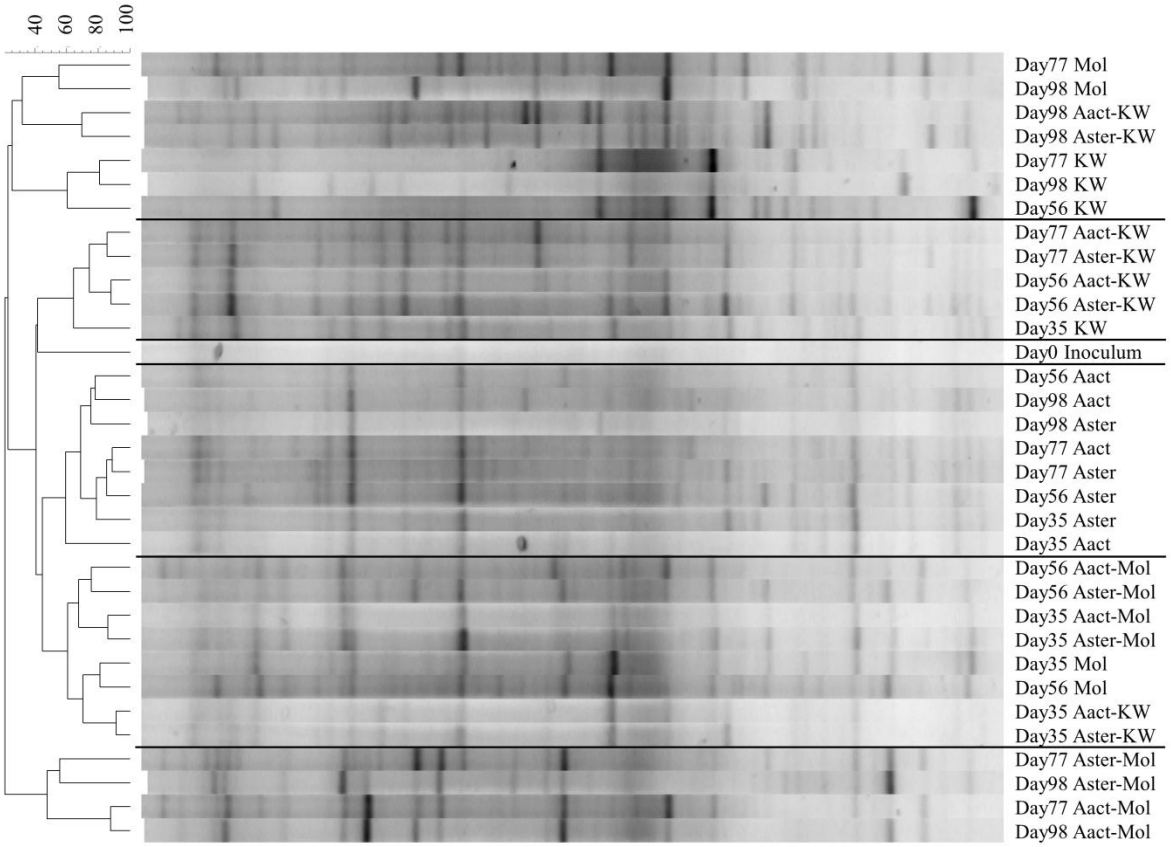

Figure 3.6 *Cluster analysis of the DGGE fingerprint of the bacterial community in the different reactors at different sampling times, i.e. at the start of the experiment (Inoculum at day 0), day 35 and day 56 (Phase 1), and day 77 and day 98 (Phase 2). Cluster analysis (WARD algorithm) of the DGGE patterns was performed based on the Pearson correlation and expressed as percentage.*

3.3.2. Methanogenic community analysis

Real-time PCR results of the Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae revealed a diverse methanogenic community in all samples, yet, a substantial evolution could be evaluated in several samples over time (Figure 3.7 and 3.8).

The inoculum sample (day 0 of the experiment) was dominated by Methanosaetaceae at 2.2 x 10^9 ± 3.8 x 10^8 copies g^{-1} of wet sludge (Figure 3.7a). Hence, acetoclastic methanogenesis in the inoculum sample was most likely the most important methanogenic pathway.

Methanosaetaceae appeared to dominate the overall methanogenesis during single A-sludge digestion (Aact and Aster), with values up to 4.3 x 10^9 ± 2.4 x 10^8 and 5.6 x 10^9 ± 1.9 x 10^9 copies g^{-1} for the Aact and Aster, respectively (Figure 3.7a). The Methanobacteriales had copy number values around 1 log unit higher in the Aact compared to the Aster, yet no other

differences were detected (Figure 3.8a). Both treatments exhibited a decreasing trend for all four methanogenic groups in the last sample (on day 98).

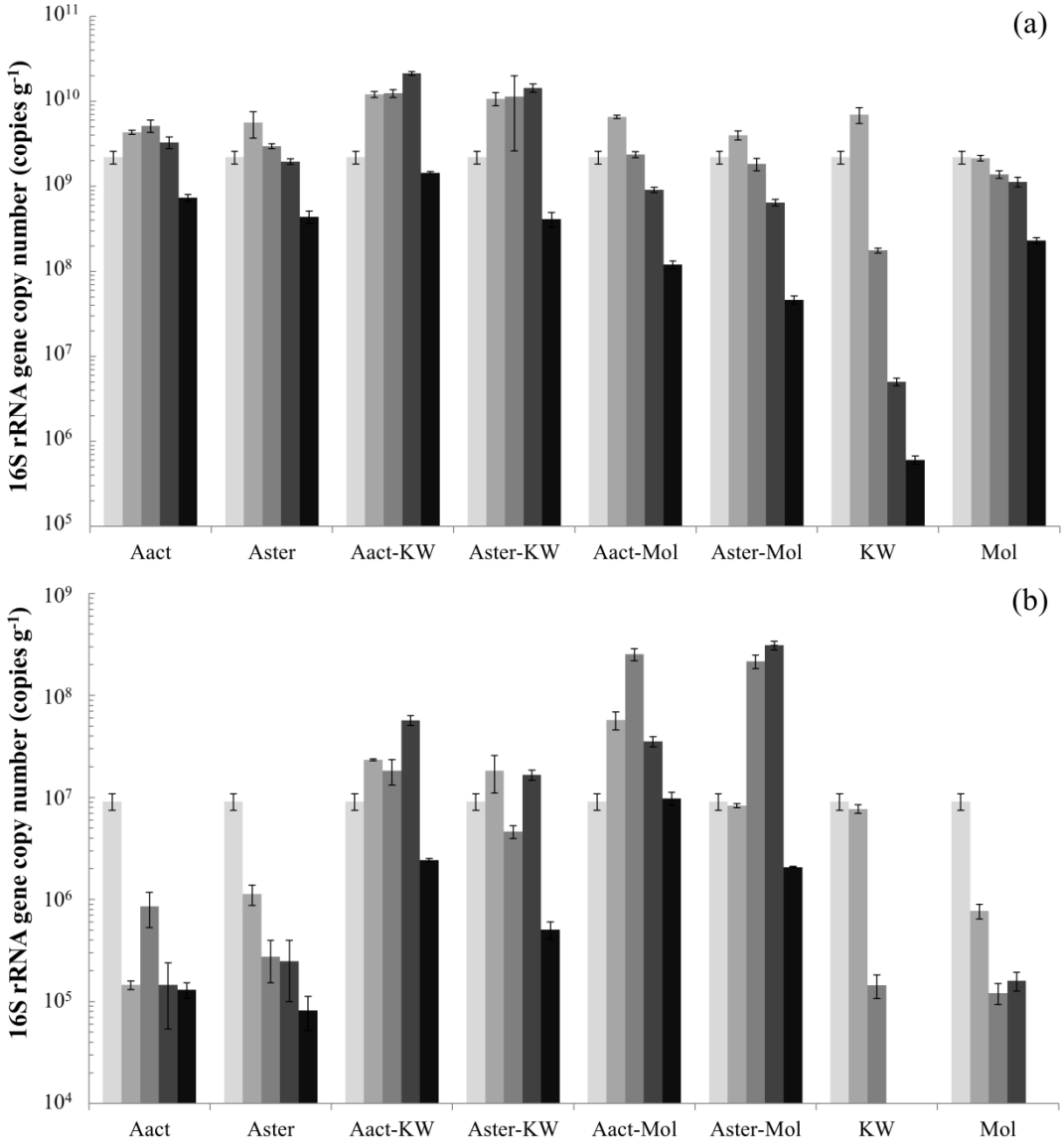

Figure 3.7 *Real-time PCR results of (a) the Methanosaetaceae and (b) the Methanosarcinaceae in the inoculum sample on day 0 (□), day 35 (■)and day 56 (■) (Phase 1), and day 77 (■) and day 98 (■) (Phase 2). Results are expressed as copies per gram of wet sludge. Average values of the triplicate analyses, together with the standard deviations are presented.*

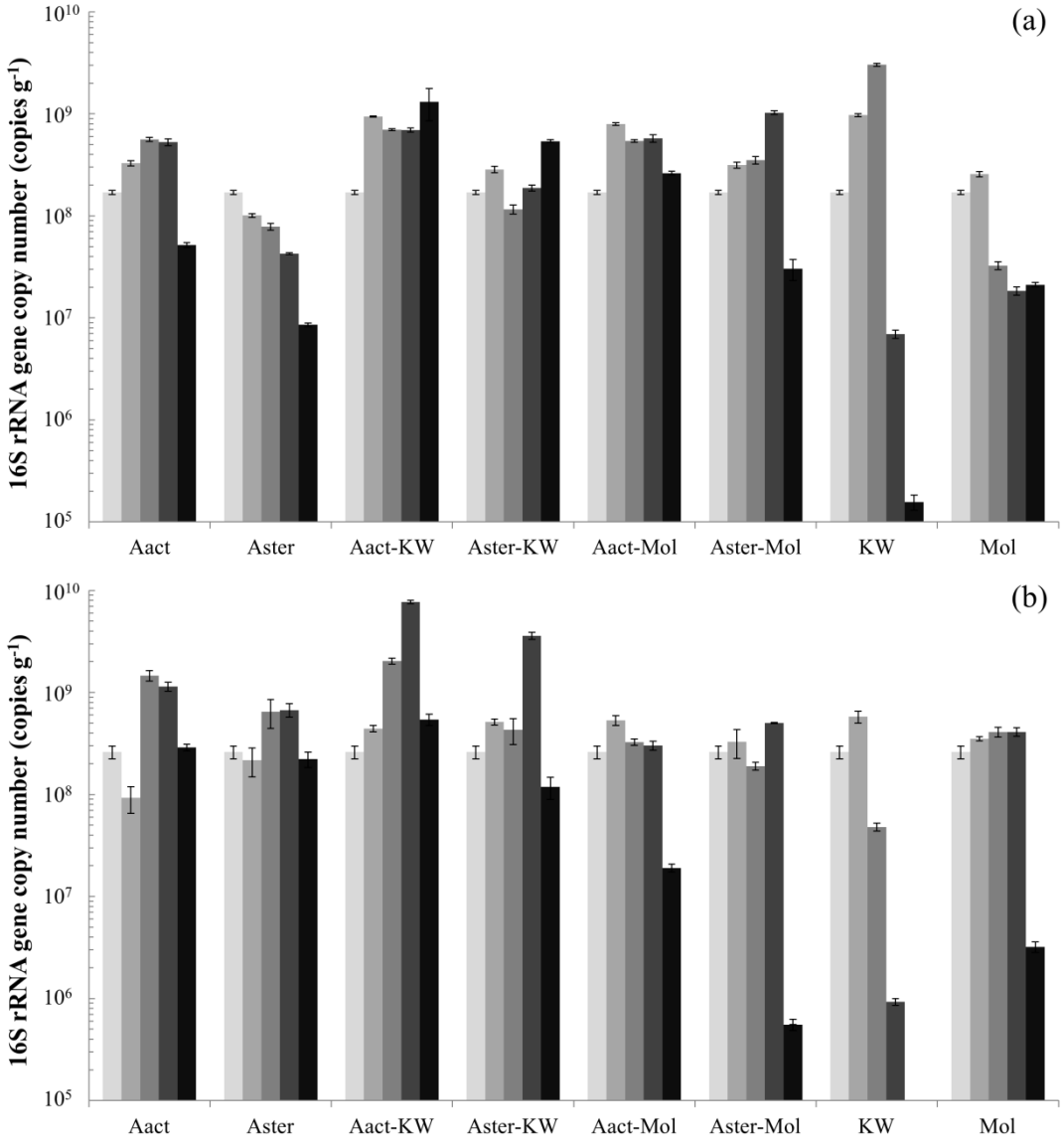

Figure 3.8 *Real-time PCR results of (a) the Methanobacteriales and (b) the Methanomicrobiales in the inoculum sample on day 0 (□), day 35 (■)and day 56 (■) (Phase 1), and day 77 (■) and day 98 (■) (Phase 2). Results are expressed as copies per gram of wet sludge. Average values of the triplicate analyses, together with the standard deviations are presented.*

Co-digestion of A-sludge and KW (Aact-KW and Aster-KW) resulted in overall higher methanogenic copy numbers, compared to the mono-digestion of A-sludge (Figure 3.7 and 3.8). Similar patterns could be observed for the Methanosaetaceae and Methanosarcinaceae in the Aact-KW and Aster-KW, with a sharp decrease in abundance in the last sample (Figure 3.7). The hydrogenotrophic groups Methanobacteriales and Methanomicrobiales did not show a decrease in the last sample, yet, total copy numbers were slightly higher in the Aact-KW compared to the Aster-KW. Both treatments had a high abundance of Methanomicrobiales on

day 77 with values of 7.7 x 10^9 ± 3.1 x 10^8 and 3.6 x 10^9 ± 2.9 x 10^8 copies g^{-1} in the Aact-KW and Aster-KW, respectively (Figure 3.8).

Both treatments in which A-sludge was co-digested with molasses had higher copy numbers of Methanosarcinaceae, compared to the other treatments, with maximum values of 2.5 x 10^8 ± 3.5 x 10^7 and 3.1 x 10^8 ± 2.9 x 10^7 copies g^{-1} for Aact-Mol on day 56 and Aster-Mol on day 77, respectively (Figure 3.7b). The other methanogenic groups demonstrated similar patterns, including a sharp decrease in Methanomicrobiales copy numbers, in both treatments on day 98 (Figure 3.8b).

Mono-digestion of KW resulted in a severe decrease from day 35 on for all methanogenic groups, with the exception of the Methanobacteriales that reached a maximum value of 3.0 x 10^9 ± 1.1 x 10^8 copies g^{-1} on day 56, after which they also decreased (Figure 3.8a), in accordance with methanogenic activity (Figure 3.3a). A similar decline took place for all methanogenic groups in the treatment with mono-digestion of molasses, yet, this decrease was not as severe as in the KW treatment.

Apart from the reactor medium, the concentrated A-sludge (A-sludge1), KW and molasses were also analysed for the different methanogenic groups. Whilst no methanogens could be detected in the KW and molasses, the A-sludge hosted methanogens nonetheless, i.e. 5.7 x 10^8 ± 6.7 x 10^7, 1.1 x 10^9 ± 3.3 x 10^7 and 2.9 x 10^6 ± 2.8 x 10^5 copies g^{-1} for the Methanomicrobiales, Methanosaetaceae and Methanosarcinaceae, respectively.

4. Discussion

4.1. Co-digestion of KW and A-sludge

Co-digestion of kitchen waste with A-sludge resulted in a stable start-up phase and constant high methane production levels, yet, only when concentrated A-sludge with a TS content of 37.2 ± 0.3 g L^{-1} (A-sludge1, Table 3.1) was used. However, when diluted A-sludge with a TS content of 19.9 ± 0.2 g L^{-1} (A-sludge2) was used in Phase 2, methane production decreased from 1.11 L L^{-1} d^{-1} to 0.06 L L^{-1} d^{-1}. Moreover, the increasing VFA levels confirmed process failure. Similar behaviour was observed either with activated or sterilized A-sludge. It was demonstrated by De Vrieze et al. (2013a) that stable methane production could be obtained during co-digestion of KW with A-sludge with a TS content of 27.6 ± 0.4 g L^{-1}. As the same amount of kitchen waste was used along the present study, it can be concluded that nutrient

limitation, rather than substrate inhibition, was the main cause for process failure (Banks et al., 2011; Zhang & Jahng, 2012; De Vrieze et al., 2013a). The substrate limitation effect could be attributed to the Fe content, and more specifically, to the very low Fe:P ratio in KW (Table 3.1). It was already observed in other studies that Fe is a crucial nutrient to ensure stable methane production (Demirel & Scherer, 2011; Schattauer et al., 2011). Anaerobic digestion of P-rich waste streams, however, leads to the formation of $FeHPO_4$ precipitates, which may cause a deficiency in Fe bioavailability (Stabnikov et al., 2004). Therefore, the Fe:P ratio can be considered a key parameter to ensure stable operation in AD.

This fact could also explain a proper performance of KW in BMP tests, as batch experiments present a sufficient nutrient load, compared to continuous reactors, thus allowing KW digestion in BMP tests, even when used as a single feeding source. Indeed, the inoculum sludge that was used to perform the BMP test had a high Fe:P ratio of 1.59, compared to the values for KW and molasses.

4.2. Co-digestion of molasses and A-sludge

A completely different pattern was, however, observed during co-digestion of molasses with A-sludge. Indeed, VFA accumulation was already observed during the start-up phase of the Aact-Mol and Aster-Mol, and the transition from concentrated to diluted A-sludge did not result in a further decrease in methane production. Residual VFA concentrations were, however, detected in both Aact-Mol and Aster-Mol throughout the entire experiment, yet, no failure took place. This state of 'inhibited steady state' that was previously described for free ammonia toxicity, may also be valid in this case (Angelidaki & Ahring, 1993). However, the maximum free ammonia concentration reached only 175 and 206 mg N L^{-1} in the Aact-Mol and Aster-Mol, respectively, values which are normally not considered to be toxic to methanogens (Appels et al., 2008; Chen et al., 2008; Rajagopal et al., 2013). The reactor in which single molasses was digested (Mol) showed a much stronger decrease in methane production and increase in VFA concentrations, compared to the Aact-Mol and Aster-Mol, although similar amounts of molasses were used in both treatments. These results therefore provide the indication that substrate inhibition, but also nutrient limitation, caused the accumulation of VFA during (co-)digestion of molasses. Indeed, the high salt levels in the molasses feed, especially potassium (81.4 g K^+ L^{-1}), can lead to methanogenesis inhibition (Satyawali & Balakrishnan, 2008). A potassium concentration of 5.85 g K^+ L^{-1} is assumed to cause 50% inhibition of acetoclastic methanogens, due to a neutralization of the cell

membrane potential from the passive influx of ions (Appels et al., 2008; Chen et al., 2008). Since the molasses feed comprised 10% of the total feed, a minimum potassium concentration of 8.14 g K^+ L^{-1} was reached in the Aact-Mol, Aster-Mol and Mol reactors, indicating potential inhibition. The gradual increase in methane production at the end of Phase 1 in the Aact-Mol and Aster-Mol is most likely due to a certain degree of adaptation of the methanogenic community to the high salt concentrations (Spanheimer & Muller, 2008).

4.3. Influence of A-sludge sterilization

Anaerobic digestion of sterilized A-sludge resulted in a small increase in methane production, compared to active A-sludge, either when A-sludge was used as a single substrate, or when it was co-digested with KW or molasses. This slight increase is in relation to the higher BMP values for the sterilized A-sludge (both concentrated and diluted), compared to the active A-sludge. The higher methane potential of the sterilized A-sludge is most likely a consequence of the autoclaving, which can, in fact, be considered a thermal pre-treatment (Ma et al., 2011; Monlau et al., 2013). The active A-sludge hosted an active indigenous methanogenic community, as methane production initiated four days after incubation at 34 °C in separate batch tests, while sterilized A-sludge had no indigenous methanogenic activity. This bioaugmenting potential of the A-sludge as a substrate led to higher concentrations of different methanogens in the treatments with active A-sludge, which was revealed by real-time PCR analysis (Figure 3.7 and 3.8). However, this had neither effect on methane production, nor on the bacterial community structure. Indeed, methane production was even slightly higher in the treatments with sterilized A-sludge (Figure 3.3) and bacterial clustering was not influenced by sterilization of the A-sludge (Figure 3.6).

These results are in contrast to the findings of Neumann and Scherer (2011), who detected a clear methanogenic population shift by adding compost to the AD. However, since in that particular study no sterilized control treatment was included, a nutrient influence could not be entirely ruled out, despite similar trace element compositions of the different inocula. Our results, hence, state that additional bioaugmentation by means of the substrate was not the main process behind the stabilizing effect of A-sludge, confirming that the stabilizing contribution of A-sludge is dominated by nutrient addition in AD.

4.4. Influence of the selected co-substrate on the microbial community

Co-digestion of A-sludge with kitchen waste and molasses resulted in a closely related bacterial community. This was rather surprising, given the large difference in composition between those two substrates. Molasses contains 11 times more P and 3 times more N than kitchen waste and is mostly sugar based, whereas kitchen waste contains more or less equal fractions of sugars, fats and proteins (Satyawali & Balakrishnan, 2008; Sirianuntapiboon & Prasertsong, 2008; Shin et al., 2010; Shen et al., 2013). Bacteria are responsible for the hydrolysis (i.e. the first step of the anaerobic digestion process), acidogenesis and acetogenesis, and usually do not pose serious problems, in contrast to the methanogens carrying out methanogenesis, the final step (Gujer & Zehnder, 1983; Sawayama et al., 2004; Chen et al., 2008; De Vrieze et al., 2012). Bacterial clustering was, nonetheless, influenced not only by substrate composition, but also by the operational conditions, at least for the KW and molasses (co-)digestion. However, it should be taken into account that only bacterial species with an abundance > 1% are visualized by means of DGGE (Boon et al., 2002), thus, only the dominant bacterial species were taken into account. This leads to the conclusion that both substrate composition and operational conditions, e.g. organic loading rate, pH and temperature, determine bacterial community organization and dynamics in AD.

There was only a limited effect of A-sludge sterilization on the methanogenic community. However, methanogenic community composition greatly differed between the reactors (co-)digesting molasses, compared to the ones (co-)digesting kitchen waste, which was in contrast to the bacterial community. Methanosaetaceae were the most abundant acetoclastic methanogens in the Aact-KW and Aster-KW, while Methanosarcinaceae reached a higher abundance in the Aact-Mol and Aster-Mol, with Methanosaetaceae, however, still showing the highest abundance. The methanogenic community remained constant, with Methanosaetaceae as the dominant (acetoclastic) methanogens, as long as optimal conditions were maintained. A deviation from these optimal conditions, as observed in the Aact-Mol, Aster-Mol, Mol and KW reactors (Phase 1 and 2) and Aact-KW and Aster-KW reactors (Phase 2) led to a shift in the methanogenic community. This demonstrates that substrate composition only determines the methanogenic community, when it leads to sub-optimal conditions for methanogenesis. This can be explained by the fact that the methanogenic community is much more susceptible to disturbances, such as elevated ammonium, VFA and salt concentrations, than the bacterial community (Chen et al., 2008; De Vrieze et al., 2012).

The dominance of Methanosaetaceae over Methanosarcinaceae during Phase 1 in the Aact-KW and Aster-KW was expected, as residual VFA remained below a value of 100 mg COD L^{-1}, thus favouring the growth of Methanosaetaceae (Gujer & Zehnder, 1983; Conklin et al., 2006; De Vrieze et al., 2012). The higher abundance of Methanosarcinaceae in the Aact-Mol and Aster-Mol, compared to the Aact-KW and Aster-KW, relates to the elevated residual VFA, most likely caused by the high concentration of potassium and other potential inhibiting compounds contained in the molasses (Conklin et al., 2006; Satyawali & Balakrishnan, 2008; Fang et al., 2011b; De Vrieze et al., 2012). Hence, it appears that kitchen waste (co-)digestion sustains Methanosaetaceae as the dominant acetoclastic methanogens, due to the maintenance of optimal conditions (in Phase 1), whereas molasses (co-)digestion creates suitable conditions for growth of Methanosarcinaceae.

5. Conclusions

Co-digestion of A-sludge with kitchen waste or molasses resulted in stable methane production, yet, in the case of kitchen waste, this stabilizing effect was reached only when using concentrated A-sludge as co-substrate. The stabilizing effect of A-sludge in anaerobic digestion could not be attributed to bioaugmentation, despite its indigenous methanogenic activity, or substrate dilution, and therefore was dominated by nutrient addition. Nonetheless, these results should be interpreted with care, since no triplicate tests were carried out. In order to validate the actual mechanism behind the stabilizing effect of A-sludge, triplicate experiments should be carried out. Molecular results revealed a constant methanogenic community with dominance of Methanosaetaceae at optimal conditions, while a shift in the methanogenic community was observed at sub-optimal conditions, irrespective of the substrate. The bacterial community was selected mainly by operational conditions and substrate composition.

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CHAPTER 4: REPEATED PULSE FEEDING INDUCES FUNCTIONAL STABILITY IN ANAEROBIC DIGESTION

This chapter has been redrafted after:

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Abstract

Anaerobic digestion is an environmental key technology in the future bio-based economy. To achieve functional stability, a minimal microbial community diversity is required. The microbial community should also have a certain 'elasticity', i.e. the ability to rapidly adapt to sub-optimal conditions or stress. In this study it was evaluated whether a higher degree of functional stability could be achieved by changing the feeding pattern, which can change the evenness, dynamics and richness of the bacterial community. The first reactor ($CSTR_{stable}$) was fed on daily basis, whereas the second reactor ($CSTR_{dynamic}$) was fed every two days. An average methane production value of 0.30 L CH_4 L^{-1} d^{-1} was obtained in both reactors, although daily variation was up to four times higher in the $CSTR_{dynamic}$ compared to the $CSTR_{stable}$ during the first 50 days. Bacterial analysis revealed that this $CSTR_{dynamic}$ had a higher degree of variation in the bacterial community dynamics, however, no statistical confirmation could be obtained, since no triplicate analyses were performed. The $CSTR_{dynamic}$ also appeared to be more tolerant to an organic shock load of 8 g COD L^{-1} and total ammonia nitrogen levels up to 8000 mg N L^{-1}. These results suggest that the regular application of a limited pulse of organic material and/or a variation in the substrate composition might promote higher functional stability in anaerobic digestion.

1. Introduction

Anaerobic digestion (AD) is a well-known and frequently used process for renewable energy production from organic waste. The European Union stated that 20% of the European energy demands should be originating from renewable energy sources by the year 2020, to which AD has to contribute for at least 25% (Holm-Nielsen et al., 2009). Anaerobic digestion will play a major role in the future bio-based economy by the conversion of low value organic products into biogas (Mata-Alvarez et al., 2000; Verstraete et al., 2005). It offers several advantages over other processes treating organic waste streams, such as the production of biogas and a substantial decrease and stabilization of the organic waste. A high loading rate, limited nutrient demands and low operational control and maintenance costs are additional advantages as well (Mata-Alvarez et al., 2000; Lesteur et al., 2011; Wijekoon et al., 2011).

A wide diversity of organic substrates can be converted to methane and CO_2 by means of AD. Stable conversion of these diverse substrates requires functional stability, i.e. constant stable methane production and a certain redundancy towards stress. It is assumed that a minimal diversity in the microbial community is necessary to achieve functional stability (Briones & Raskin, 2003; Riviere et al., 2009). Each step in the degradation pathway of the organic compounds of the substrate is conducted by at least one micro-organism. The first three steps of the AD system (hydrolysis, acidogenesis and acetogenesis) are carried out by bacteria, whereas archaea are responsible for methanogenesis, the last step (Gerardi, 2003b). This bacteria - archaea succession normally yields an almost four times higher bacterial diversity compared to the archaeal diversity in stable anaerobic digesters (Fernandez et al., 1999; Briones & Raskin, 2003). Both bacterial and archaeal diversity are of major importance, because they contribute to the stability of the digesters. A higher diversity creates the potential of multiple pathways for the degradation of a certain organic compound, hence yielding functional redundancy (Peterson et al., 1998; Briones & Raskin, 2003; Carballa et al., 2011).

It is important to indicate that microbial diversity as such does not necessarily imply functional stability, i.e. high methane production, as also the ability of the microbial community to rapidly adapt to sub-optimal conditions is of crucial importance (Briones & Raskin, 2003; Dearman et al., 2006; Carballa et al., 2011). Low microbial diversity can coincide with a high functional stability, indicating that the flexibility of the community, instead of its diversity, is crucial to ensure stable operation (Haruta et al., 2002; Dearman et al., 2006). A dynamic microbial community, together with a high initial evenness are considered to be of vital importance to guarantee functional stability in microbial

communities (Fernandez et al., 1999; Wittebolle et al., 2009a; Boon et al., 2011; Carballa et al., 2011). The evenness, dynamics and diversity of a microbial community in AD greatly depend on the reactor conditions (e.g. pH, TAN and salt concentration or conductivity), feed composition (e.g. total nitrogen and organic matter content) and feeding pattern (e.g. pulse or continuous feeding) (Conklin et al., 2006; Dearman et al., 2006; Krakat et al., 2011).

The objective of this study was to evaluate whether a higher degree of functional stability could be achieved by changing the feeding pattern, which may influence the evenness, dynamics and diversity of the microbial community in AD. To achieve this, the effect of a difference in the feeding pattern in AD on (1) methane production, (2) bacterial community evenness, dynamics and richness and (3) tolerance of the reactor to several impairments, by means of a short-term stress test, was investigated. The microbial resource management (MRM) approach was implemented to gain insight in the microbial community organization in the anaerobic digesters (Marzorati et al., 2008; Read et al., 2011). The microbial community parameters range-weighted richness Rr (the amount of species), dynamics Dy (number of species that on average come to significant dominance during a defined time interval, in this case 7 days) and community organization Co (which indicates the evenness of the community) were determined, based on the bacterial DGGE-profile (denaturing gradient gel electrophoresis), and linked to the reactor performance and stress tolerance (Marzorati et al., 2008; Read et al., 2011).

2. Material and methods

2.1. Experimental set-up and operation

Two anaerobic lab-scale continuously stirred tank reactors (CSTR), each with a total volume of 10 L and a working volume of 8 L, were operated for 73 days under mesophilic conditions, (34 ± 1 °C) at a hydraulic retention time (HRT) of 20 days. An operational volume of 8 litres was chosen for the reactors, since these reactors are reproducible, as indicated in earlier preliminary research (data not shown) and other similar studies (Wittebolle et al., 2008; Carballa et al., 2011; Zamalloa et al., 2012). The reactors were inoculated with mesophilic sludge, which originated from a domestic wastewater treatment plant (Ossemeersen, Belgium). This sludge was diluted with tap water until a volatile suspended solids (VSS) concentration of 10 g VSS L^{-1} was obtained. The two reactors were both subjected to a daily pulse loading rate of 1 g COD L^{-1} d^{-1} (chemical oxygen demand) during the first 24 days of

the experiment. After 24 days, this daily feeding pattern was continued in reactor one ($CSTR_{stable}$), whereas the second reactor ($CSTR_{dynamic}$) was fed every two days with the same average loading rate of 1 g COD L^{-1} d^{-1}. The composition of the synthetic feed was based on the SYNTHES feed (Table 4.1) (Aiyuk & Verstraete, 2004). This SYNTHES feed is a synthetic raw domestic sewage suitable for AD and was developed to apply a feed with constant stable characteristics (Aiyuk & Verstraete, 2004). This SYNTHES feed contains all components necessary to ensure stable growth and metabolic activity of the microbial community in AD. A synthetic feed was selected to ensure a constant and well-defined composition of the substrate, to make sure that no unforeseen (negative) effects of the substrate on the AD process could take place.

Table 4.1 *Composition of the synthetic feed*

Component	Concentration
Carbon source	(mg L^{-1})
Starch	18000
Milk powder	2000
Yeast extract	200
Tryptic soy	200
Buffer	(mM)
KH_2PO_4	10
K_2HPO_4	10
$NaHCO_3$	20
Macronutrients	(mg L^{-1})
NH_4Cl	500
$CaCl_2.2H_2O$	200
$MgCl_2.6H_2O$	100
$Fe_2(SO_4)_3$	100
Trace elements	(µg L^{-1})
$NiSO_4.6H_2O$	500
$MnCl_2.4H_2O$	500
$FeSO_4.7H_2O$	500
$ZnSO_4.7H_2O$	100
H_3BO_3	100
$Na_2MoO_4.2H_2O$	50
$CoCl_2.6H_2O$	50
$CuSO_4.5H_2O$	5

The pH of both reactors was monitored and adjusted on daily basis to a value of 7.2 with a NaOH solution of 2 M. The biogas production and content were measured on daily basis and reported at STP (standard temperature and pressure) conditions. Total biogas production was monitored by means of a gas meter. Effluent samples were taken three times a week for volatile fatty acids (VFA) analysis and once a week for soluble COD (COD_{sol}) and total ammonia nitrogen (TAN). From day 24 on, a sample of 10 mL of the anaerobic biomass was taken every week to examine the bacterial community. These samples were subsequently stored at -20°C until DNA extraction was performed.

2.2. Short-term stress test

The short-term stress test at the end of the experiment, i.e. after 73 days, was implemented to estimate the tolerance of both reactors to high concentrations of TAN and sulphate, low pH values and high organic loading rates. Several sub-samples were taken of the two main reactors on day 73 and all treatments were performed on three samples, which can be considered biological replicates, from each reactor. Ammonium was added as NH_4Cl, sulphate as Na_2SO_4 and the pH was lowered with a 2 M HCl solution. The same feed as during operation of the main experiment was used for both the normal feeding and the high OLR treatment. All treatments for both reactors received a daily feeding of 1 g COD L^{-1} d^{-1}, with the exception of the high organic loading rate treatment in which the OLR was raised every day (Table 4.2). The test was carried out in airtight penicillin bottles with a volume of 120 mL, which contained 50 mL of biomass from the $CSTR_{stable}$ or $CSTR_{dynamic}$, during a period of four days. Both biogas production and composition and pH were measured on daily basis. Feeding was performed and samples were taken by means of a syringe to keep the bottles air-tight. Gas production was monitored by means of a gas syringe.

2.3. Microbial community analysis

Total DNA was extracted from the sludge samples and subsequently purified according to the method of Boon et al. (2000). Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 2 g of sample was taken for DNA extraction. Denaturing gradient gel electrophoresis (DGGE) on the total bacterial community was performed following the PCR protocol of Boon et al. (2002), using the primers P338f-GC and P518r,

targeting total bacteria (Muyzer et al., 1993). The PCR was run with a 2720 thermal cycler (Applied Biosystems). The quality of the PCR product was verified on a 1% agarose gel. An INGENY phorU2X2 DGGE-system (Goes, The Netherlands) was subsequently used to run a 8% (w/v) polyacrylamide DGGE gel with a denaturing gradient ranging from 45% to 60%, consistent with the protocol of Boon et al. (2002). The obtained DGGE gel was processed using the Bionumerics software 5.1 (Applied Maths, Kortrijk, Belgium). Only bands with an intensity higher than 1% were considered. The DGGE results were used to estimate the theoretical ecological parameters range-weighted richness (Rr), dynamics (Dy) and community organization (Co), as stated above, of the bacterial communities in both reactors (Marzorati et al., 2008; Read et al., 2011). The Rr values were determined based on the number of bands in the DGGE pattern and the percentage of the denaturing gradient between the first and the last band of the pattern, as described by Marzorati et al. (2008). A matrix of similarities between the densiometric curves of the band patterns was calculated by means of the Pearson product-moment correlation coefficient, from which the Dy values were deducted (Marzorati et al., 2008). The Co value was determined based on the number and the intensity of the bands in the DGGE pattern. This value is deducted from the Gini coefficient, which describes a specific degree of evenness, by means of a measurement of the normalized area between a given Pareto-Lorenze curve and the perfect evenness line. The higher the Co value, the more uneven the community is (Marzorati et al., 2008; Wittebolle et al., 2009a).

Table 4.2 *Short-term stress test set-up. The values presented for ammonium, sulphate and acidification are final concentrations in the reactor (n=3).*

Stressor	Day 1	Day 2	Day 3	Day 4
Control	-	-	-	-
Ammonium (mg N L^{-1})	1000	2000	4000	6000
Sulphate (mg L^{-1})	500	1000	2000	4000
High OLR (g COD L^{-1} d^{-1})[a]	2	4	6	8
Acidification with HCl (mmol L^{-1})	2	6	12	18
Acidification with HCl (final pH)	7.27 ± 0.05	6.97 ± 0.06	6.72 ± 0.04	6.44 ± 0.02

[a] In every treatment, the OLR was 1 g COD L^{-1} d^{-1}, except for the high organic loading rate treatment, in which the OLR was raised every day, as presented in the table.

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Analytical triplicates of a 10 to 100-fold dilution of the DNA-samples were analysed for Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and

Methanosaetaceae, using the primer sets described by Yu et al. (2005). A reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, Wis), and contained 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure for all 4 groups which consisted of a predenaturation step of 10 min at 94°C, followed by 40 cycles of 10 s at 94°C and 1 min at 60°C. The qPCR data were represented as copies of the target gene per gram of wet sludge.

2.4. Analytical techniques

Total suspended solids (TSS), VSS, TAN, total COD (COD_{tot}) and COD_{sol} were determined according to Standard Methods (Greenberg et al., 1992). The VFA were extracted with diethyl ether and measured in a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands), equipped with a DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector. Biogas composition was analysed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), which was equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH_4, CO_2 and H_2 were determined by means of a thermal conductivity detector. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium).

2.5. Statistical analysis

Correlation coefficients between the ecological parameters Rr, Dy and Co and the variation of the 7-days moving window average methane production were determined by means of the two-tailed Spearman's Rank Order Correlation test, for which the statistical software SPSS (IBM SPSS Statistics, Version 20, IBM Corp., Armonk, New York, USA) was used. This software was applied to estimate whether there was a significant linear correlation. A statistical significance level of 5 % was applied.

3. Results

3.1. Anaerobic reactors performance

During the first 24 days of the experiments, both reactors were operated under similar conditions, i.e. a daily loading rate of 1 g COD L^{-1} d^{-1}. An average methane production rate of

0.31 ± 0.07 L L^{-1} d^{-1} was achieved in both reactors, which corresponds to a removal efficiency of 86.6 %. On day 24, both reactors were mixed to start phase 2 with the same inoculum in the 2 reactors. From day 24 until day 73, both reactors were run at a different feeding pattern, daily vs. every two days feeding. A 7-days moving window, together with the in-window variation of the methane production was determined for the $CSTR_{stable}$ (Figure 4.1a) and the $CSTR_{dynamic}$ (Figure 4.1b), for each day of operation. Each value represents the average and the variation of the value on the day itself and the 6 previous days. This 7-days moving window of the methane production was deviated to achieve an accurate comparison between methane production and the ecological parameters, which were determined every seven days.

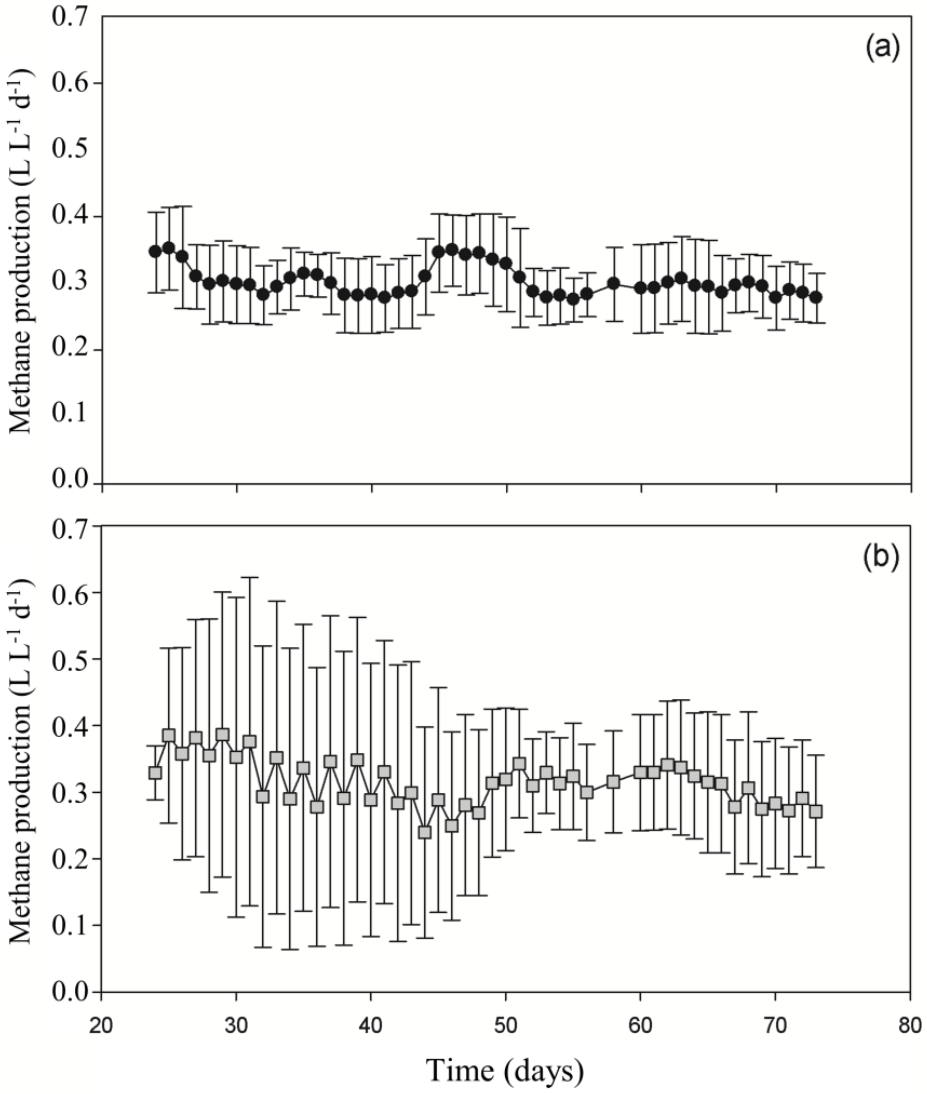

Figure 4.1 *Performance of the $CSTR_{stable}$ (●) and $CSTR_{dynamic}$ (■) in terms of methane production. A 7-days moving window, together with the in-window variation of the methane production is visualized for (a) the $CSTR_{stable}$ and (b) the $CSTR_{dynamic}$, for each day of operation. Each value represents the average and the variation of the value on the day itself and the 6 previous days.*

The average methane production was 0.28 ± 0.06 L L^{-1} d^{-1} in the $CSTR_{stable}$ and 0.29 ± 0.15 L L^{-1} d^{-1} in the $CSTR_{dynamic}$. Both reactors thus demonstrated an equal average methane production, yet with elevated daily variations in the $CSTR_{dynamic}$ compared to the $CSTR_{stable}$. These daily variations were highest in the beginning, but slowly declined towards the end of the experiment (Figure 4.1). The average COD removal efficiency was 77.8 en 81.2 % over time in the $CSTR_{stable}$ and $CSTR_{dynamic}$, respectively.

The $CSTR_{stable}$ and $CSTR_{dynamic}$ had an average pH of 7.11 ± 0.07 and 7.10 ± 0.08 respectively, indicating that the average pH, as well as this variation was similar between the two reactors, although the $CSTR_{dynamic}$ demonstrated more daily variation. Soluble COD (COD_{sol}) remained below 300 mg COD L^{-1} in the $CSTR_{stable}$, with an average value of 221 ± 47 mg COD L^{-1}. This was in contrast to the $CSTR_{dynamic}$, which demonstrated a maximum COD_{sol} concentration of 613 mg COD L^{-1} on day 73 and an average value of 347 ± 130 mg COD L^{-1}. The residual VFA concentration remained below the detection limit of 2 mg L^{-1} in both reactors for the entire period of the experiment. The TAN concentration remained below 523 mg N L^{-1} in the $CSTR_{stable}$ and 506 mg N L^{-1} in the $CSTR_{dynamic}$. COD_{tot}, VS and TS gave similar results for both reactors (data not shown).

3.2. Short-term stress test

A substantial difference in tolerance to ammonium was observed between the two reactors, since the relative methane production (the relation between the methane production of the treatment and the control) was 10 to 50% higher in the $CSTR_{dynamic}$ compared to the $CSTR_{stable}$ (Figure 4.2a), which indicates that the $CSTR_{dynamic}$ is more tolerant to high ammonium concentrations. No differences in pH were detected.

An elevated organic loading rate had a different effect on the different reactors as well (Figure 4.2b). During day 1 and 2, which corresponds to an OLR of 2 and 4 g COD L^{-1} d^{-1} respectively, no differences in terms of methane production could be detected between both reactors. On day 3 and 4, however, during which an OLR of 6 and 8 g COD L^{-1} d^{-1}, respectively, was applied, methane production was 27% higher on day 3 and even 57% higher on day 4 in the $CSTR_{dynamic}$ compared to the $CSTR_{stable}$. These results are also reflected in the pH, which was 6.22 ± 0.03 in the $CSTR_{dynamic}$ and 5.04 ± 0.12 in the $CSTR_{stable}$ on day 4, indicating severe acidification of the $CSTR_{stable}$. Elevated concentrations of sulphate and the induction of acidification by means of HCl yielded no effect on methane production in and

between both reactors. There was no difference in methane production and pH between the sulphate and control treatment. Acidification by means of HCl did decrease the pH to a value of 6.43 ± 0.01 in the $CSTR_{stable}$ and 6.44 ± 0.03 in the $CSTR_{dynamic}$ on day four. This resulted in a decrease of 0.3 pH units compared to the control treatments (6.69 ± 0.05 and 6.74 ± 0.04 for the $CSTR_{stable}$ and $CSTR_{dynamic}$, respectively), yet methane production decreased with only 10% compared to the control treatment.

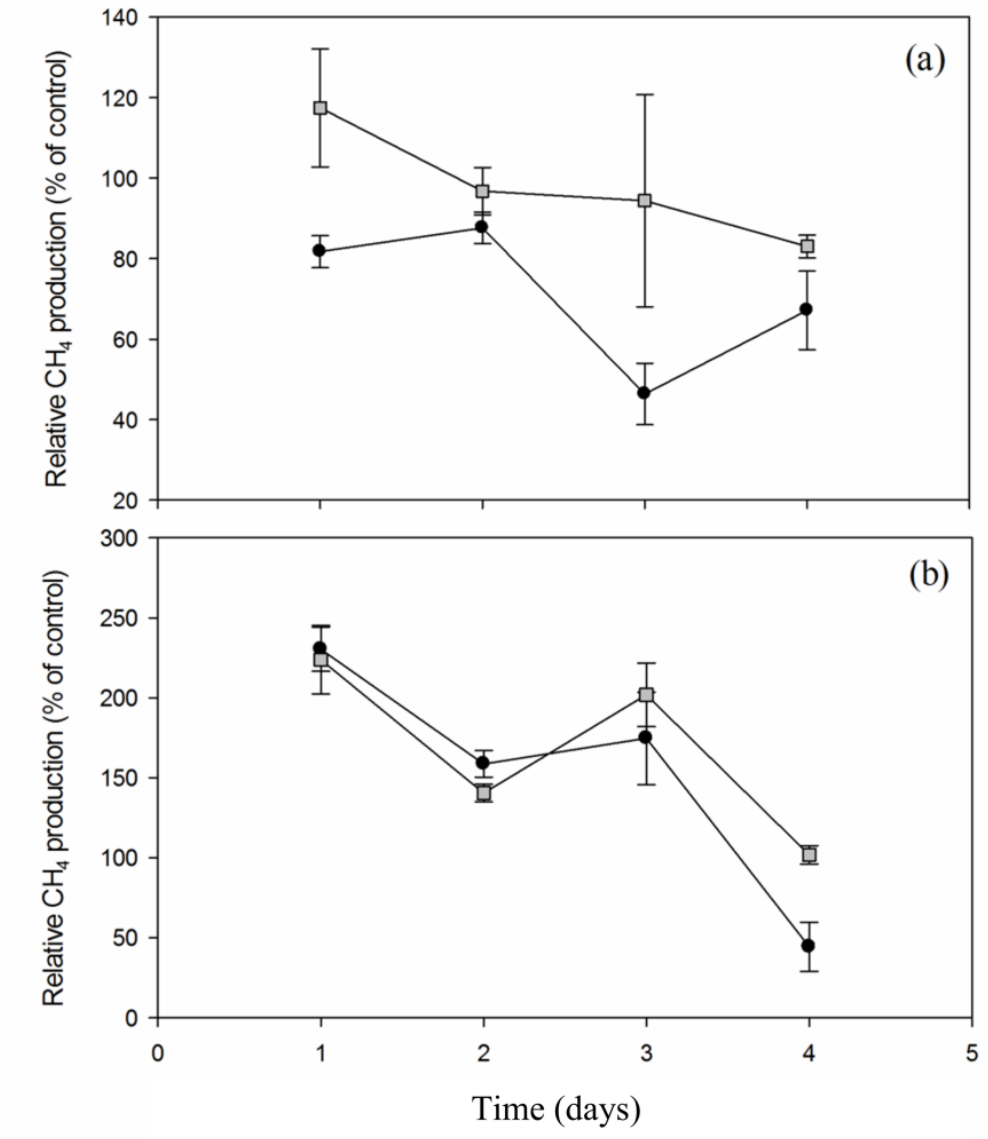

Figure 4.2 *Results of the short-term stress test at the end of the experiment in terms of the tolerance of the $CSTR_{stable}$ (●) and $CSTR_{dynamic}$ (■) to high concentrations of ammonium (a) and elevated organic loading rates (b). Average values of the three replicates per treatment are represented together with the values of the standard deviation as error bars.*

3.3. Microbial community analysis

The ecological parameters range-weighted richness (Rr), dynamics (Dy) and community organization (Co) in the $CSTR_{stable}$ and $CSTR_{dynamic}$ were deducted based on the DGGE profile of the bacterial community in both reactors (Figure 4.3).

Bacterial diversity was estimated by means of the Rr value. Both reactors started with an equal Rr value of 93 on day 24. Further operation of the two reactors in a different feeding pattern led the Rr to increase to higher levels in the $CSTR_{dynamic}$ compared to the $CSTR_{stable}$, although both reactors exhibited a higher Rr value at the end of the experiment (Figure 4.4a). The final Rr values of the $CSTR_{dynamic}$ and the $CSTR_{stable}$ were 250 and 182, respectively. The average Rr value was higher for the $CSTR_{dynamic}$ in comparison to the $CSTR_{stable}$, with values of 140 ± 55 and 119 ± 36, respectively.

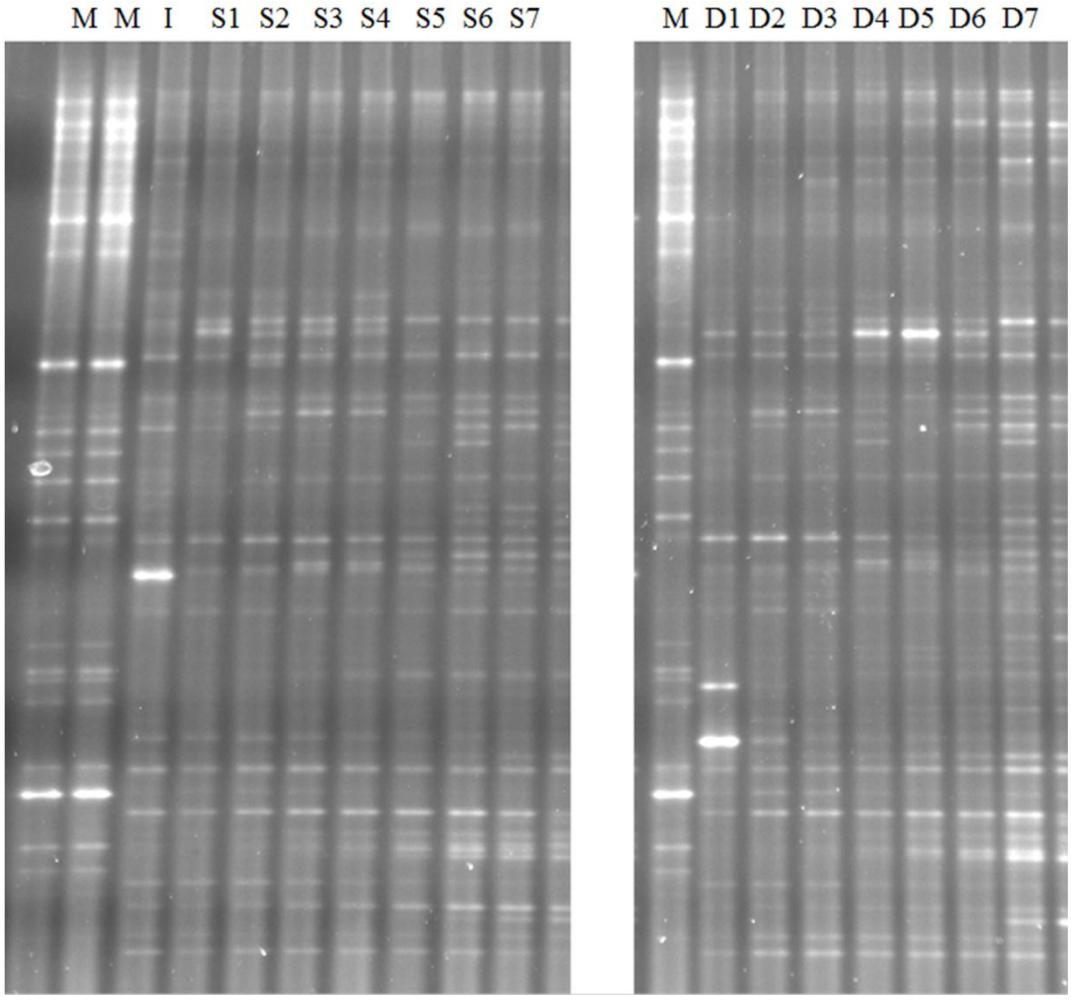

Figure 4.3 *DGGE profile of the bacterial community in the $CSTR_{stable}$ (S1 - S7) and $CSTR_{dynamic}$ (D1 – D7) from day 31 to day 73 of the experiment. Both reactors started with the same sludge inoculum on day 24 (I). The markers are given by the letter M.*

The bacterial community dynamics were evaluated using the Dy coefficient. Both reactors demonstrated very high bacterial community dynamics after the first 7 days, i.e. 76% for the $CSTR_{stable}$ and 97% for the $CSTR_{dynamic}$ (day 31), following the introduction of the different feeding pattern (Figure 4.4b). The 7-days community change decreased to a value < 16% within 21 days after the change of the feeding pattern in the $CSTR_{stable}$. The $CSTR_{dynamic}$ still demonstrated 7-days changes up to 50% after 21 days and there was a also substantial variation in the 7-days change pattern. Indeed, the 7-days evolution of dynamics was more variable as well, which was in contrast to the $CSTR_{stable}$.

The bacterial community evenness was reflected by means of the Co coefficient. A lower value of Co corresponds to a more even community. The two reactors each started from a Co value of 63, and evolved towards a lower Co value at the end of the experiment, i.e. 44 for the $CSTR_{stable}$ and 39 for the $CSTR_{dynamic}$ (Figure 4.4c). Both reactors thus evolved towards a more even community.

The qPCR results of the Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae revealed no differences between the $CSTR_{stable}$ and $CSTR_{dynamic}$. The Methanosaetaceae are the dominating methanogens and remained constant throughout the entire experiment, with on average $2.2 \times 10^{10} \pm 1.7 \times 10^{9}$ and $2.3 \times 10^{10} \pm 2.1 \times 10^{9}$ copies g^{-1} sludge in the $CSTR_{stable}$ and $CSTR_{dynamic}$, respectively. The Methanobacteriales showed a slight increase from $3.8 \times 10^{8} \pm 2.5 \times 10^{7}$ copies g^{-1} in both reactors on day 24 to $2.2 \times 10^{9} \pm 1.2 \times 10^{8}$ and $2.7 \times 10^{9} \pm 2.4 \times 10^{8}$ copies g^{-1} sludge on day 73 in the $CSTR_{stable}$ and $CSTR_{dynamic}$, respectively. The Methanomicrobiales showed similar values compared to the Methanobacteriales, yet copy numbers remained stable in both reactors in the entire experiment with average values of $1.4 \times 10^{9} \pm 1.3 \times 10^{8}$ copies g^{-1} in the $CSTR_{stable}$ and $1.1 \times 10^{9} \pm 1.4 \times 10^{8}$ copies g^{-1} in the $CSTR_{dynamic}$. Methanosarcinaceae copy numbers also remained stable and similar in the $CSTR_{stable}$ and $CSTR_{dynamic}$ with average values of $2.1 \times 10^{6} \pm 2.7 \times 10^{5}$ and $1.9 \times 10^{6} \pm 3.5 \times 10^{5}$ copies g^{-1}, respectively.

3.4. Correlations between methane production variation and the bacterial community

A moving window value of the methane production was determined of the seven days preceding each microbial community sampling time point (every 7 days). To correlate methane production variation to the ecological parameters Rr, Dy and Co, the variation of this 7-days moving window methane production was determined. The correlations between the

ecological parameters and the moving window methane production variation were subsequently determined (Table 4.3). There was a significant negative correlation ($p < 0.05$) between the bacterial community richness and organization in both reactors and there also was a significant positive correlation ($p < 0.05$) between the bacterial community organization and the in-window variation of methane production in the $CSTR_{stable}$ only (Table 4.3).

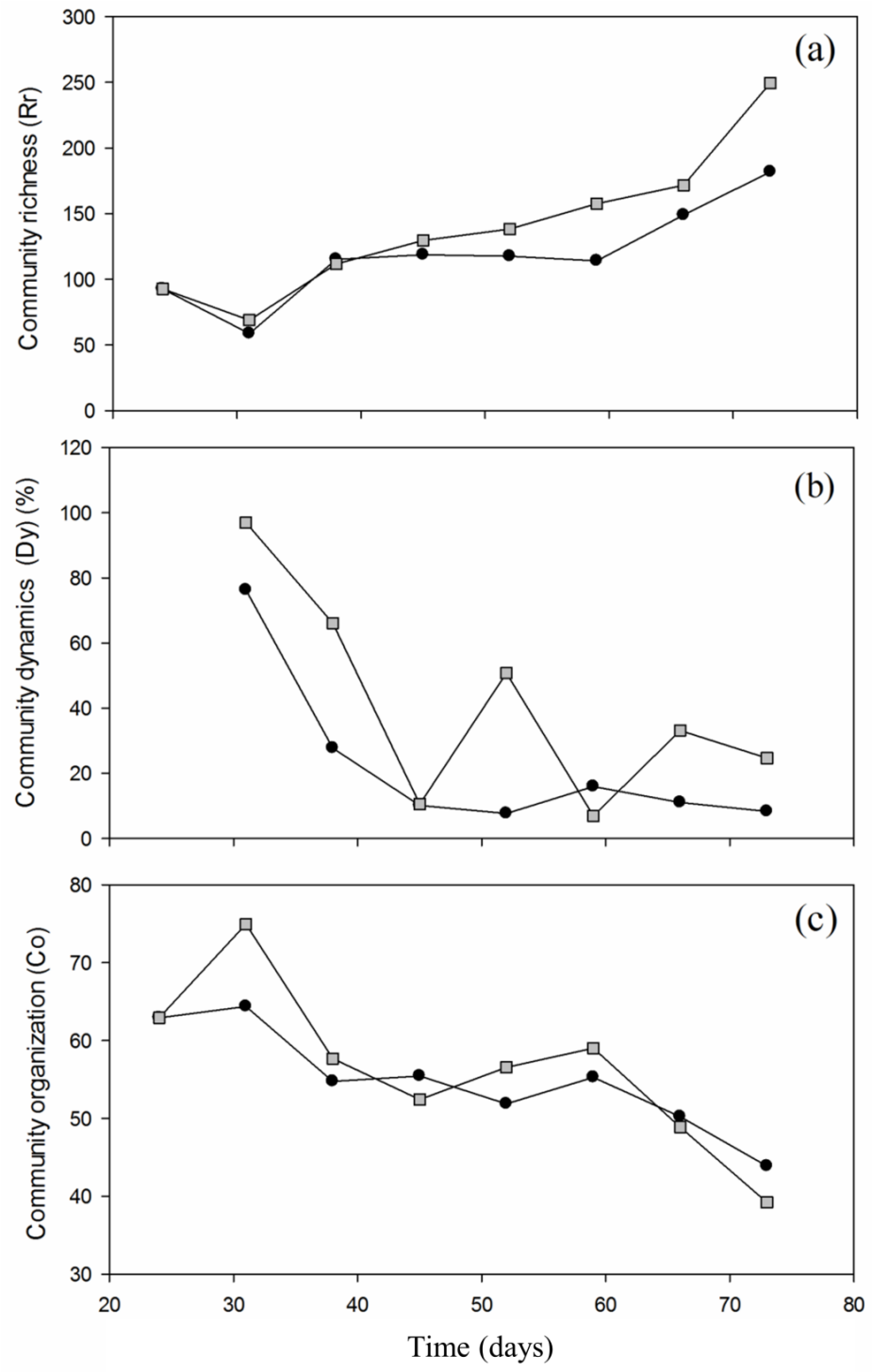

Figure 4.4 *Ecological parameters (a) range-weighted richness, (b) dynamics and (c) community organization of the bacterial communities in the $CSTR_{stable}$ (●) and $CSTR_{dynamic}$ (■).*

Table 4.3 *Correlations between the ecological parameters Rr, Dy and Co and the moving window methane production variation (Var CSTR), determined by means of the Spearman rank order correlation analysis, for the $CSTR_{stable}$ and $CSTR_{dynamic}$.*

$CSTR_{stable}$		Var $CSTR_{stable}$	Dy $CSTR_{stable}$	Co $CSTR_{stable}$	Rr $CSTR_{stable}$
Var $CSTR_{stable}$	Corr. Coeff	1.000	0.536	0.714*	-0.548
	Sign. level	.	0.215	0.047	0.160
Dy $CSTR_{stable}$	Corr. Coeff	0.536	1.000	0.607	-0.714
	Sign. level	0.215	.	0.148	0.071
Co $CSTR_{stable}$	Corr. Coeff	0.714*	0.607	1.000	-0.857*
	Sign. level	0.047	0.148	.	0.007
Rr $CSTR_{stable}$	Corr. Coeff	-0.548	-0.714	-0.857*	1.000
	Sign. level	0.160	0.071	0.007	.
$CSTR_{dynamic}$		Var $CSTR_{dynamic}$	Dy $CSTR_{dynamic}$	Co $CSTR_{dynamic}$	Rr $CSTR_{dynamic}$
Var $CSTR_{dynamic}$	Corr. Coeff	1.000	0.536	0.048	-0.286
	Sign. level	.	0.215	0.911	0.493
Dy $CSTR_{dynamic}$	Corr. Coeff	0.536	1.000	0.357	-0.607
	Sign. level	0.215	.	0.432	0.148
Co $CSTR_{dynamic}$	Corr. Coeff	0.048	0.357	1.000	-0.833*
	Sign. level	0.911	0.432	.	0.010
Rr $CSTR_{dynamic}$	Corr. Coeff	-0.286	-0.607	-0.833*	1.000
	Sign. level	0.493	0.148	0.010	.

*Correlation is significant at the 0.05 level.

4. Discussion

A higher degree of functional stability was achieved by changing the feeding pattern, which altered the evenness, dynamics and diversity of the bacterial community, yet the archaeal community was not influenced. A short-term stress test revealed that the $CSTR_{dynamic}$ was more tolerant to high levels of TAN and high organic loading rates. The bacterial community in the $CSTR_{dynamic}$ demonstrated a higher degree of dynamics, yet both reactors evolved towards a more even bacterial community.

Average methane production and yield remained the same in both reactors, indicating that the stronger pulse feeding pattern of the $CSTR_{dynamic}$ (fed every two days) did not cause an organic overloading of the reactor, as no fatty acids were detected and the pH remained stable. These results are in agreement with an earlier study, in which only little difference in average biogas production was detected between an hourly and a daily fed reactor (Conklin et al., 2006). Daily variation in methane production was however much higher in the $CSTR_{dynamic}$, compared to the $CSTR_{stable}$, which is reflected in the in-window variation of the methane production of both reactors. This higher degree of variation in the $CSTR_{dynamic}$ was also reflected in a higher degree of variation in the pH and COD_{sol} in this reactor, compared to the $CSTR_{stable}$. These observations correspond with the study of Conklin et al. (2006), who had a higher standard deviation of methane production and a higher degree of variation in pH in the daily fed reactor, compared to the hourly fed reactor.

Bacterial community analysis revealed Rr values reaching 250 and 182 at the end of the experiment in the $CSTR_{dynamic}$ and $CSTR_{stable}$, respectively, while in other anaerobic CSTR reactors the bacterial richness never exceeded a Rr value of 40 (Carballa et al., 2011; Pycke et al., 2011). The difference in bacterial richness is quite low and despite the fact that it diverges towards the end of the experiment, it can be stated that bacterial richness is similar in both reactors. When comparing these results to the Rr values microbial communities in different environments, as listed by Marzorati et al. (2008), it is clear that bacterial richness was very high in the reactors in this study. This can be correlated to the diversity of the substrate, which consisted of several different organic compounds (Table 4.1), as the application of only one substrate to the anaerobic digester strongly reduces or limits bacterial richness (Fernandez et al., 1999; Delbes et al., 2000; Zamalloa et al., 2012).

Bacterial community dynamics in the $CSTR_{dynamic}$ demonstrated 7-days changes up to 50%, which can be considered a high value of dynamics, when compared to other (anaerobic)

ecosystems that only had an average 7-days dynamics of 25% (Marzorati et al., 2008; Wittebolle et al., 2009a; Carballa et al., 2011; Pycke et al., 2011). This higher degree of dynamics is however not negatively correlated to operational stability, since the $CSTR_{dynamic}$ produced equal levels of methane as the $CSTR_{stable}$. This was also reflected in the study of Fernandez et al. (1999), who stated that extremely dynamic communities can still maintain high functional stability and that a high degree of bacterial diversity, which is also the case in our reactors, can contribute to high levels of dynamics. This high level of dynamics, as well as the high variation in the weekly estimated dynamics, in correlation with a high bacterial diversity also implies that the $CSTR_{dynamic}$ could be able to rapidly respond to changing conditions (Dearman et al., 2006; Verstraete et al., 2007).

The bacterial community evolved towards a more even community in both reactors. This community organization can be considered a measure of the degree of functional organization of the bacterial community, i.e. the higher the Co value, the more specialized the bacterial community (Marzorati et al., 2008; Read et al., 2011). A very uneven community, however, can be considered less resilient to changing conditions, because of its high level of specialization (Wittebolle et al., 2009a). A stable community therefore needs to contain a certain level of organization (more uneven) but also a level of functional resilience (more even), to which both community richness and dynamics can contribute (Fernandez et al., 1999; Marzorati et al., 2008; Wittebolle et al., 2009a). The evolution of both reactors towards a more even community, compared to community at the start of the experiment, might be attributed to the diversity of the substrate, which requires multiple bacterial species to degrade all compounds. Nonetheless, the specific contribution of the substrate to bacterial richness, organization and diversity remains to be confirmed, since several other factors can also influence the bacterial community (Krakat et al., 2011; Zhang et al., 2014b).

Although the ecological parameters, based on the DGGE profile, represent valuable information concerning the bacterial community, caution should be taken with the interpretation of these data, since the DGGE method has some well-known limitations. The number and abundance of bacterial species in the anaerobic digester is not exactly reflected by the number and intensity of the bands (Boon et al., 2002). One bacterial species may demonstrate more than one band, one band may represent multiple species and species which have an abundance < 1% cannot be visualized by means of DGGE (Boon et al., 2002), thus only dominant species were taken into account, which was the goal of this research. Moreover, no replicates of the two different treatments were included, which did not allow

statistical comparison of the (molecular) results. Hence, when interpreting these ecological parameters, deducted from any molecular analysis, one should be aware of the limitations of the techniques used, and the amount of replicates included in the analysis.

Real-time PCR results demonstrated that there was no difference in methanogenic community composition between the two reactors, and that there was only a slight increase in Methanobacteriales copy numbers. The other methanogenic populations remained constant. This is in contrast to the bacterial community that showed a substantial change throughout the experiment, with different levels of dynamics in the two reactors. The presence of the different methanogenic groups, however, demonstrates that both acetoclastic and hydrogenotrophic methanogenesis took place in both reactors, yet the dominance of the Methanosaetaceae in the two reactors assigns acetoclastic methanogenesis as the dominant pathway. This is, however, to be expected, since residual VFA concentrations were below detection limit at all times in the two reactors. Since *Methanosaeta sp.* show a high affinity for acetate compared to *Methanosarcina sp.*, they tend to be the dominant acetoclastic methanogens at low acetate concentrations, which immediately also explains the low Methanosarcinaceae copy numbers (De Vrieze et al., 2012). It was shown in the study of Conklin et al. (2006) that there was a clear shift from a *Methanosaeta* to a *Methanosarcina* dominated methanogenic community at higher interval feeding, which was not the case in this research, because of the very low residual acetate concentrations.

The strong negative correlation between the bacterial community richness and organization in both reactors indicated that a higher degree of bacterial community evenness might be directly correlated to a higher bacterial richness, a similar result which was obtained in the research of Carballa et al. (2011). Unfortunately, our results could not be related to the in-window methane production variation. However, these results, together with the results of Carballa et al. (2011) indicated that bacterial richness in AD can be predicted by the bacterial community organization and vice versa, which does not particularly seem to be the case in other bacterial ecosystems. It can be deducted from the positive correlation between community organization and operational variation in the $CSTR_{stable}$ that a bacterial community with high evenness (low Co value) causes limited process variation. Hence, a community with a few dominant species and several other species present in lower abundance, i.e. a more uneven community (high Co value) may lead to more process variation. This might attribute an extra dimension to the findings of Wittebolle et al. (2009a), who reported that initial evenness contributes to functional stability. Community unevenness may lead to operational variation under normal

or optimal conditions and when the community evolves towards a more even community, process variation declines.

The higher tolerance of the $CSTR_{dynamic}$ to higher levels of TAN and OLR is in agreement to the study of Conklin et al. (2006) that demonstrated that daily feeding compared to hourly feeding in AD led to a higher tolerance to organic overloading. Yet, they did not detect a higher tolerance to ammonium stress and no relation with the bacterial community was established. The elevated tolerance of the $CSTR_{dynamic}$ to ammonium stress can be related to its more variable methane production profile. Indeed, a higher resistance to ammonium stress can be induced by means of a pulse feeding pattern and a subsequent higher degree of methane production variation can be a sign of the latter. This elevated ammonium tolerance can be correlated to the higher degree of variation in the dynamics of the bacterial community as well, which is also shown in the study of Fernandez et al. (1999). That study demonstrated that a more flexible microbial community is correlated to a higher degree of stability when exposed to a shock load of glucose, thus connecting process stability to bacterial community dynamics. Our study demonstrated that the elevated resistance to impairments can be reflected in the variation in methane production and community dynamics. This supports the hypothesis of Verstraete et al. (2007) that stable processes do not host a stable climax community but that there is always a certain degree of dynamics required to ensure continuous stable operation. However, only an evolution in the bacterial, but not in the methanogenic community could be observed. A higher degree of process stability, i.e. higher tolerance to common forms of stress, can, thus, be achieved by introducing a pulse feeding pattern in AD.

5. Conclusions

This study hypothesized that stable operation and higher stress tolerance can be obtained in AD when stronger pulse feeding patterns are applied, although at the cost of more daily operational variation. A pulse feeding pattern leads to a higher degree of variation in bacterial dynamics, which can be correlated to a higher tolerance to high levels of ammonium and organic overloading in AD. The methanogenic community remained stable in both reactors, with a clear dominance of the Methanosaetaceae. These results suggest that the regular application of a limited (to avoid overloading) pulse of organic material, such as glycerol or molasses, and/or a variation in the substrate might allow the microbial community to adapt to low levels of stress. That way the microbial community should be able to respond when

exposed to higher stress levels which would allow the system to obtain a higher degree of functional stability. This would then increase functional stability in AD. However, these results should be interpreted with a certain level of care, since no triplicate experiments were carried out. Hence, no statistical confirmation of the applied feeding pattern on operational performance, nor on the microbial community or MRM parameters could be obtained. Molecular fingerprinting techniques, e.g. DGGE, could provide valuable information concerning the microbial community in AD. Further research concerning the role of initial evenness of the bacterial and archaeal community and its evolution in terms of process stability, by means of triplicate experiments, will, however, be required to provide more valuable and statistically confirmed information to further steer AD. The application of next-generation sequencing techniques might also provide interesting information concerning the identity of the dominant species and the role of species present at low abundance.

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CHAPTER 5: INOCULUM SELECTION IS CRUCIAL TO ENSURE OPERATIONAL STABILITY IN ANAEROBIC DIGESTION

This chapter has been redrafted after:

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Abstract

Anaerobic digestion is considered a key technology for the future bio-based economy. The microbial consortium carrying out the anaerobic digestion process is complex, and its exact role in terms of 'elasticity', i.e. the ability to rapidly adapt to changing conditions, is still unknown. In this study the role of the initial microbial community in terms of operational stability and stress tolerance was evaluated during a 175 days experiment. Five different inocula from stable industrial anaerobic digesters were fed a mixture of waste activated sludge and glycerol. Increasing ammonium pulses were applied to evaluate stability and stress tolerance. A different response in terms of start-up and ammonium tolerance was observed between the different inocula. Methanosaetaceae were the dominant acetoclastic methanogens, yet, Methanosarcinaceae increased in abundance at elevated ammonium concentrations. A shift from a Firmicutes to a Proteobacteria dominated bacterial community was observed in failing digesters. Methane production was strongly positively correlated with Methanosaetaceae, but also with bacteria related to Anaerolinaceae, Clostridiales and α-Proteobacteria. Volatile fatty acids were strongly positively correlated with β-Proteobacteria and Bacteroidetes, yet, ammonium concentration only with Bacteroidetes. Overall, these results indicate the importance of inoculum selection to ensure stable operation and stress tolerance in anaerobic digestion.

1. Introduction

Renewable energy production has been at the forefront of modern science, and can be considered one of the major aspects within the future bioeconomy. Anaerobic digestion (AD) is an established technology that can be considered the first microbial technology to allow energy recovery from low-value organic by-products and wastes. Therefore, it has the potential to become a key technology for renewable energy production (Mata-Alvarez et al., 2000; Verstraete et al., 2005; Holm-Nielsen et al., 2009; Tyagi & Lo, 2013). Indeed, AD has several advantages over aerobic microbial technologies, such as biogas production, low energy requirements and a substantial decrease and stabilization of organic waste (Mata-Alvarez et al., 2000; Angenent et al., 2004a).

One of the major drawbacks of AD can be found in the susceptibility of the microbial community to impairments, mainly in the final step of the process, methanogenesis. Hydrolysis, acidogenesis and acetogenesis, the first three steps of the AD process, are carried out by different groups of bacteria and do not pose serious problems, in contrast to methanogenesis (Gujer & Zehnder, 1983; Sawayama et al., 2004; Chen et al., 2008; De Vrieze et al., 2012). Methanogenic archaea carry out the final step in the AD process, producing the methane. These methanogens are, however, most vulnerable to different environmental factors, including abrupt pH changes, organic overloading and high salt and total ammonia nitrogen (TAN) concentrations, leading to the accumulation of volatile fatty acids (VFA) and subsequent process failure (Chen et al., 2008; De Vrieze et al., 2012). High TAN concentrations in AD often result from feeding the reactor with substrates with high protein content, such as slaughterhouse waste and manure (Hansen et al., 1998; Bayr et al., 2012). The toxic effect of TAN in anaerobic digestion can be attributed to the free ammonia (NH_3) fraction, which increases with increasing pH and temperature (Hashimoto, 1986; Chen et al., 2008; Schnurer & Nordberg, 2008). Free ammonia (FA) diffuses through the cell membrane, and subsequently interferes with proton transport across the membrane, and/or causes potassium deficiencies, especially in methanogens. (Gallert et al., 1998; Chen et al., 2008; Pitk et al., 2013). A wide range of TAN levels causing 50% inhibition of methane production has been reported in literature, ranging from 1.7 to 14 g N L^{-1} (Chen et al., 2008). This high level of variation in tolerance to ammonia can depend on differences in the composition of the substrate, environmental conditions (temperature, pH), acclimation periods and the selected inoculum (De Vrieze et al., 2012; Rajagopal et al., 2013).

The composition and organization of the microbial community plays an important role in the tolerance to high TAN levels in AD. A well-organized bacteria – archaea community with a certain 'elasticity', i.e. the ability to adapt to changing conditions, is required to ensure steady conversion of organic substrates to CH_4 and CO_2 (Fernandez et al., 2000; Riviere et al., 2009). Functional redundancy positively correlates with microbial diversity, as the presence of a more diverse microbial community directly relates to a higher availability of potential metabolic conversion pathways (Peterson et al., 1998; Briones & Raskin, 2003; Carballa et al., 2011). Nevertheless, a high microbial diversity as such is not sufficient to ensure stable methane production during high TAN levels, since the presence of specific tolerant species is required. The overall tolerance of the system is partially determined either by the treated waste stream (nitrogen content), the frequency of feeding or the operational conditions of the reactor (Dearman et al., 2006; Krakat et al., 2011; De Vrieze et al., 2013b). Inoculum selection might also be crucial to ensure stable operation, as it determines the initial operating potential of the anaerobic digester (Wittebolle et al., 2009b; Dechrugsa et al., 2013).

In this research the functionality and microbial community structure in five AD reactors, inoculated with five different inocula, were correlated with the methane production, VFA and TAN concentration during stable and high TAN concentration conditions. The role of the microbial community structure was investigated, and key microbial players for the stability and robustness of the digester were identified. Moreover, a correlation between microbiome adaptation under high VFA and TAN concentration in AD reactors was assessed.

2. Materials and methods

2.1. Inoculum and substrate characterization

Five different anaerobic inoculum sludge samples were selected, originating from full-scale mesophilic AD installations treating different waste streams. One inoculum was granular sludge that originated from an upflow anaerobic sludge blanket (UASB) reactor treating potato wastewater (Myd). The other inocula were collected from a continuous stirred tank reactor (CSTR) digester, treating a combination of energy maize, lipid and fruit waste (Agri), maize and manure (Vce) and waste activated sludge (Oss). The last inoculum was a mixture that consisted of 25% (w/w) on VSS (volatile suspended solids) basis of each of the four inocula from the full-scale digesters (Mix). The characteristics of these five inocula can be found in Table 5.1.

Table 5.1 *Characteristics of the five different original undiluted inoculum sludge samples (FW = fresh weight, COD = chemical oxygen demand).*

Parameter	Unit	Myd	Agri	Vce	Oss	Mix
pH	-	7.12	8.19	8.52	7.48	7.77
TSS	g kg^{-1} FW	30.3	133.0	127.1	45.6	62.7
VSS	g kg^{-1} FW	20.4	48.2	77.0	24.3	32.2
Conductivity	mS cm^{-1}	8.0	37.5	28.6	7.3	14.9
Total VFA	mg COD kg^{-1} FW	325	912	1211	0	408
Acetic acid	mg COD kg^{-1} FW	0	676	882	0	205
Propionic acid	mg COD kg^{-1} FW	0	0	0	0	0
TAN	mg N kg^{-1} FW	836	2904	4647	953	1620
FA[a]	mg N kg^{-1} FW	12	410	1460	30	95

[a] The free ammonia (FA) concentration was calculated based on the TAN concentration, pH and temperature in the full-scale installation.

Waste activated sludge, used as a feed source during the experiments, was collected from the municipal wastewater treatment plant the Ossemeersen, Ghent (Belgium), with characteristics described in Table 5.2.

2.2. Experimental set-up and operation

Five anaerobic lab-scale CSTR units with a total volume of 1 L and a working volume of 800 mL were operated at mesophilic conditions (34 ± 1 °C) in a temperature controlled room for 175 consecutive days. Each unit was connected to a water column to collect the produced biogas by means of the water displacement method (Figure 2.1). A sludge retention time (SRT) of 20 days was maintained. In each reactor a different inoculum (Myd, Agri, Vce, Oss or Mix) was added. Each inoculum was diluted with tap water until a VSS concentration of 10 g L^{-1} was obtained in all reactors. Feeding of the reactors took place by means of the fed-batch principle, and was carried out three times per week. Fresh feed was prepared for every feeding.

Four periods were defined for the study: a start-up phase, a phase with stable feeding (Phase 1), a phase with increasing ammonium addition (Phase 2), and a regeneration phase (Phase 3). During the start-up phase the organic loading rate (OLR) was slowly increased. From day 1 to 7 only waste activated sludge was used as feed, resulting in an OLR of 1.5 g COD L^{-1} d^{-1}. From day 8 to 14 glycerol was added to the waste activated sludge to increase the OLR to 2.0

g COD L^{-1} d^{-1}. During Phase 1 and 2 a mixture of waste activated sludge and glycerol was used as feed, resulting in an OLR of 2.5 g COD L^{-1} d^{-1}, whereas only waste activated sludge was used from day 120 to 146 in Phase 3, with a lower OLR of 1.5 g COD L^{-1} d^{-1}, to allow regeneration of the reactors. Afterwards (from day 147 to 175) the OLR was increased again to 2.5 g COD L^{-1} d^{-1} by adding glycerol to the feed (Table 5.3). Increasing amounts of NH_4Cl were added every week during Phase 2, to increase the TAN concentration in the reactors, which resulted in a final concentration of 4000 mg N L^{-1} on day 112.

Table 5.2 *Characteristics of the waste activated sludge used to prepare the feed of the reactors. All analyses were carried out in triplicate, except for the pH and conductivity measurement. FW = fresh weight.*

Parameter	Unit	Waste activated sludge
Total COD	g kg^{-1} FW	47.6 ± 1.6
Soluble COD	mg kg^{-1} FW	1427 ± 14
Total solids	g kg^{-1} FW	52.6 ± 0.2
Volatile solids	g kg^{-1} FW	32.9 ± 0.2
Total ammonia nitrogen (TAN)	mg N kg^{-1} FW	143 ± 8
Kjeldahl nitrogen, (KjN)	mg N kg^{-1} FW	2916 ± 297
Total phosphorous (TP)	g P kg^{-1} FW	1.07 ± 0.13
Conductivity	mS cm^{-1}	2.09
pH	-	6.98
Total VFA	mg kg^{-1} FW	0 ± 0
COD:N ratio	-	16.3 ± 1.8
COD:P ratio	-	44.6 ± 5.7
TS:VS ratio	-	1.60 ± 0.01
COD:VS ratio	-	1.45 ± 0.05

During the entire experimental period biogas production and content were determined three times a week, and reported at STP (standard temperature and pressure) conditions. Effluent samples were taken three times a week for analysis of pH, VFA and once a week for TAN. Biomass samples of 10 mL were taken from the inoculum samples and from each reactor at the end of Phase 1 to 3 for microbial community analysis. These samples were subsequently stored at −20 °C until DNA extraction was performed.

Table 5.3 *Operational conditions in the reactors during the four different phases of the experiment. WAS = Waste activated sludge.*

Phase	Period	Substrate	OLR (g COD L^{-1} d^{-1})
Start-up phase	Day 1-14	WAS + glycerol	1.5 - 2.0
Phase 1	Day 15-77	WAS + glycerol	2.5
Phase 2	Day 78-119	WAS + glycerol + NH_4Cl	2.5
Phase 3	Day 120-175	WAS + glycerol	1.5 - 2.5

2.3. DNA extraction and amplicon sequencing

Microbial community analysis was applied to the inoculum sludge samples and the reactor samples at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175). Total DNA extraction from the sludge samples was performed using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 200 mg of sample was taken for DNA extraction.Total DNA concentration was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands), by measuring the absorbance ratios at 260 nm and 280 nm. The quality of the extracted DNA was evaluated on a 1% agarose gel. Libraries for the Illumina platform (MiSeq) were prepared as previously described (Camarinha-Silva et al., 2014), using the primers 807F and 1050R (Bohorquez et al., 2012) for the V5-V6 region of the 16S rRNA gene. Definition of operational taxonomic units (OTUs) and data-set quality filtering were performed as previously described (Camarinha-Silva et al., 2014).

2.4. Sequence analysis

The 16S rRNA gene sequences from the closest taxonomic relatives assigned to each of the phylotypes using RDP/NCBI were obtained as a pre-aligned set of manually curated sequences from the SILVA database (Pruesse et al., 2007). A maximum likelihood tree was constructed using MEGA5 (Tamura et al., 2011). The Jukes-Cantor model with branch support values calculated from 1000 bootstrap re-samplings was used to calculate evolutionary distances across all sites (Jukes & Cantor, 1969). Phylogenetic trees with

correlations between OTUs were created using iTol (http://itol.embl.de) for data visualization (Letunic & Bork, 2011).

2.5. Statistical analysis

A data-set containing the relative abundance of each phylotype in each of the 20 samples was analysed using the software R, version 3.0.2. (http://www.r-project.org) (R Development Core Team, 2013). Rarefaction curves were created for each sample to evaluate if the sampling depth was sufficient (Sanders, 1968; Hurlbert, 1971). A table with the abundance of different OTUs and their taxonomic assignments in each sample was generated. Pearson correlations between functional parameters and relative abundances of OTUs were calculated using the software R. Relative abundances of OTUs were used to generate a heatmap.

2.6. Real-time PCR analysis

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Analytical triplicates of a 100-fold dilution of the DNA-samples were analysed for the acetoclastic methanogenic populations Methanosarcinaceae and Methanosaetaceae. The primer sets used for the methanogenic populations Methanosarcinaceae (Msc) and Methanosaetaceae (Mst) were previously described by Yu et al. (2005). The reaction mixture of 20 µL was prepared using the GoTaq qPCR Master Mix (Promega, Madison, WIS, USA), and contained 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water, 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure, which consisted of a predenaturation step of 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. The qPCR data were represented as copies per gram of wet sludge.

2.7. Analytical techniques

Total suspended solids (TSS), VSS, total and volatile solids (TS, VS), TAN, Kjeldahl nitrogen (KjN) and COD were determined according to Standard Methods (Greenberg et al., 1992). Total P analysis was carried out by means of a Jenway 6400 spectrophotometer (Keison Products, Essex, UK). The FA concentration was calculated based on the TAN concentration, pH and temperature. Biogas composition was analysed by means of a Compact

GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH_4 and CO_2 were determined using a thermal conductivity detector with a lower detection limit of 1 ppmv for each gas component. The volatile fatty acid (VFA) concentrations were measured using gas chromatography (GC-2014, Shimadzu®, The Netherlands) with a DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector (FID). Liquid samples were conditioned with sulphuric acid and sodium chloride, and 2-methyl hexanoic acid was used as internal standard for quantification of further extraction with diethyl ether. The prepared sample (1 µL) was injected at 200 ºC with a split ratio of 60 and a purge flow of 3 mL min^{-1}. The oven temperature increased by 6 ºC min^{-1} from 110 ºC to 165 ºC, where it was kept for 2 min. The FID had a temperature of 220 ºC. The carrier gas was nitrogen at a flow rate of 2.49 mL min^{-1}. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium), and conductivity (EC) was determined using a C833 conductivity meter (Consort, Turnhout, Belgium).

2.8. Data deposition

The sequences reported in this paper have been deposited in the European Nucleotide Archive (ENA) database (accession no. LK055288-902, WEBIN ID no. Hx2000040310).

3. Results

3.1. Effect of inoculum selection on methane production and ammonia tolerance

Methane production showed a similar increasing trend in all five AD reactors, i.e. Myd, Agri, Vce, Oss and Mix, during the start-up phase, although methane production was consistently lower in the Vce reactor (Figure 5.1). In all five AD reactors an increasing trend in total VFA could be detected during start-up (Figure 5.2).

At the start of Phase 1 total VFA decreased to a value below detection limit in all reactors, with the exception of the Vce reactor (Figure 5.2). Methane production results were in accordance to this, as methane production values of 513 ± 55, 506 ± 68, 509 ± 66 and 520 ± 55 mL L^{-1} d^{-1} were obtained for the Myd, Agri, Oss and Mix reactor, respectively, in Phase 1. Methane production in the Vce reactor decreased to an average value of 99 ± 80 mL L^{-1} d^{-1} in Phase 1 (Figure 5.1).

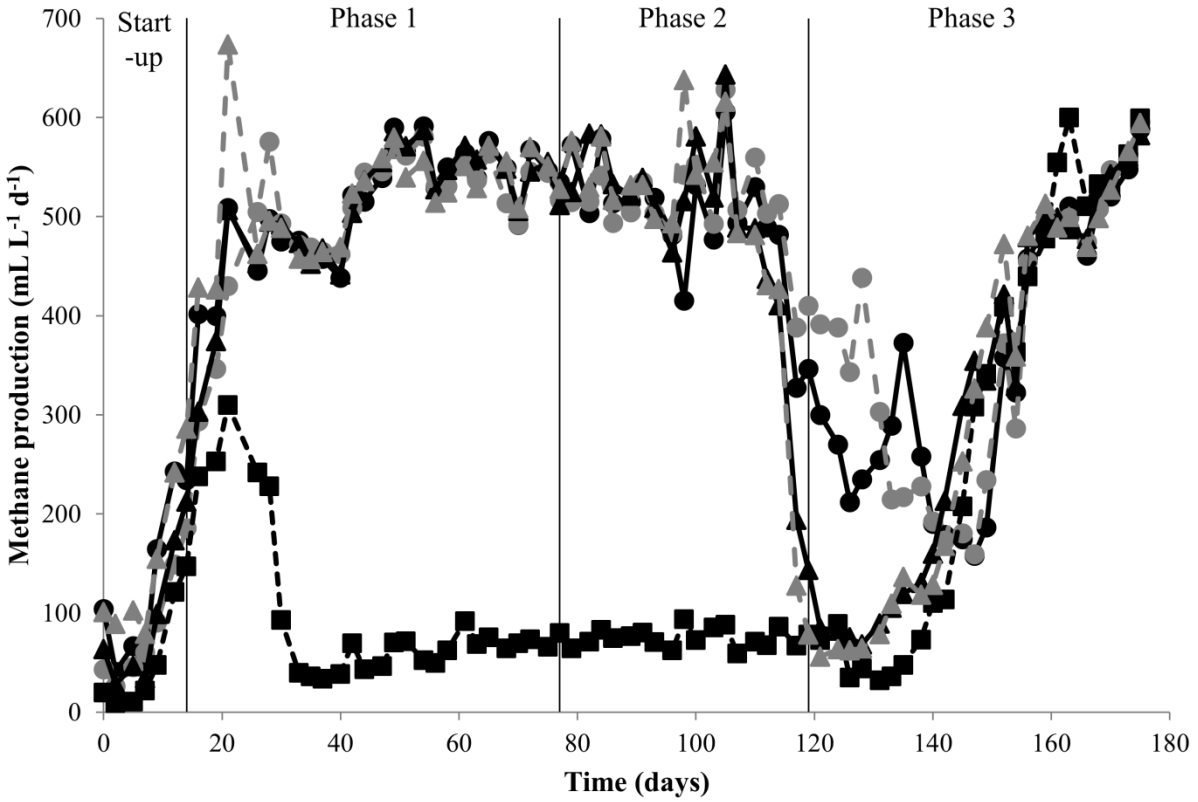


Figure 5.1 *Methane production profiles in the four different phases, i.e. Start-up phase (day 0 -14), Phase 1 (day 15 – 77), Phase 2 (day 78 – 119) and Phase 3 (day 120 – 175) for the Myd (●), Agri (●), Vce (■), Oss (▲) and Mix (▲) reactor.*

In Phase 2 subsequent pulses of ammonia were applied, resulting in a final average TAN concentration of 4000 mg N L^{-1} on day 112 (Figure 5.3). An abrupt decrease in methane production was observed on day 112, following the increased ammonium concentrations, with the exception of the Vce reactor. Methane production decreased to a value of 144 mL L^{-1} d^{-1} in the Oss and 78 mL L^{-1} d^{-1} in the Mix reactor, while higher values of 346 and 410 mL L^{-1} d^{-1} were maintained in the Myd and Agri reactor, respectively, at the end of Phase 2 (Figure 5.1). In parallel, increased VFA levels were detected (Figure 5.2). The main fraction (81.0 ± 13.2%) of total VFA consisted of propionic acid in the Myd, Agri, Oss and Mix reactor. The Vce reactor had a similar low methane production rate and high VFA concentration, mostly valeric acid (45.9%), propionic acid (18.3%) and butyric acid (14.3%), compared to Phase 1. Consecutively to decreasing methane production and increasing VFA concentration, pH values decreased to 6.22 in the Oss and 6.03 in the Mix reactors, whereas values of 6.73 and 6.88 were maintained in the Myd and Agri reactor, respectively, at the end of Phase 2 (Figure 5.4). The pH in the Vce reactor remained at a low value of 5.13 at the end of Phase 2.

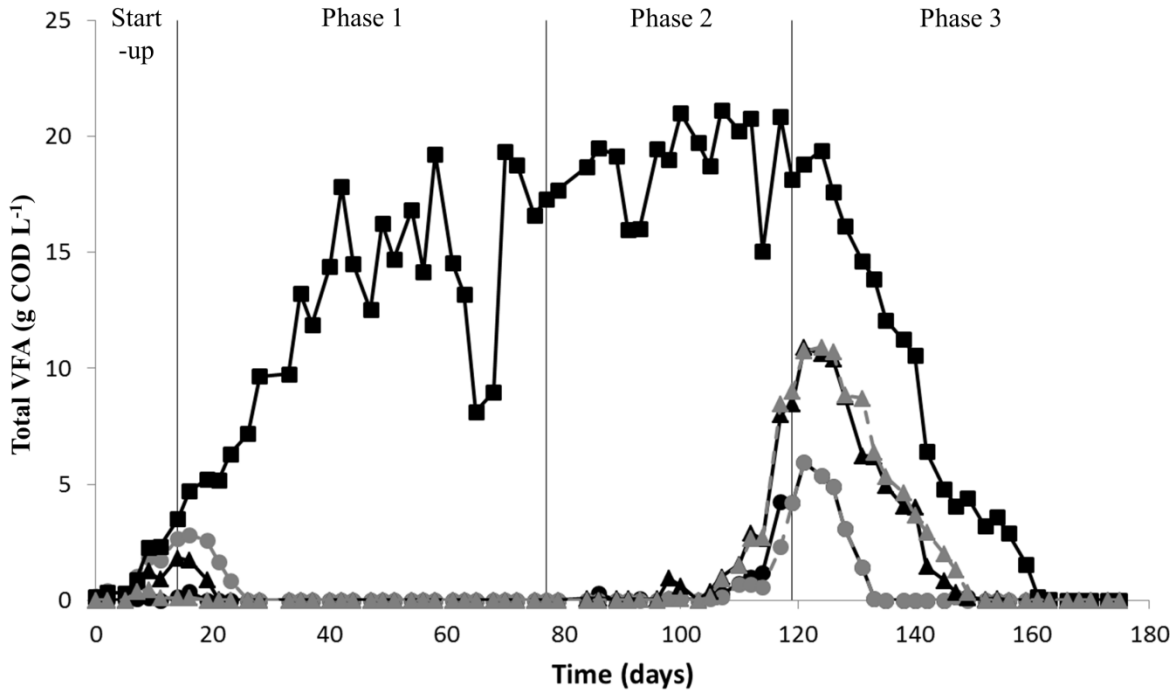


Figure 5.2 *Total VFA concentration profiles in the four different phases, i.e. Start-up phase (day 0 - 14), Phase 1 (day 15 – 77), Phase 2 (day 78 – 119) and Phase 3 (day 120 – 175) for the Myd (●), Agri (●), Vce (■), Oss (▲) and Mix (▲) reactor.*

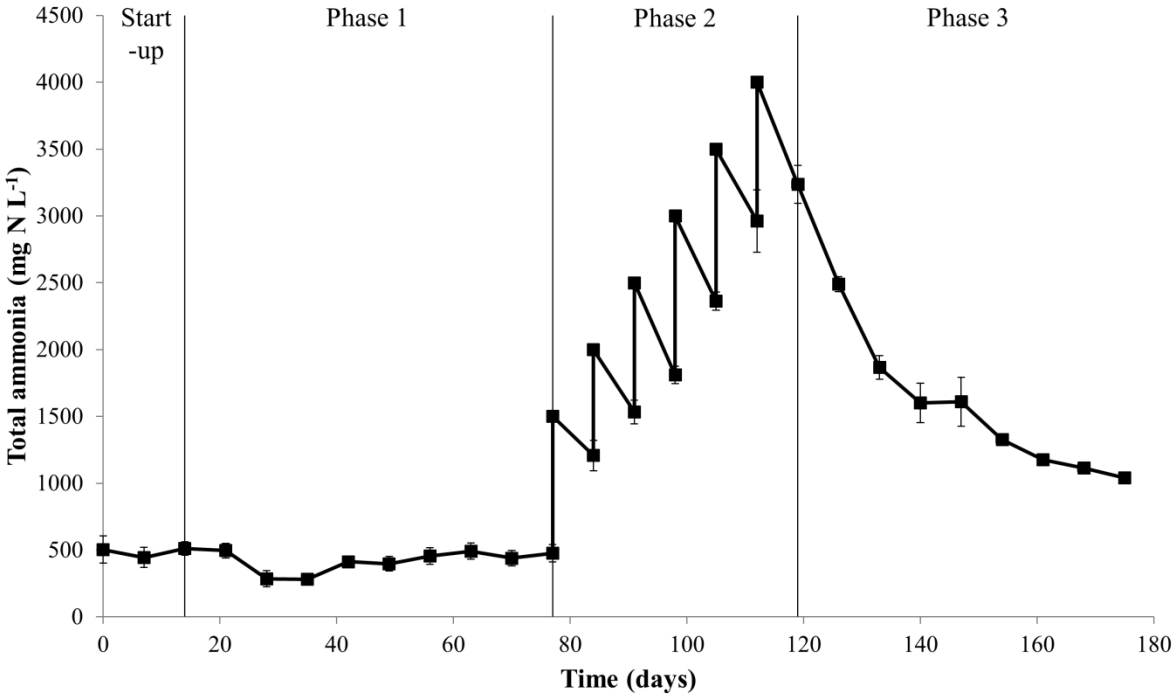


Figure 5.3 *Average total ammonia concentration in the five anaerobic digestion reactors in the four phases.*

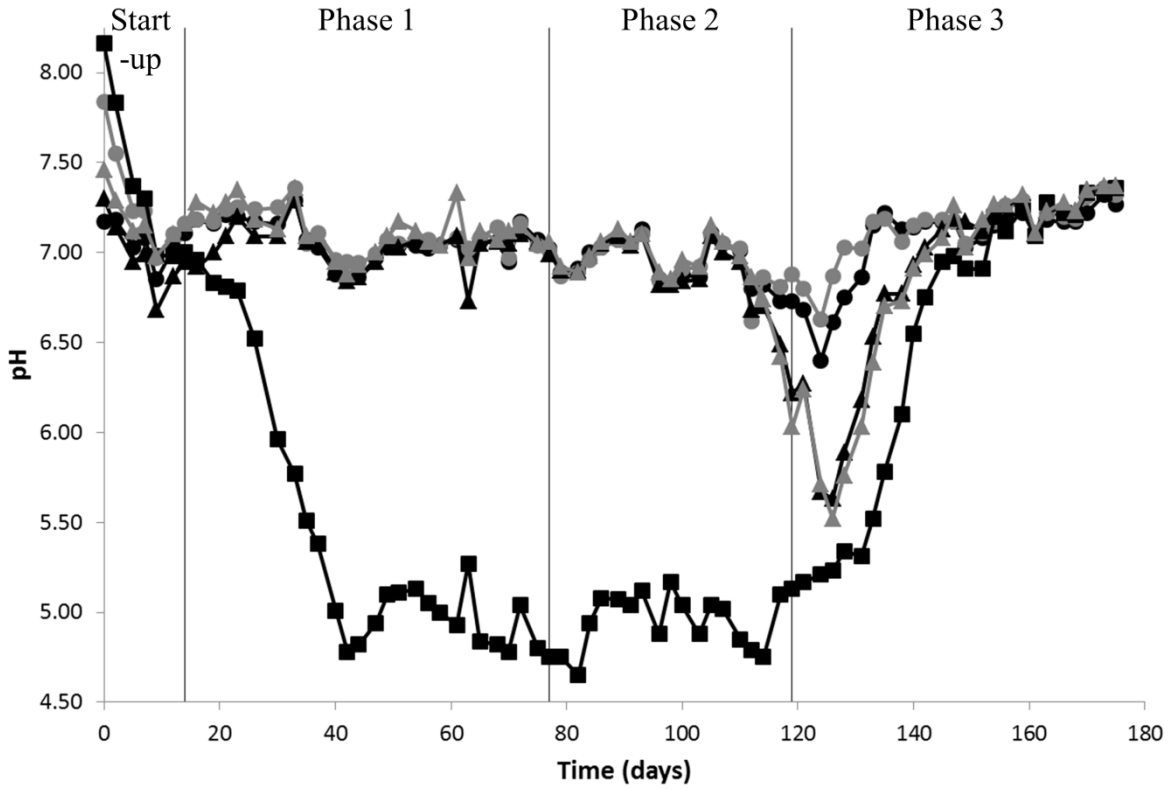


Figure 5.4 *pH profile in the four different phases, i.e. Start-up phase (day 0 -14), Phase 1 (day 15 – 77), Phase 2 (day 78 – 119) and Phase 3 (day 120 – 175) for the Myd (●), Agri (●), Vce (■), Oss (▲) and Mix (▲) reactor.*

In Phase 3 the OLR was lowered and ammonium was no longer added to the reactor, hence, the TAN concentration slowly decreased (Figure 5.3). This resulted in an increase in methane production in all five AD reactors, even in the Vce reactor that had low methane production values during Phase 1 and 2, with an average final methane production value of 592 ± 7 mL L^{-1} d^{-1} (Figure 5.1). Total VFA were below detection limit in all five reactors at the end of Phase 3, and stable pH values of 7.34 ± 0.04 were measured (Figure 5.2 and 5.4).

3.2. Taxonomic profiles of the different reactors

The microbial community was analysed at the beginning of the experiment, before inoculation (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175) to estimate the effect of the operational regime of each phase on the community structure in the different reactors (Figure 5.5 and 5.6).

An average of 74510 ± 16956 reads was obtained per sample, resulting in a total number of 718 different OTUs. Rarefaction curves, generated to estimate the coverage of the microbial community in the samples by the created dataset, showed that the plateau phase was reached

for all samples, indicating sufficient coverage of the microbial community (Figure 5.7). The inoculum samples of the Myd (315 OTUs), Agri (312 OTUs) and Vce (322 OTUs) reactor had a much lower species richness compared to the Oss (582 OTUs) and Mix (636 OTUs) samples, but at the end of Phase 1, similar high species richness values (627 ± 33 OTUs) were detected in all AD reactors. These high species richness values were maintained during Phase 2 and 3.

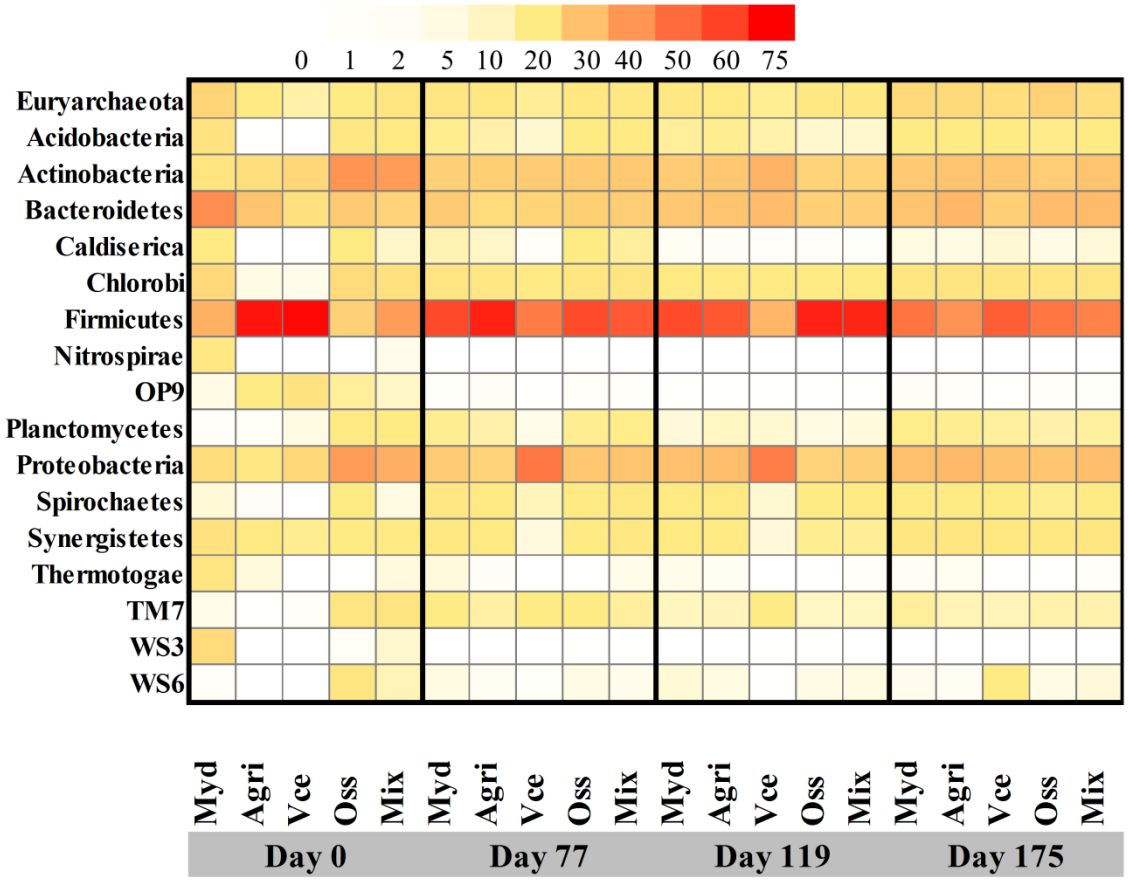


Figure 5.5 *Heatmap representing the phyla present at a relative abundance ≥ 1% in at least one of the samples. Samples are presented at the beginning of the experiment (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175) for the five AD reactors. The colour scale ranges from 0 to 75% relative abundance.*

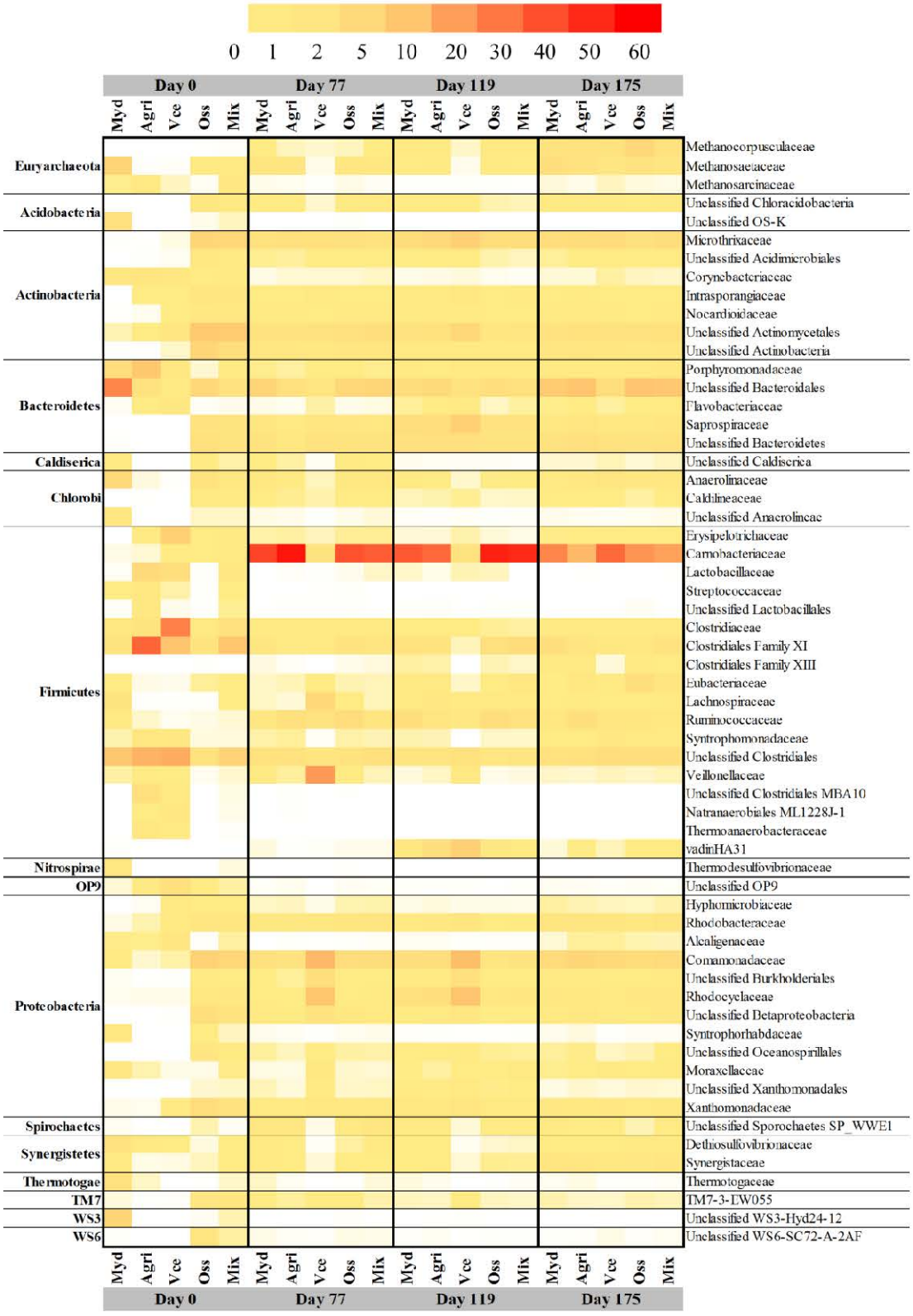


Figure 5.6 *Heatmap representing the families present at a relative abundance ≥ 1% in at least one of the samples. The colour scale ranges from 0 to 60% relative abundance. Samples are presented at the beginning of the experiment (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175) for the five AD reactors. Taxonomy is shown at the phylum level (left column) and at the lowest determined level, i.e. family (right column).*

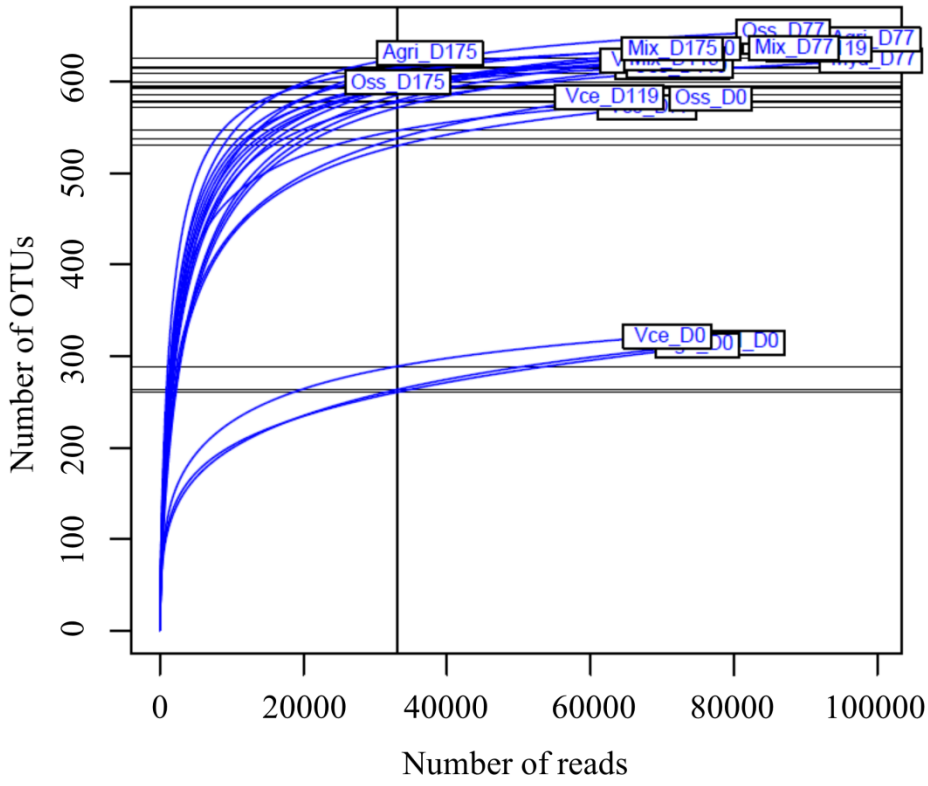


Figure 5.7 *Rarefaction curves indicating the number of resolved phylotypes against sampling depth of each of the samples of the five AD reactors, at the beginning of the experiment (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175).*

The phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were mostly represented, covering over 74% of the microbial community in all samples, with the exception of the Myd reactor on day 0 (Figure 5.5). There were, however, substantial differences on phylum level between the five samples at the start of the experiment. Indeed, whereas several other phyla were also represented in the Myd, Oss and Mix reactor, the Agri and Vce reactor hosted a lower initial phylum richness. Towards the end of Phase 1, the phylum composition in the five AD reactors was similar, with the exception of the Vce reactor, presenting a lower relative abundance of Firmicutes (35.8% vs. an average value of 53.7 ± 7.4% for the other 4 reactors), yet a higher abundance of Proteobacteria (37.1% vs. an average value of 11.2 ± 2.1% for the other 4 reactors). These results can be related to the methane production values, reaching high levels in the Myd, Agri, Oss and Mix reactor, compared to the Vce reactor at the end of Phase 1. A similar pattern was observed at the end of Phase 2, i.e. an increased relative abundance of Proteobacteria (35.6% vs. an average value of 11.8 ± 3.3% for the other 4 reactors) and a decreased relative abundance of Firmicutes (17.3% vs. an average value of

56.7 ± 8.6% for the other 4 reactors) in the Vce reactor, compared to the other four AD reactors. The microbial community evolved towards a similar composition in all five AD reactors at the end of the experiment (end of Phase 3), with an overall increase in relative abundance of Euryarchaeota in all five AD reactors, which also correlated to similar levels of methane production.

In accordance with the microbial community composition at phylum level, the composition on family level also greatly varied between the different reactors and in the different phases (Figure 5.6). The methanogenic community (phylum of the Euryarchaeota) highly differed between the different reactors in the inoculum samples, yet, in all five AD reactors a methanogenic community dominated by the Methanocorpusculaceae (3.3 ± 1.4% of the total community) and Methanosaetaceae (2.4 ± 0.8% of the total community) was obtained at the end of the experiment. The Actinobacteria phylum was dominated by the Microthrixaceae (3.8 ± 1.1% of the total community) and an unclassified Actinomycetales family (3.0 ± 0.8% of the total community) throughout the entire experiment, with the exception of the inoculum samples. The Firmicutes phylum mostly contained representatives of the Carnobacteriaceae family (up to 54.7% of the total microbial community), with the exception of the Vce sample at the end of Phase 1 that was dominated by the Veillonellaceae family, with a value of 21.0% of the microbial community. The high relative abundance of the Proteobacteria phylum in the Vce reactor at the end of Phase 2 and 3 was due to the Comamonadaceae (13.0 and 11.8% for Phase 2 and 3) and Rhodocyclaceae (9.8 and 10.1% for Phase 2 and 3) families. Overall, in accordance to the phylum level, the bacterial community composition on family level also finally evolved to a similar pattern in all five AD reactors at the end of the experiment.

3.3. Quantitative analysis of the acetoclastic methanogenic community

Methanosaetaceae were the dominant acetoclastic methanogens in all five AD reactors (Figure 5.8), which corresponded with the 16S rRNA gene sequence results. At the end of Phase 1 the Myd, Agri, Oss and Mix reactors had a similar profile, with Methanosaetaceae as the dominant methanogens, reaching an average value of $1.9 \times 10^{11} \pm 7.0 \times 10^{10}$ copies g^{-1}. In the Vce reactor the Methanosaetaceae remained the dominant acetoclastic methanogens, yet, at a lower absolute value of $5.7 \times 10^{9} \pm 6.3 \times 10^{8}$ copies g^{-1}. The increased TAN concentration during Phase 2 resulted in all five AD reactors in an increase in Methanosarcinaceae abundance to an average value of $6.2 \times 10^{6} \pm 4.6 \times 10^{6}$ copies g^{-1} at the end of Phase 2, compared to $1.7 \times 10^{5} \pm 1.4 \times 10^{5}$ copies g^{-1} at the end of Phase 1, yet, Methanosaetaceae

remained the dominant acetoclastic methanogens. At the end of Phase 3, all five AD reactors reached similar profiles, which is in correlation to a similar methane production, with Methanosaetaceae as the prevailing acetoclastic methanogens. However, a clear decrease in Methanosarcinaceae was observed in the Myd and Agri reactors, compared to Phase 2, which was not the case in the Vce, Oss and Mix reactor.

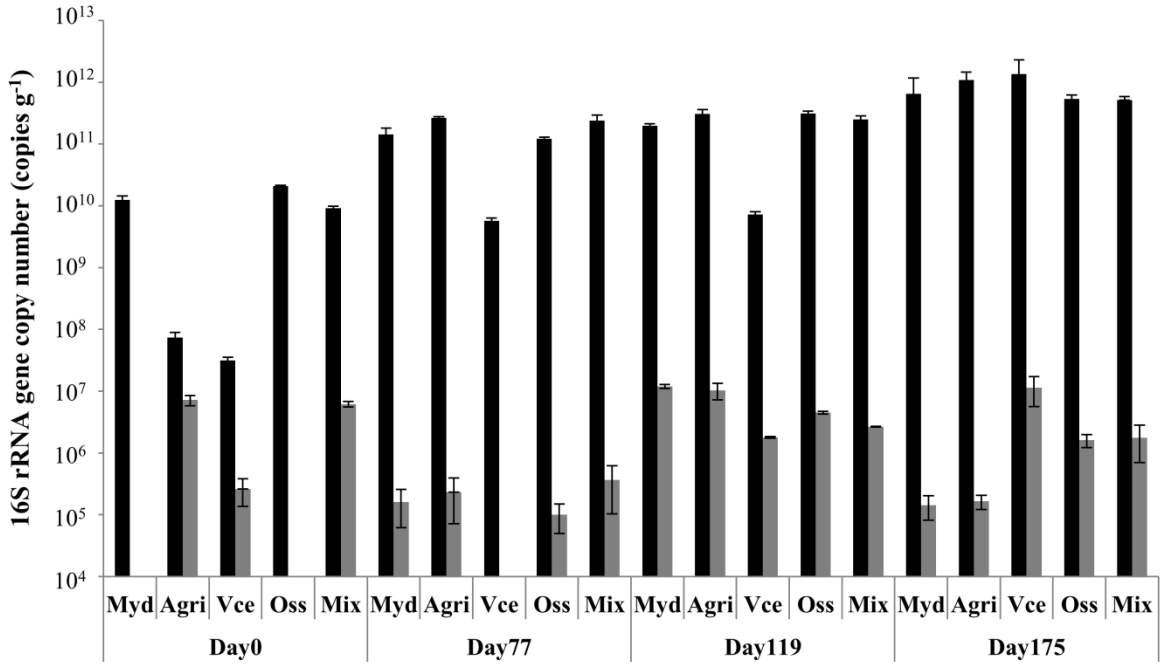


Figure 5.8 *Real-time PCR results of the Methanosaetaceae (■) and Methanosarcinaceae (■) at the beginning of the experiment (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175) for the five AD reactors. The data are represented as copies of the target gene per gram of wet sludge. Average values of the triplicate analyses, together with the standard deviations are presented.*

3.4. Microbial community correlated with methane production, VFA and TAN concentrations

A strong positive correlation ($P < 0.005$) was detected between the OTUs belonging to the acetoclastic Methanosaetaceae and hydrogenotrophic Methanomicrobiaceae and methane production in all five AD reactors during the entire experiment (Figure 5.9). The hydrogenotrophic Methanocorpusculaceae were also positively correlated ($P < 0.05$) to methane production. The OTU12 (ρ=0.8), closely related to *Methanosaeta concilii* DQ150255, showed the highest role in methane production in all five AD reactors.

On the level of bacterial domain, 76 bacterial OTUs, most of which belonged to the Anaerolinaceae and Clostridiales were positively correlated ($P < 0.05$) with methane production (Figure 5.9 and 5.10). Other OTUs contained within the families Bradyrhizobiaceae and Rhodobacteraceae (α-Proteobacteria), Planctomycetaceae and the Bacteroidetes phylum were also positively correlated ($P < 0.05$) with methane production. All OTUs positively correlated to methane production were neither positive nor negative correlated with any other functional parameters (TAN or VFA concentration), with the exception of 8 OTUs that were negatively correlated with VFA concentration. A community that consisted of 13 non-related OTUs was negatively correlated with methane production.

The majority of the core microbiome positively correlated with VFA concentration, a total of 85 OTUs, was related to Bacteroidetes and β-Proteobacteria and to lesser extend to Clostridiales (Figure 5.10). Surprisingly, the OTUs belonging to the Methanobacteriaceae were not positively correlated with methane production, but with VFA concentration. All OTUs positively correlated with VFA concentration were neither positively or negatively correlated with methane production, nor showed a negative correlation with TAN concentration. However, a total of 34 OTUs were also positively correlated with TAN concentration, of which most were related to Bacteroidetes. The OTUs 12 and 7, belonging to the Methanosaetaceae and Methanomicrobiaceae, respectively, were negatively correlated with VFA concentration, but also a non-related core of bacteria with the same negative correlation with VFA concentration was detected.

4. Discussion

In this research, the role of initial inoculum selection and the adaptation of the core microbiome to changes of operational parameters in anaerobic digestion in terms of start-up, stable operation and stress tolerance were investigated. Methane production revealed that the selected inoculum had a clear contribution to methane production and stress tolerance. Phylogenetic sequencing analysis revealed the prevalence of specific microbial communities at different operational conditions, with clear correlations between both domains of bacteria and archaea and operational parameters. Nonetheless, despite the long-term operation of the experiment, care should be taken with the interpretation of the results and the drawing of conclusion, since no triplicate experiments were carried out.

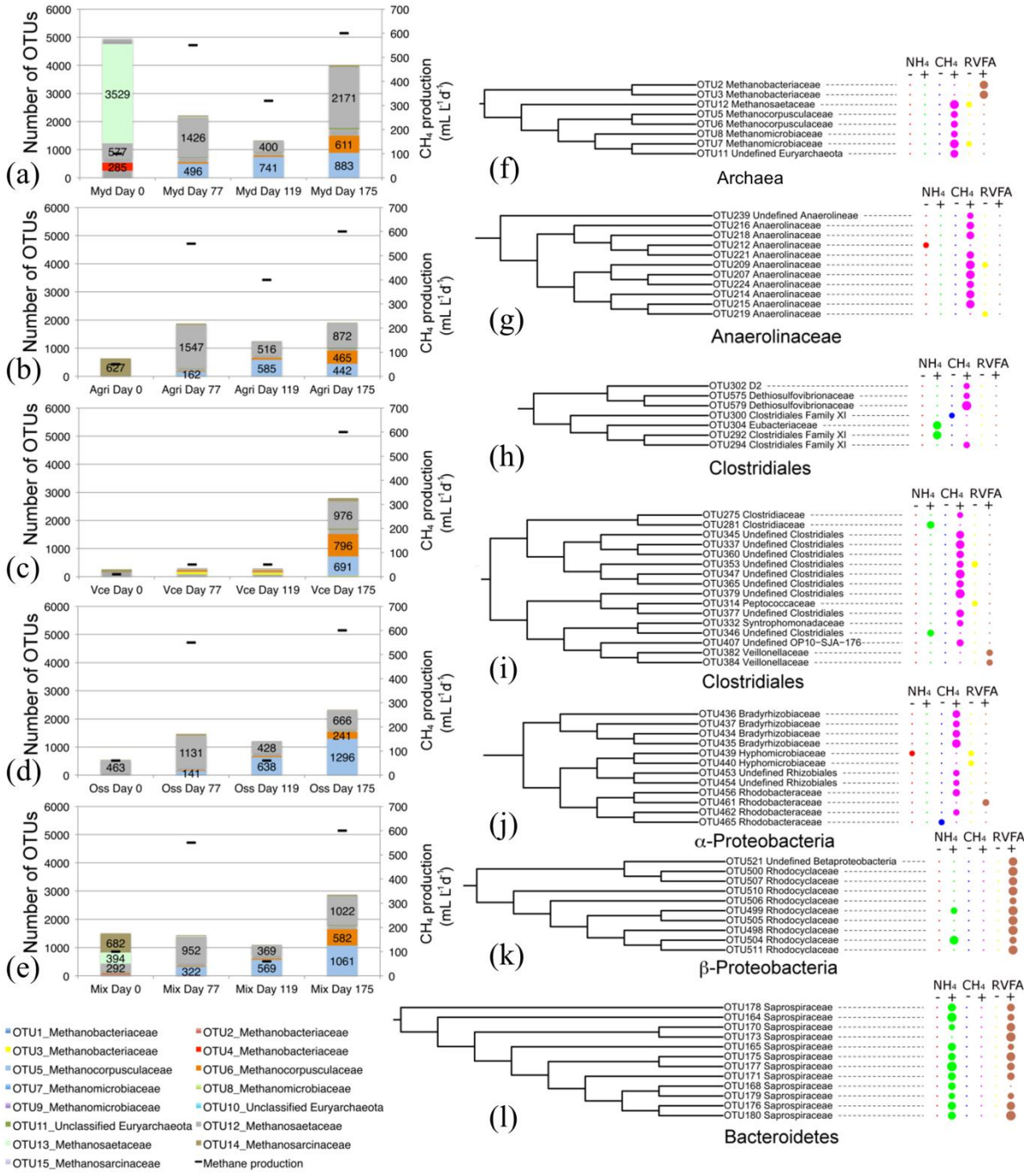
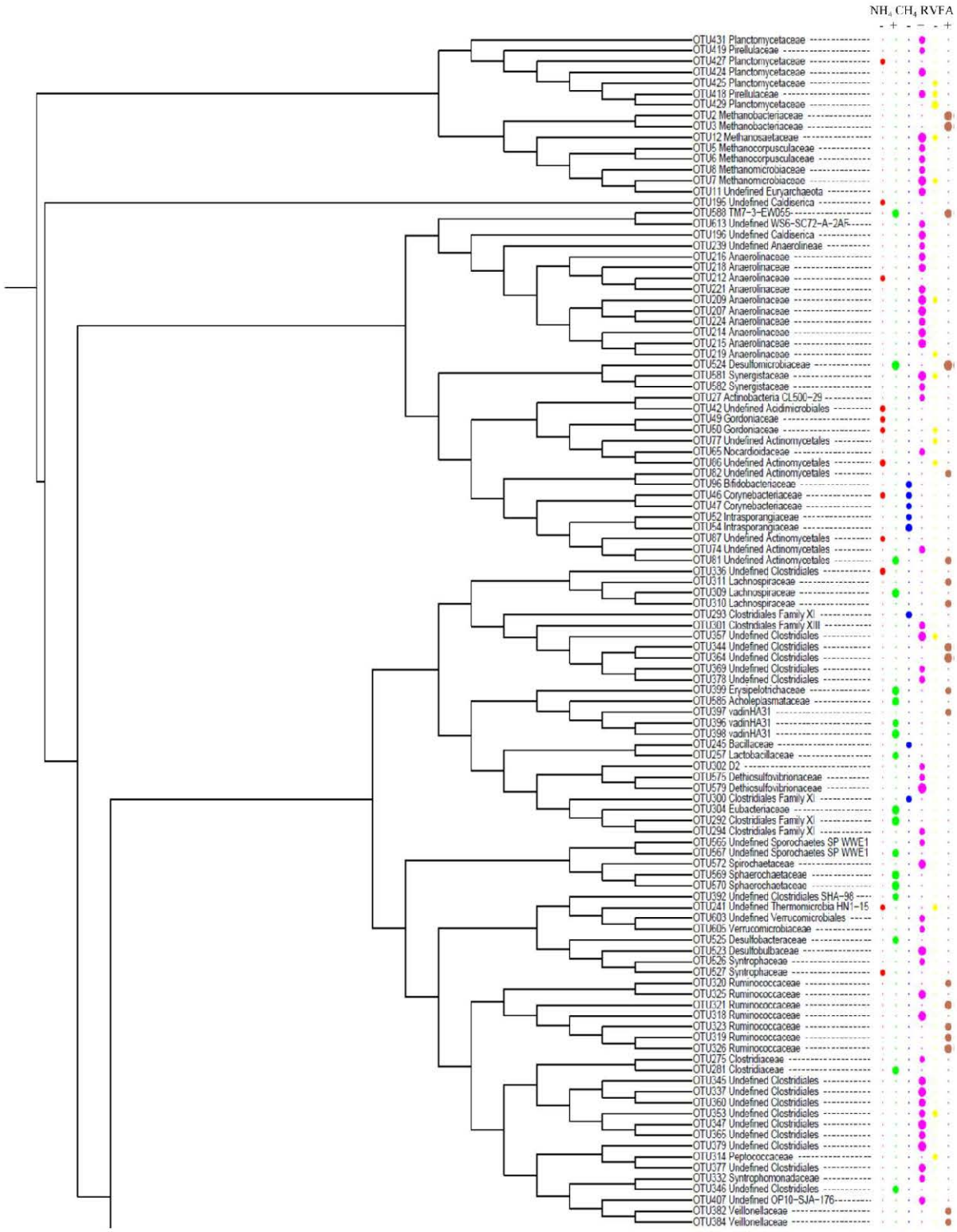


Figure 5.9 Pearson c*orrelation between methane production, VFA and TAN concentration and the microbial community. Methane production (—) is represented in the five reactors, i.e. (a) Myd, (b) Agri, (c) Vce, (d) Oss and (e) Mix at the beginning of the experiment (day 0) and at the end of Phase 1 (day 77), Phase 2 (day 119) and Phase 3 (day 175) together with the relative abundance (number of reads) of the OTUs belonging to the archaea domain. Phylogenetic trees with correlation between OTUs were created using iTol (http://itol.embl.de) for data visualization (Letunic & Bork, 2011), resulting in archaeal and bacterial OTUs positively correlated to methane production (●), VFA (●) and TAN concentration (●) and negatively correlated to methane production (●), VFA (●) and TAN*

concentration (●) (f-l). Large dots represent a strong significant correlation with P<0.01, while small dots represent a correlation with P<0.05.



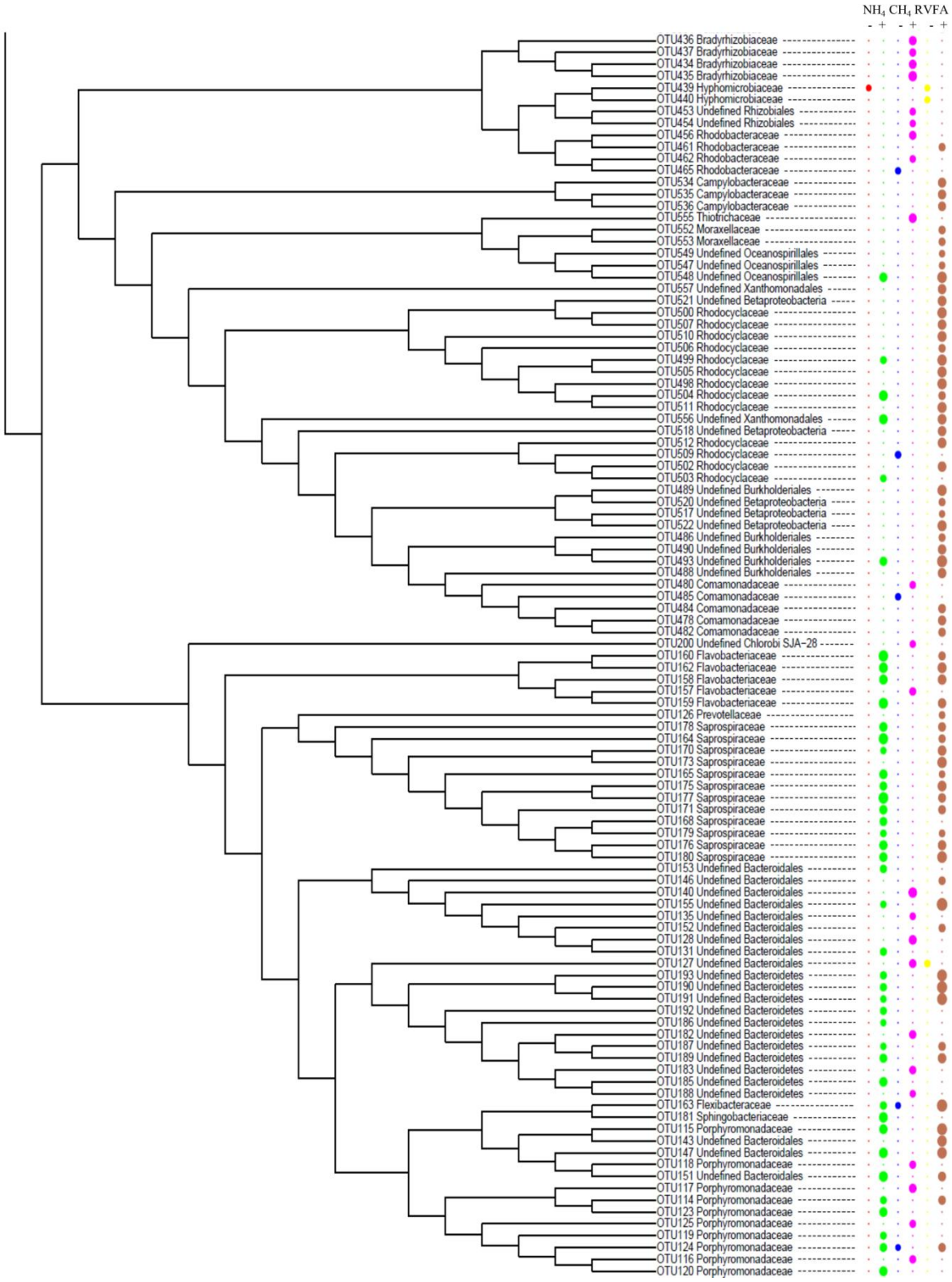


Figure 5.10 *Phylogenetic tree of the bacterial 16S rRNA gene sequences highlighting archaeal and bacterial OTUs positively correlated (P < 0.05) to methane production (●), VFA (●) and TAN concentration (●) and negatively correlated (P < 0.05) to methane production (●), VFA (●) and TAN concentration (●). Phylogenetic trees with correlation between OTUs were created using iTol (http://itol.embl.de) for data visualization (Letunic & Bork, 2011).*

Methane production results revealed a clear effect of the selected inoculum on anaerobic digester performance. During Phase 2, only four inocula (Myd, Agri, Oss and Mix) presented stable methane production, i.e. residual VFA remained below 1.0 g COD L^{-1} d^{-1}, whereas the Vce inoculum caused process failure, i.e. a 90% decrease in methane production was observed compared to the other four AD reactors. The inability of the Vce inoculum to reach stable methane production could be attributed to its high initial TAN and FA content (Table 5.1). Indeed, elevated FA concentrations are known to negatively affect methanogenesis, as FA concentrations between 220 and 1100 mg N L^{-1} can inhibit methanogenesis at mesophilic conditions, depending on the reactor conditions and the degree of adaptation of the microbial community (Gallert & Winter, 1997; Hansen et al., 1998; Chen et al., 2008; Rajagopal et al., 2013). Since the Vce inoculum originated from a full-scale AD plant treating manure, which contains high TAN concentrations, adaptation of the microbial community already took place for several years. The high FA concentration of 1460 mg N L^{-1}, measured in the Vce inoculum, was, however, far above the maximum concentration causing inhibition, hence, the methanogenic community was most likely already (partially) inhibited at the start of the experiment.

The increased TAN concentration during Phase 2 also had a variable impact, depending on the selected inoculum, as the Myd and Agri reactor maintained higher levels of methane production at the maximum TAN concentration on day 112, compared with the Oss and Mix reactor. The higher tolerance of the Agri inoculum to high TAN levels can be attributed to a certain level of adaptation of the microbial community due to an elevated TAN concentration of 2904 mg N L^{-1} in the initial inoculum (Hashimoto, 1986; Angelidaki & Ahring, 1993; Hansen et al., 1998; Schnurer & Nordberg, 2008). The higher ammonium tolerance of the Myd reactor, compared to the Oss and Mix reactor was rather unexpected, especially considering the fact that the Mix reactor contained 25% (w/w) of each of the four inocula. However, the structural organization of the microbial biomass in the granules of the Myd sludge, in contrast to the other inocula, might have enhanced its tolerance to ammonia stress (Satoh et al., 2007).

The initial phylum richness was lower in the Agri and Vce reactor, compared to the Myd, Oss and Mix reactor (Figure 5.5). This could be correlated to the high TAN and FA concentrations and high conductivity in these inocula, as these led to unfavourable conditions for the AD microbial community, thus, reducing phylum richness (Chen et al., 2008; Marzorati et al., 2008). Feeding of waste activated sludge and glycerol resulted in similar TAN concentrations

(475 ± 65 mg N L^{-1}) at the end of Phase 1, and this led to a similar phylum composition in the Myd, Agri, Oss and Mix reactor. There was a substantial difference in community composition between the latter four reactors and the Vce reactor at the end of Phase 1 and 2. Members of the Firmicutes phylum dominated the microbial community in the Myd, Agri, Oss and Mix reactor, while the Proteobacteria phylum was dominant in the Vce reactor at the end of Phase 1 and 2. Several members of the Firmicutes phylum are syntrophic bacteria responsible for the degradation of VFA, such as propionic and butyric acid to acetic acid and H_2, which are the main precursors for methane (Riviere et al., 2009; Vanwonterghem et al., 2014). The presence of syntrophic bacteria in AD is essential, as they ensure, amongst other degradation processes, one of the most critical aspects in the AD process, the degradation of VFA, hereby preventing inhibition of methanogenesis (Krakat et al., 2011). Although not all representatives of the Firmicutes phylum are syntrophic bacteria, the decreased abundance of Firmicutes in relation to the increased abundance of the Proteobacteria phylum in the Vce reactor might, therefore, directly relate to enhanced residual VFA concentrations and reduced methane production. More in-depth microbial community analysis is, however, required to confirm whether the Firmicutes present in the different reactors are syntrophic bacteria. Indeed, stable AD systems with efficient COD conversion to methane often show a domination of the Firmicutes phylum over the Proteobacteria phylum (Krober et al., 2009; Liu et al., 2009; Bengelsdorf et al., 2013; Sundberg et al., 2013), although in several other cases a dominance of the Proteobacteria phylum over the Firmicutes phylum was observed (Riviere et al., 2009; Lee et al., 2012). The shift in dominance from the Firmicutes to the Proteobacteria phylum in the Vce reactor can therefore not be related directly to its failure. However, in this research, a shift from a Firmicutes to a Proteobacteria dominated bacterial community could only be detected in the failed digester and after remediation (end of Phase 3) the Firmicutes phylum again became dominant in the Vce reactor. An increased abundance of the Proteobacteria phylum has been related to dysbiosis in the microbial community in the human gut, a similar principle that may apply for AD as well (Mondot et al., 2011; Li et al., 2012; Michail et al., 2012).

In addition to the bacterial community, the methanogenic community was influenced by the reactor conditions as well. The overall dominance of the Methanosaetaceae in the Myd, Agri, Oss and Mix reactor can be attributed to their ability to outcompete other acetoclastic methanogens at low residual VFA concentrations, due to their high affinity for acetate (Gujer & Zehnder, 1983; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b). The absolute

increase in Methanosarcinaceae abundance at the end of Phase 2 relates to the elevated ammonium concentration, as they tend to be more resilient to ammonia stress, compared to Methanosaetaceae (Calli et al., 2005a; Demirel & Scherer, 2008; Schnurer & Nordberg, 2008; Nettmann et al., 2010; Williams et al., 2013).

Both acetoclastic (Methanosaetaceae) and hydrogenotrophic (Methanomicrobiaceae and Methanocorpusculaceae) methanogens positively correlated to methane production, yet Methanobacteriaceae positively correlated to VFA accumulation. The increased abundance of Methanobacteriaceae has often been observed in AD reactors with high residual VFA concentrations and decreased pH values (Delbes et al., 2001; McMahon et al., 2004; Steinberg & Regan, 2011). The positive correlation of several OTUs belonging to the Clostridiales order and Firmicutes phylum with methane production can point to a syntrophic interaction between hydrogenotrophic methanogens and syntrophic acetogenic bacteria and syntrophic acetate oxidizing bacteria (Zinder & Koch, 1984; Schink, 1997; Hattori, 2008; Kato & Watanabe, 2010; Westerholm et al., 2010). Hence, both acetoclastic methanogenesis and hydrogenotrophic methanogenesis, in combination with syntrophic acetate oxidation, could take place in the different reactors in the different phases. The dominating methanogenic pathway in each phase depended on the conditions in the reactor, with the syntrophic acetate oxidation and subsequent hydrogenotrophic methanogenesis pathway usually dominating at sub-optimal conditions (Karakashev et al., 2006; Schnurer & Nordberg, 2008; Hao et al., 2011; Lu et al., 2013a).

5. Conclusions

This research demonstrated the importance of the selection of a suitable inoculum to initiate an anaerobic digester. A shift from a Firmicutes to a Proteobacteria dominated bacterial community was observed in a failing digester. The acetoclastic Methanosaetaceae remained the main methanogens in each reactor, irrespective of the present conditions. Acetoclastic and hydrogenotrophic methanogens, as well as several bacterial groups positively correlated with methane production, indicating the necessity for close microbial cooperation to obtain high methane production rates. Nevertheless, to extrapolate these results to full-scale application, more and different inocula should be analysed in terms of stable operation and stress tolerance in triplicate experiments to allow statistical confirmation of the results.

Acknowledgements

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CHAPTER 6: BIOMASS RETENTION ON ELECTRODES PROVED TO BE THE MAIN STABILIZING MECHANISM RATHER THAN ELECTRICAL CURRENT DURING ANAEROBIC DIGESTION OF MOLASSES

This chapter has been redrafted after:

De Vrieze, J., Gildemyn, S., Arends, J.B.A., Vanwonterghem, I., Verbeken, K., Boon, N., Verstraete, W., Tyson, G.W., Hennebel, T., Rabaey, K. 2014. Biomass retention on electrodes rather than electrical current enhances stability in anaerobic digestion. Water Research, 54, 211-221.

Abstract

Anaerobic digestion is a well-established technology for energy recovery from organic waste streams. Several studies noted that inserting a bioelectrochemical system inside an anaerobic digester can increase biogas output, however, the mechanism behind this was not explored and primary controls were not executed. Here, we evaluated whether a bioelectrochemical system could stabilize anaerobic digestion of molasses. Lab-scale digesters were operated in the presence or absence of electrodes, in open (no applied potential) and closed circuit conditions. In the control reactors without electrodes methane production decreased to 50% of the initial rate, while it remained stable in the reactors with electrodes, indicating a stabilizing effect. After 91 days of operation, the now colonized electrodes were introduced in the failing anaerobic digestion reactors to evaluate their remediating capacity. This resulted in an immediate increase in methane production and volatile fatty acids removal. Although a current was generated in the bioelectrochemical system operated in closed circuit, neither direct effect of applied potential, nor current was observed. A high abundance of *Methanosaeta* was detected on the electrodes, however, irrespective of the applied cell potential. This study demonstrated that, in addition to other studies reporting only an increase in methane production, a bioelectrochemical system can also remediate anaerobic digestion systems that exhibited process failure. However, the lack of difference between current driven and open circuit systems indicates that the key impact is through biomass retention, rather than electrochemical interaction with the electrodes.

1. Introduction

Bio-refineries produce sidestreams with high organic content (Verstraete et al., 2005). The success rate of most bio-refineries depends on the full utilization of all resources present in the original biomass, including these sidestreams. In this study, molasses was used to mimic sidestreams originating from bio-refineries. The direct discharge of untreated molasses wastewaters may cause serious environmental issues, due to their high concentration of organic matter, high salt content and low pH (Sirianuntapiboon & Prasertsong, 2008). Anaerobic digestion (AD) is an established technology, and can be considered the first microbial technology to allow energy recovery from complex organic waste streams. AD therefore has the potential to become a key technology to treat these sidestreams, and generate heat and electricity for the refinery (Verstraete et al., 2005). AD can also deal with high loading rates, has limited nutrient demands and low operational control and maintenance costs (Mata-Alvarez et al., 2000; Verstraete et al., 2009). Methanogenic archaea are responsible for the final and most critical step of AD, i.e. the production of methane. One of the main drawbacks of AD is a sometimes-observed process failure, due to sensitivity of these methanogens to different environmental factors, such as abrupt pH changes, organic overloading and high salt concentrations, leading to the accumulation of volatile fatty acids (VFA) (Gujer & Zehnder, 1983; Ahring et al., 1995; Chen et al., 2008; De Vrieze et al., 2012).

Bioelectrochemical systems (BESs) are an alternative technology to AD, capable of directly producing electrical power from liquid organic waste streams. Contrary to AD, very few BESs exist beyond the lab-scale, hence, their competitiveness with AD remains thus far unproven (Pham et al., 2006; Arends & Verstraete, 2012). On the other hand, BESs are highly versatile in terms of potential application, ranging from energy production from organic substrates to product generation and specific environmental niche creation (Rabaey & Rozendal, 2010; Arends & Verstraete, 2012; Logan & Rabaey, 2012). These last two processes are of main interest to AD, due to their possible influence on process stability and microbial activity.

It has been postulated that a BES can be used to alter and/or control the main processes in AD (Sasaki et al., 2010b; Arends & Verstraete, 2012). Several studies already highlighted that combining anaerobic digesters with a BES resulted in a higher level of biogas production (Rabaey et al., 2005; Sasaki et al., 2010b; Vijayaraghavan & Sagar, 2010; Sasaki et al., 2011a; Tartakovsky et al., 2011; Weld & Singh, 2011). Different AD-BES configurations,

such as the utilization of a BES as pre- or post-treatment device outside the AD reactor, or the direct application of a BES in the digester, may lead to enhanced methane production. The introduction of a BES in the recirculation loop of a thermophilic UASB (upflow anaerobic sludge bed) resulted in a higher tolerance of the digester to a severe drop in pH due to the addition of an acetate pulse to the system (Weld & Singh, 2011). The direct application of the cathode in an AD reactor resulted in enhanced COD (chemical oxygen demand) removal and methane production during AD of filter paper and garbage slurry, respectively (Sasaki et al., 2010b; Sasaki et al., 2011a). The introduction of both the anode and cathode of a BES in the sludge bed of an UASB reactor (Tartakovsky et al., 2011) or in a CSTR (continuous stirred tank reactor) (Vijayaraghavan & Sagar, 2010) also resulted in increased methane production. A BES can also be used for post-digestion polishing of highly loaded wastewaters, leading to side products such as H_2 (Rabaey et al., 2005).

The objective of this study was (1) to evaluate whether a BES could stabilize AD (AD-BES) of molasses leading to higher COD removal and methane production, and (2) if a BES could remediate systems that have experienced severe process failure and (3) how this influences the microbial community composition of the entire system. The term ‘stable’ was used as long as total residual VFA remained below 1.0 g COD L^{-1}, whereas the term ‘failure’ referred to a 50% decrease in methane production compared to the initial value. To achieve these goals, different lab-scale anaerobic digesters were operated in the presence or absence of a BES to evaluate the stabilizing potential of a BES in AD. The cell potentials were selected to avoid direct electrochemical production of H_2 at the cathode or O_2 at the anode but to potentially stimulate biologically catalysed H_2 production, which could lead to an increased methane production. The BESs were also introduced in failing AD reactors to evaluate their remediating capacity.

2. Material and methods

2.1. Experimental set-up and operation

2.1.1. Reactor set-up and operation

Seven lab-scale CSTR vessels with a liquid volume of 800 mL were each connected to a gas column to collect the produced biogas (Figure 6.1). These reactors are considered reproducible, as indicated in earlier preliminary research (data not shown). Moreover, each reactor was considered a time series in accordance with the research of Wittebolle et al.

(2008), Carballa et al. (2011) and Zamalloa et al. (2012). A pair of carbon felt electrodes were introduced in three vessels, each with a surface area of 60 cm² (projected area; BET 2 m^2 g^{-1}; Carbon felt, 3.18 mm thickness, Alfa Aesar, Ward Hill, MA, USA), which corresponded to a projected surface area to volume ratio of 0.015 m^2 L^{-1} reactor (a). The electrodes were fixed in parallel at a distance of 1 cm. The reactors with electrodes contained a Ag/AgCl reference electrode (MF-2052, BASi, West Lafayette, IN, USA) (b) and were connected to a power source (3030D, Protek, USA) (c) via a stainless steel wire and a 1 Ω resistor (d). The reactor itself was a glass Schott Bottle (Duran Group GmbH, Mainz, Germany), closed with a rubber stopper. Volumetric biogas production was evaluated by means of water displacement (e). Mixing of the reactors was carried out using a magnetic stirrer (f).

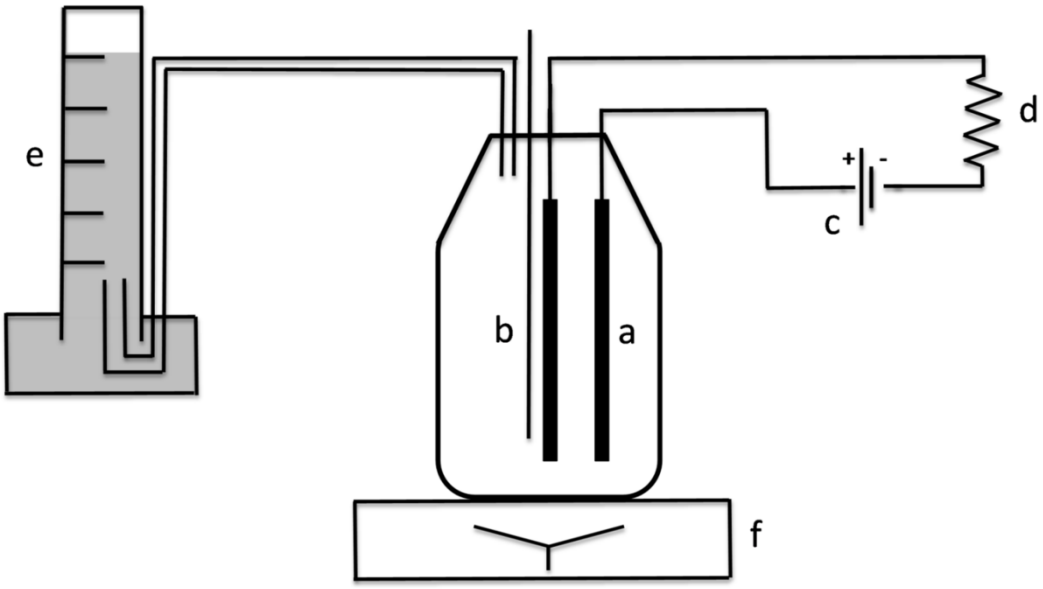

Figure 6.1 *Schematic overview of the reactor set-up. The set-up consisted of a reactor, in which an electrode pair (a) and a reference electrode (b) were introduced. The electrodes were connected to a power source (c) and a resistor (d). Biogas was collected by means of a gas collection device (e), and stirring took place by means of a magnetic stirrer (f).*

2.1.2. Reactor operation

All seven reactors were inoculated with anaerobic sludge from a municipal sludge digester (Ossemeersen, Ghent, Belgium). The sludge was diluted with tap water to obtain an initial sludge concentration of 10 g L^{-1} volatile suspended solids (VSS). All reactors were operated at 34 °C in fed batch mode, and fed 3 times a week (Monday, Wednesday and Friday) for a total period of 154 days. Fresh feed was prepared for every feeding. A sludge retention time

(SRT) of 20 days was maintained. During the start-up phase (Phase 1) waste activated sludge (collected from the Ossemeersen, Ghent, Belgium) was used as feeding source (Table 6.1), whereas during Phase 2 and 3 diluted molasses, originating from potato processing (AVEVE, The Netherlands), was used as feed (Table 6.1 and 6.2). Waste activated sludge (WAS) was selected to feed the reactor during Phase 1, since this was the same feed that was used in the full-scale reactor, from which the anaerobic inoculum sludge sample originated. Phase 1 was considered as an adaptation period for the anaerobic sludge to adapt to laboratory conditions. Molasses was selected as a *proxy* of bio-refinery sidestreams, because of its high COD and salt content (Sirianuntapiboon & Prasertsong, 2008). Reactor nomenclature was set based on the cell potential during Phase 2 and 3. The first letter (Phase 2) and last letter (Phase 3) of the name show whether a cell potential was applied in the presence of an electrode pair, V(oltage), or whether no electrode pair was present, C(ontrol). The number or letter in the middle of the name indicates the cell potential (1 = 1V cell potential, 0.5 = 0.5V cell potential, O = open circuit potential and N = new electrode pair at open circuit potential) in Phase 2 or 3. During Phase 1 and 2, one reactor (V1C) was operated at a fixed potential of 1 V, a second reactor (V0.5C) at 0.5 V and a third reactor (VOC) at open circuit potential (OCP). The 4 reactors without electrodes (C1V, C0.5V, COV and CNV) were operated in parallel as control reactors.

Table 6.1 *Operational conditions in the reactors during the different phases of the experiment.*

Phase	Period	Substrate	OLR (g COD L^{-1} d^{-1})	Buffer	Electrodes
1	Day 1-27	WAS	1.5 - 2	Yes	V1C, V0.5C and VOC
2	Day 28-91	Molasses	2	No	V1C, V0.5C and VOC
3	Day 92-154	Molasses	2	No	C1V, C0.5V, COV and CNV

On day 91 (start of Phase 3), the electrodes were removed from the reactors V1C, V0.5C, VOC and inserted in the reactors C1V, C0.5V, COV, respectively. A piece of 5 cm^2 was cut from each electrode for molecular and electrochemical analysis. The reactors V1C, V0.5C and VOC were further operated at a liquid volume of 730 mL without electrodes. The content of the four control reactors (C1V, C0.5V, COV and CNV) was mixed and redistributed over the four reactors for a liquid volume of 730 mL per vessel. The liquid volume in the reactors was reduced from 800 to 730 mL to maintain a constant projected surface area to volume ratio of 0.015 m^2 L^{-1}. The electrode pair previously belonging to V1C was inserted in C1V. The

reactor was connected to the power source and operated at 1V for the remainder of the experiment. The electrode pair of V0.5C was inserted in C0.5V and operated at 0.5 V, while the electrode pair of VOC was inserted in COV and operated at OCP. A new electrode pair (projected surface area of 55 cm^2) was inserted in CNV at the start of Phase 3, and also operated at OCP. During the entire experiment, methane production was measured three times a week and reported at STP (standard temperature and pressure) conditions. Samples were taken three times a week for analysis of pH, VFA and once a week for volatile solids (VS), conductivity and total ammonia nitrogen (TAN).

Table 6.2 *Characteristics of the molasses feed applied during Phase 2 and 3. All analyses were carried out in triplicate, except for the K^+ analysis.*

Parameter	Value
pH	5.44 ± 0.10
Conductivity (mS cm^{-1})	14.7 ± 0.3
Total COD (g L^{-1})	44.7 ± 0.9
Total solids (g L^{-1})	47.7 ± 5.6
Volatile solids (g L^{-1})	33.6 ± 5.2
Total ammonia nitrogen, TAN (mg N L^{-1})	122 ± 1
Kjeldahl nitrogen, KjN (mg N L^{-1})	2746 ± 25
Acetate (mg L^{-1})	173 ± 1
PO_4^- (mg P L^{-1})	797 ± 51
Cl^- (mg L^{-1})	805 ± 127
NO_3^- (mg L^{-1})	398 ± 93
SO_4^- (mg L^{-1})	1034 ± 136
K^+ (g L^{-1})	6.90
COD:N ratio	16.3 ± 0.4
COD:P ratio	56.1 ± 3.8
TS:VS ratio	1.4 ± 0.3
COD:VS ratio	1.3 ± 0.2

2.2. Electrochemical characterization

Cell voltages were applied using a portable power supply (3030D, Protek, NJ, USA). Applied cell voltage (dV) and cathode potential (E_{cat}) versus a Ag/AgCl reference electrode were measured continuously at 5 min intervals (34972A, Agilent, MetricTest, CA, USA). The anode potential (E_{an}) was estimated as E_{cat}-dV. The resulting current was logged as the

potential difference over a 1Ω resistor at 5 min intervals. The potentials of the reference electrodes were regularly monitored relative to a calomel electrode (+244 mV vs. Standard Hydrogen Electrode (SHE); QIS, the Netherlands) for correct conversion of the electrode potentials, compared to the SHE. Electrochemical calculations were performed according to Logan et al. (2006), and were based on hourly averages. Current and power density are reported normalized to the projected electrode area (60 cm^2 in Phase 1 and 2 and 55 cm^2 in Phase 3). Electrode potentials are reported versus the SHE.

2.3. Microbial community analysis

Microbial community analysis was applied to the inoculum sludge sample and the planktonic (liquid phase) and electrode biofilm (if present) samples of each reactor (V1C, V0.5C, VOC, C1V, C0.5V, COV and CNV) after 91 and 154 days. For the planktonic samples a 10 mL sample was taken, whereas for the electrode biofilm a 1 cm^2 piece was cut of the electrode. The extraction of total DNA from the sludge sample was performed by means of the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), following the manufacturer's instructions. Prior to extraction, the samples were thawed, and the planktonic samples were homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 200 mg of sample was taken for DNA extraction for the planktonic samples and 1 cm^2 for the electrode samples. The DNA concentration in the extracts was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands), by measuring the absorbance ratios at 260 nm and 280 nm. The quality of the extracted DNA was evaluated on a 1% agarose gel.

PCR amplification of the universal 16S rRNA genes was carried out according to the protocol as described by Dennis et al. (2013). The PCR reaction mixture (50 µL) contained 20 ng of template DNA, 5 µL 10x buffer, 1 µL dNTP mix (10 mM each), 4 µL 25 mM MgCl, 0.2 µL Taq polymerase, 1.5 µL BSA (Invitrogen, US) and 8 µM of each of the primers 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') modified on the 5' end to contain the 454 FLX Titanium Lib L adapters B and A, respectively (Engelbrektson et al., 2010). The reverse primers also contained a 5–6 base sample unique bar-code. The PCR protocol consisted of an initial denaturation step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s, with a final elongation step of 72 °C for 10 min. Amplifications were performed using a Veriti® 96-well

thermocycler (Applied Biosystems). 16S rRNA gene amplicons were sequenced using the Roche 454 GS-FLX Titanium Platform at the Australian Centre for Ecogenomics (ACE).

Amplicon sequences were quality filtered, trimmed to 250 base pairs, and dereplicated using the QIIME pipeline (Caporaso et al., 2010). Chimeric sequences were removed with UCHIME (Edgar et al., 2011), and homopolymer errors were corrected using Acacia (Bragg et al., 2012). The number of sequences per sample was normalized to 2100 (minimum number of sequenced per sample) to allow comparison of diversity without bias from unequal sampling effort. CD-Hit OTU (operational taxonomic unit) was used to cluster sequences at 97% similarity (Wu et al., 2011) and cluster representatives were selected. GreenGenes taxonomy (DeSantis et al., 2006) was assigned to each cluster representative based on BLASTn comparison (Altschul et al., 1990). A table with the abundance of different operational units (OTUs) and their taxonomic assignments in each sample was generated. The number of OTUs observed at equal number of sequences between samples (richness) and Simpsons Diversity Index (evenness) were calculated.

All statistical analyses were implemented using R Studio (version 2.15.0) and R packages vegan (Oksanen et al., 2012) and RColorBrewer. The effects of position within the reactor (planktonic, anode, cathode) on richness and evenness were investigated using Tukey Honestly Significant Differences tests (TukeyHSD). The effects of position within the reactor on the community composition were determined using Permutational Multivariate Analysis of Variance on Hellinger transformed OTU abundances. These results were visualized by means of redundancy analysis and principle coordinate analysis (PCA), which were applied as described by (Zuur et al., 2007). Relative abundances of OTUs were used to generate a heatmap.

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Analytical triplicates of a 100-fold dilution of the DNA-samples were analysed for total bacteria, total archaea and the methanogenic populations Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae. Total archaea can be considered a valid estimation of total methanogens in AD, because of the highly unfavourable conditions for non-methanogenic archaea in AD (Woese et al., 1990; Raskin et al., 1995). To quantify total bacteria, the general bacterial primers P338F (5'-ACTCCTACGGGAGGCAGCAG-3') and P518r (5'-ATTACCGCGGCTGCTGG-3'), as described by Ovreas et al. (1997), were used. The primer sets used for total archaea (ARC) and the methanogenic populations Methanobacteriales (MBT), Methanomicrobiales (MMB),

Methanosarcinaceae (Msc) and Methanosaetaceae (Mst) were previously described by Yu et al. (2005). The reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, WIS, USA) and consisted of 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure, which consists of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C for total bacteria. The qPCR program for total archaea, Methanobacteriales, Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae consisted of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. The qPCR data were represented as copies per gram of wet sludge or per square centimetre of carbon felt.

2.4. Analytical techniques

Sludge samples and headspace gas samples were taken three times a week and were analysed immediately, or stored at -20 °C for further analysis. Biogas composition was analysed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH_4, CO_2 and H_2 were determined using a thermal conductivity detector with a lower detection limit of 1 ppmv for each gas component. The VFA were extracted with diethyl ether and measured in a GC-2014 gas chromatograph (Shimadzu, ‘s-Hertogenbosch, the Netherlands), which was equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (dimensions: 25 mm x 0.53 mm, film thickness 1.2 µm; Alltech, Laarne, Belgium), a split injector and a flame ionization detector.

Chemical oxygen demand (COD) was determined using Nanocolor COD 1500 Tube test kits, according to the manufacturer’s instructions (Machery-Nagel, Düren, Germany). Total solids (TS), VS, total suspended solids (TSS), VSS, total Kjeldahl nitrogen (KjN) and TAN were determined according to Standard Methods (Greenberg et al., 1992). Anions (PO_4^{3-}, NO_3^-, NO_2^-, SO_4^{2-}) were analysed using a metrosep A Supp 5-150 column after a metrosep A 4/5 guard column in a 761 Compact IC with a conductivity detector (Metrohm, Switzerland). Potassium was determined using a flame photometer (Eppendorf ELEX6361, Hamburg, Germany). The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium) and conductivity (EC) was determined by means of a C833 conductivity meter (Consort, Turnhout, Belgium).

Scanning electron microscopy (SEM) analysis was carried out on the anodes and cathodes in V1C, V0.5C and VOC after Phase 2. A piece of 1 cm^2 was cut from each electrode at the end of Phase 2. Before SEM analysis, samples were coated with a thin gold layer with a SCD005 Sputter Coater (Bal-Tec AG, Principality of Liechtenstein). The samples were subsequently studied by means of a FEI XL30 scanning electron microscope (FEI, The Netherlands), equipped with a LaB_6 filament.

3. Results

3.1. Reactor performance

Methane production values were similar in all seven reactors during the start-up phase (Phase 1). Molasses was used as feed from day 28 on, yet, no differences in methane production could be detected up to day 38. The seven reactors showed an average methane production of 577 ± 11 mL L^{-1} d^{-1} between day 28 and 38 (Figure 6.2), with an average pH value of 7.67 ± 0.09 on day 38.

The control reactors without electrodes (C1V, C0.5V, COV and CNV) showed decreasing performance from day 40, while the three reactors containing electrodes (V1C, V0.5C and VOC) maintained a similar level of methane production (Figure 6.2a). The methane production in the control reactors declined, with a simultaneous increase in total VFA concentration and decrease in pH. On day 91, the average methane production in the control reactors decreased to 265 ± 97 mL L^{-1} d^{-1} (Figure 6.2b), which corresponds to a decrease of 50% compared to the initial methane production during day 28 to 38. Total VFA concentrations on day 91 increased to 11.5 ± 3.2 g COD L^{-1} in the four control reactors (Figure 6.3b), with propionate and acetate as the most important components with 72 ± 14 and 18 ± 12%, respectively. In the three reactors containing electrodes the total VFA concentration remained below 0.32 g COD L^{-1} (Figure 6.3a). The pH of V1C, V0.5C and VOC increased to 7.85 ± 0.05 on day 91, while the control reactors showed a gradual decrease to 7.42 ± 0.18.

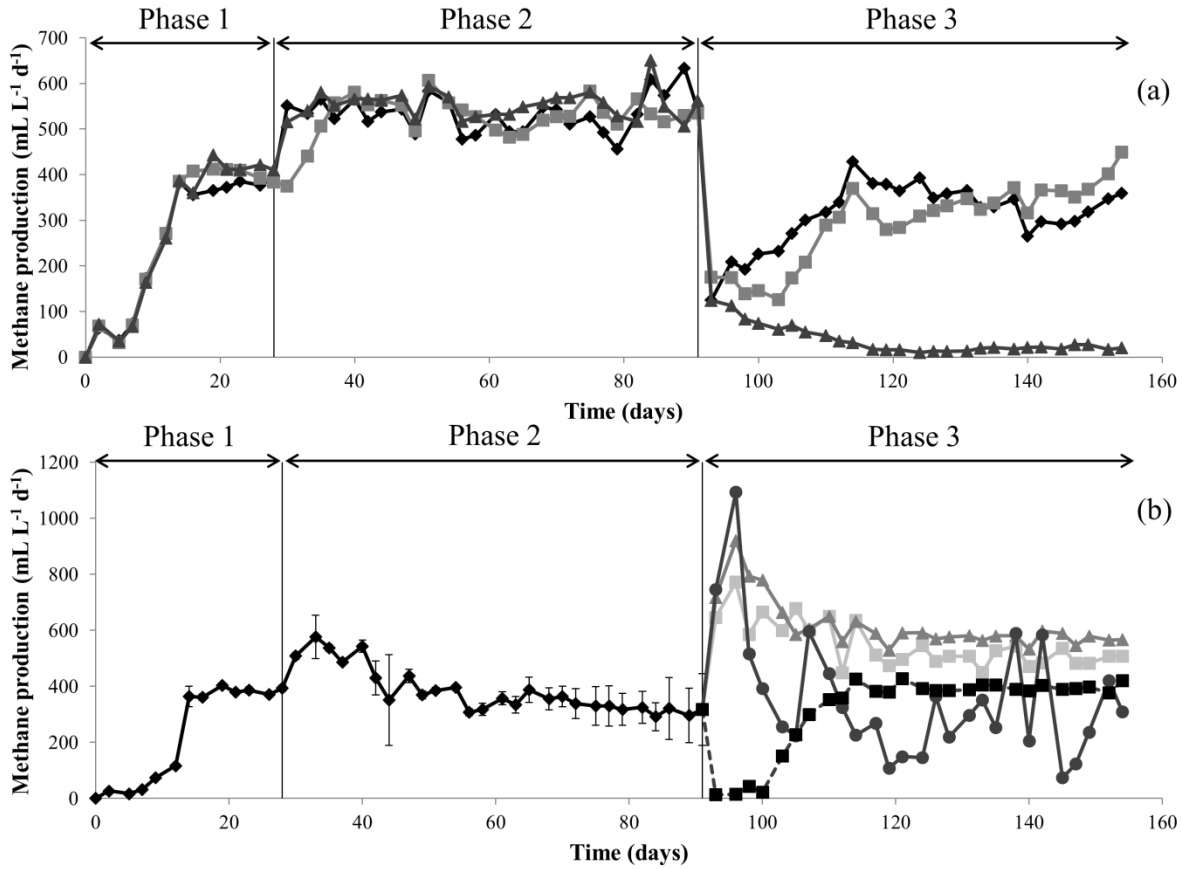

Figure 6.2 *Methane production of (a) V1C (♦), V0.5C (■) and VOC (▲) and (b) C1V-CNV (♦), C1V after introduction of the electrodes (■), C0.5V after introduction of the electrodes (▲), COV after introduction of the electrodes (■) and CNV after introduction of the electrodes (■).*

After 91 days the electrodes were removed from V1C, V0.5C and VOC and inserted in C1V, C0.5V and COV. This led to an immediate 3- to 4-fold decrease in methane production in V1C, V0.5C and VOC and a subsequent increase in total VFA concentrations to values between 2.0 and 2.5 g COD L^{-1}. After the decrease in methane production that followed electrode removal on day 91, reactor V1C (previously at 1 V cell potential) showed an increased methane production rate from day 97 on. Methane production in V0.5C (previously at 0.5 V cell potential) initially decreased, but regained from day 105 on. Reactors V1C and V0.5C had a similar methane production profile from day 112 on, i.e. 344 ± 39 and 343 ± 41 mL L^{-1} d^{-1}, respectively (Figure 6.2a). Methane production, hence, only partially recovered compared to the average stable methane production of 546 ± 15 mL L^{-1} d^{-1} on day 91, with residual VFA levels up to 10.3 g COD L^{-1} on day 154 in V1C and V0.5C (Figure 6.3a). Propionate was the main component of the total VFA, reaching 86% in V1C and 89% in V0.5C on day 154.

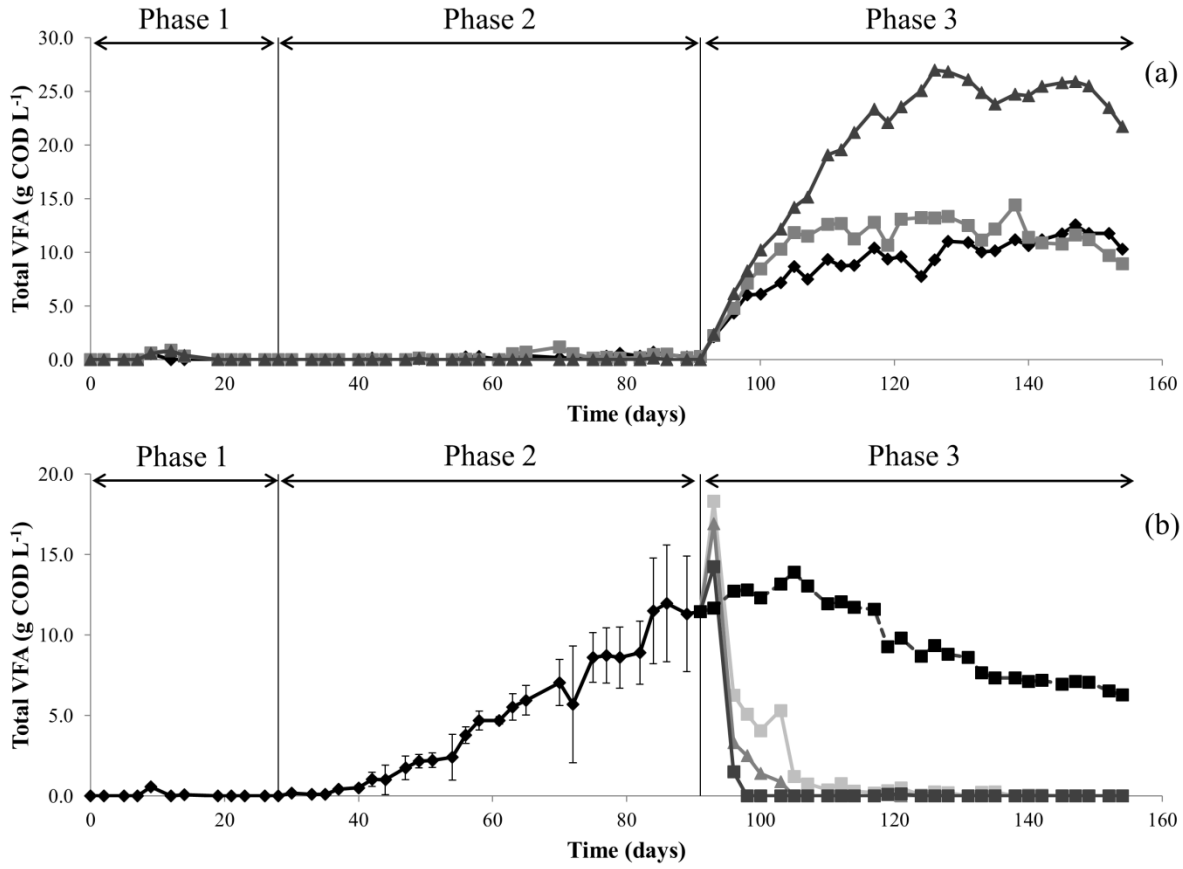

Figure 6.3 *Total VFA concentration of (a) V1C (♦), V0.5C (■) and VOC (▲) and (b) C1V-CNV (♦), C1V after introduction of the electrodes (■), C0.5V after introduction of the electrodes (▲), COV after introduction of the electrodes (■) and CNV after introduction of the electrodes (■).*

The methane production in VOC decreased further during the entire Phase 3, while the VFA concentration increased to a maximum of 27.0 g COD L^{-1}, which consisted mostly of acetate (44%) and propionate (33%) (Figure 6.2a and 6.3a). This build-up of VFA was also reflected in the pH values in Phase 3. The pH in V1C and V0.5C was lower compared to Phase 2 (7.47 ± 0.07 and 7.42 ± 0.07, respectively), yet, remained stable from day 105 on, while in VOC the pH decreased from 7.52 on day 91 to 6.16 on day 154.

The reactors C1V, C0.5V and COV showed an increase in methane production and an immediate decrease in VFA levels on day 93, i.e. directly after the introduction of the electrodes. The methane yield was higher than the theoretical maximum of 350 mL CH_4 g^{-1} COD in the week following the electrode switch, as a result of the removal of residual VFA in the reactors (Figure 6.2b and 6.3b). The methane production remained stable for reactor C1V and C0.5V, with values of 501 ± 28 and 571 ± 20 mL L^{-1} d^{-1}, respectively, from day 119 to 154, until the end of Phase 3, reaching similar stable methane production levels as V1C,

V0.5C and VOC in Phase 2. Reactor COV (OCP) showed more variation in methane production in Phase 3 although VFA concentrations remained below detection limit (Figure 6.2b). During Phase 3, reactor CNV, in which a new electrode pair was introduced, was also operated under the same conditions as COV. From day 91 to 100, this reactor produced the least methane and showed the highest VFA concentrations (> 14 g COD L^{-1}, 64% propionate and 25% acetate), compared to C1V, C0.5V and COV. After day 100 the methane production increased, and the VFA content decreased to 6.3 g COD L^{-1} (88% propionate) on day 154 (Figure 6.3b). From day 105 the performance of the CNV reactor was similar to V1C and V0.5C (Figure 6.2b). The methane production rate of CNV, however, did not reach the same stable levels as C1V and C0.5V.

3.2. Electrochemical performance

The reactors containing an electrode pair were operated at fixed cell potentials (Table 6.1). The fixed cell potentials resulted in an average current density of 6.8 ± 2.6 A m^{-2} and 3.4 ± 3.4 A m^{-2} for V1C and V0.5C, respectively, before removing the electrodes (Phase 2). The replacement of the electrodes from V1C and V0.5C to C1V and C0.5V respectively (on day 91), resulted in an average current density of 3.8 ± 1.8 A m^{-2} and 6.4 ± 4.8 A m^{-2} in C1V and C0.5V, respectively (Table 6.3). As a result of the applied cell potential of 1 V, potentially a more oxidizing environment at the anode electrode surface was created in V1C and C1V (Table 6.3) than commonly occurring in AD (E_h~ -0.25 to -0.35 V vs. SHE) (Thauer et al., 1977). The application of a cell potential of 1 and 0.5 V led to more reducing conditions at the cathode than commonly occurring during AD.

3.3. Microbial community analysis

3.3.1. Qualitative microbial community composition based on 16S rRNA gene sequences

The microbial community was characterized at two time points to compare community diversity (Figure 6.4 and 6.5). Averaged over both time points and all reactors, the number of observed OTUs (operational taxonomic units) was lower in the planktonic samples compared to the anodes (P = 0.003) and cathodes (P = 0.004). The relative abundance of methanogens was higher on the anode and cathode (30 ± 10), compared to the planktonic samples (5 ± 4). The methanogenic community was dominated by *Methanosaeta* (relative abundance of up to ~29%).

Table 6.3 *Measured electrical parameters during the experimental phases (Phase 2 and 3). Cell potential (dV), anode (E_{an}) and cathode (E_{cat}) are expressed in volts (V). Electrode potentials are expressed relative to the SHE. Current density (J, A m^{-2}) is expressed per unit of projected electrode area (60 cm^2 in Phase 2 and 55 cm^2 in Phase 3). Input power (P, mW L^{-1}) is expressed per unit of liquid volume (800 mL in Phase 2 and 730 mL in Phase 3). Theoretical methane production M (mL L^{-1} d^{-1}) is based on measured current and expressed per unit of liquid volume (800 mL in Phase 2 and 730 mL in Phase 3). Solidus: no data since the reactor is operated in open circuit.*

	Phase 2		
	V1C	V0.5C	VOC
dV (V)	0.97 ± 0.12	0.51 ± 0.03	0.00 ± 0.00
E_{an} (V)	0.14 ± 0.24	-0.24 ± 0.10	-0.30 ± 0.03
E_{cat} (V)	-0.84 ± 0.22	-0.75 ± 0.10	-0.30 ± 0.02
J (A m^{-2})	6.78 ± 2.58	3.36 ± 3.44	/
P input (mW L^{-1})	50.2 ± 21.5	12.8 ± 11.1	/
M (mL L^{-1} d^{-1})	127.5 ± 48.6	65.5 ± 64.8	/

	Phase 3			
	C1V	C0.5V	COV	CNV
dV (V)	0.98 ± 0.14	0.51 ± 0.04	0.01 ± 0.01	0.00 ± 0.01
E_{an} (V)	-0.09 ± 0.08	-0.26 ± 0.03	-0.29 ± 0.02	-0.06 ± 0.04
E_{cat} (V)	-1.06 ± 0.06	-0.76 ± 0.04	-0.30± 0.01	-0.06 ± 0.03
J (A m^{-2})	3.80 ± 1.75	6.44 ± 4.77	/	/
P input (mW L^{-1})	13.4 ± 0.0	24.1 ± 16.4	/	/
M (mL L^{-1} d^{-1})	71.8 ± 33.1	111.1 ± 82.2	/	/

The dominant bacterial populations belonged to the orders Actinomycetales, Lactobacillales, Clostridiales and Sphaerochaetales. Visualization of the variability in community composition between samples, using principal component analysis (Figure 6.6) and Tukey HSD tests, indicated that there was a significant difference in composition between day 91 and day 154 (PC1 scores: $P < 0.001$, PC2 scores: $P = 0.033$). At both time points, the microbial planktonic communities of the reactors with and without electrodes were similar ($P > 0.100$; Figure 6.5). There were no significant differences between the communities associated with the anode and cathode (Day 91: $P = 0.670$, day 154: $P = 1.000$). The community profile of the planktonic samples in both the reactors with and without electrodes differed significantly from the anodic and cathodic samples (Day 91: $P < 0.040$, day 154: $P < 0.003$). The applied potential did not

have a significant effect on the composition of the microbial communities. Environmental parameter fitting showed a correlation between higher methane yield and the surface-attached communities in the reactors with electrodes, and more specifically with a higher abundance of *Methanosaeta*, at day 91 (P = 0.001). Higher VFA and TAN concentrations were correlated to populations in the planktonic samples of reactors without electrodes, such as *Trichococcus* and *Peptoniphilus*, at days 91 and 154 (VFA: P = 0.001, TAN: P > 0.018).

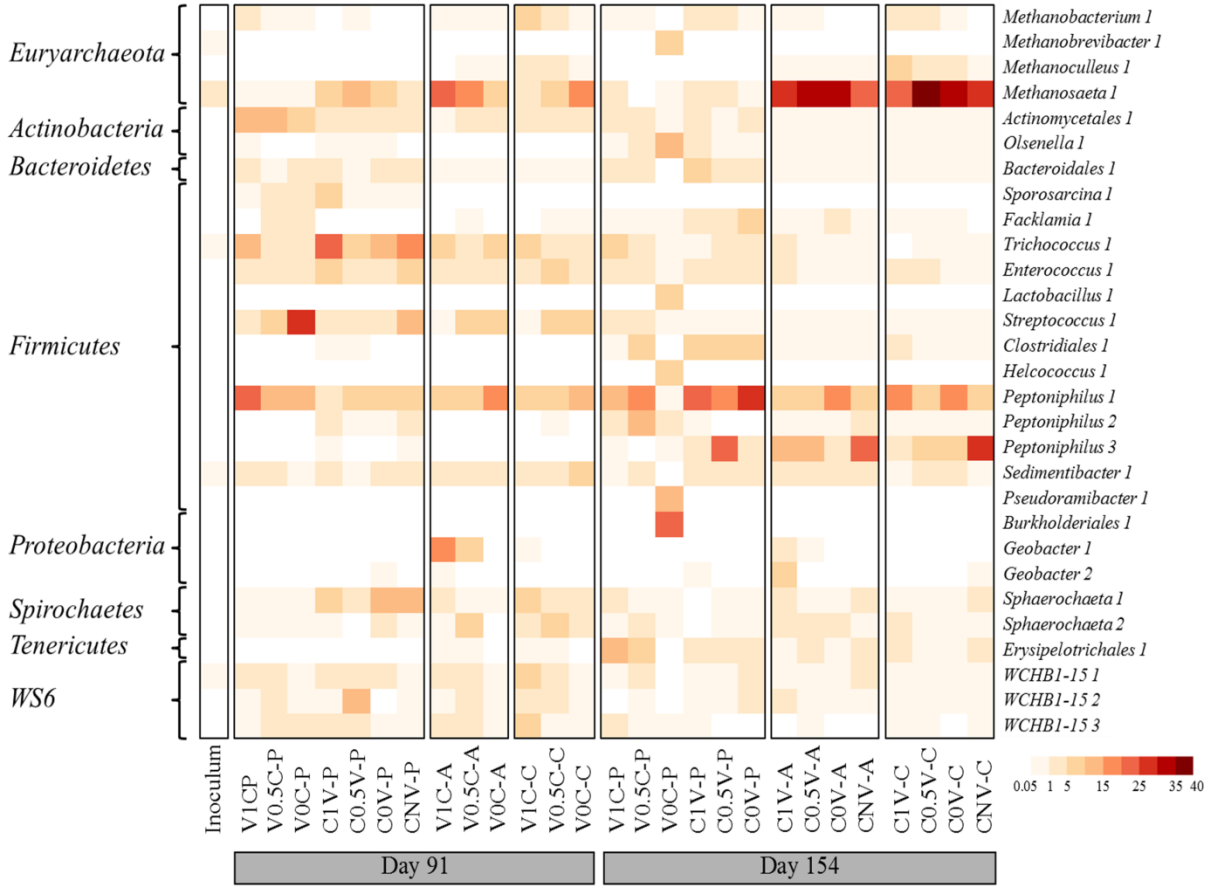

Figure 6.4 *Heatmap representing all OTUs present at a relative abundance ≥ 5% in at least one of the samples. The colour scale ranges from 0 to 40% relative abundance. Planktonic (P), anodic (A) and cathodic (C) samples are presented at the end of Phase 2 and 3, i.e. after 91 and 154 days, respectively. Taxonomy is shown at the phylum level (left column) and at the lowest determined level, i.e. order or genus (right column).*

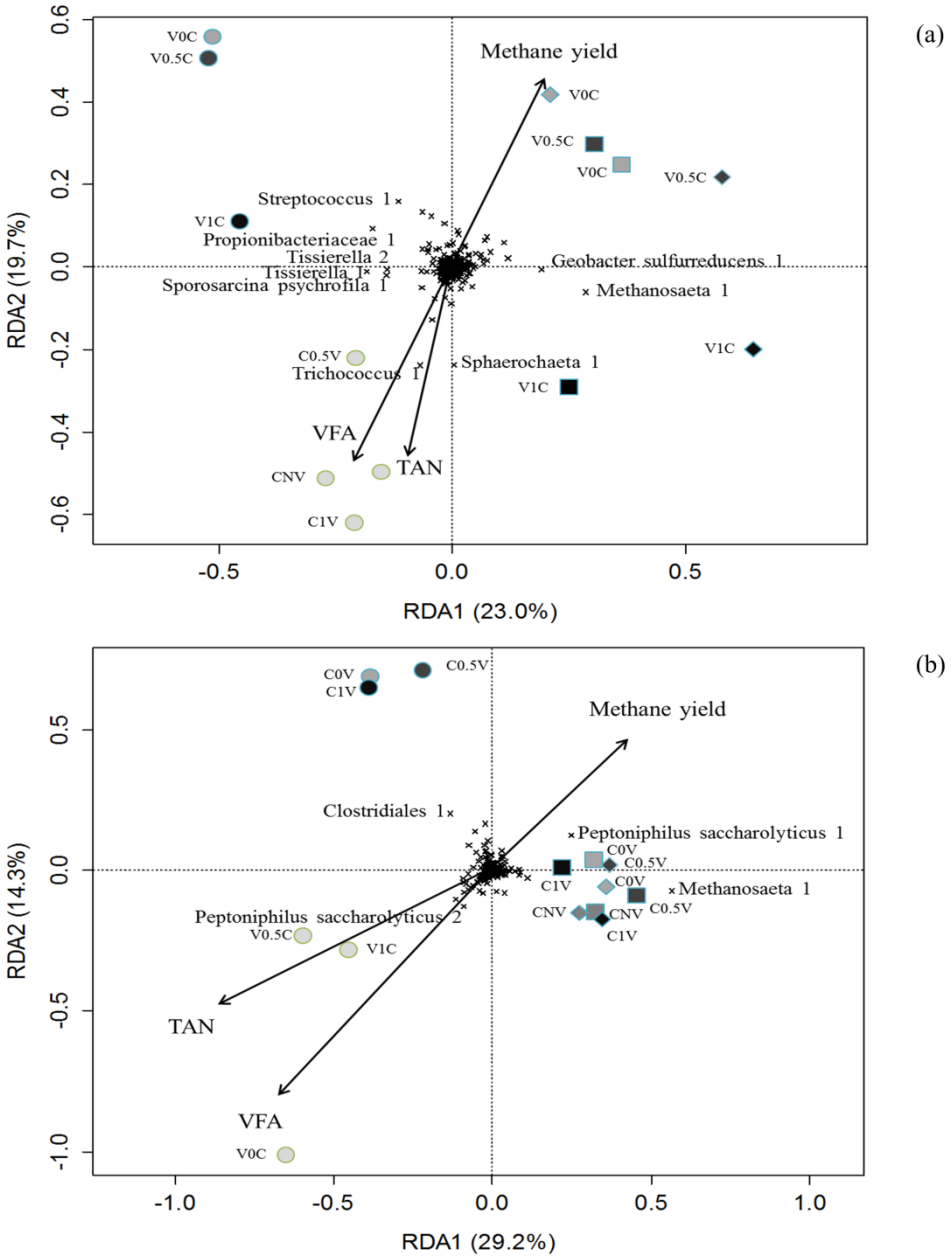

Figure 6.5 *Redundancy analysis showing the microbial community composition at the OTU level (Hellinger transformed) constrained by type of reactor (with/without BES), potential (no potential, 0.5V, 1.0V and OCP) and type of sample (planktonic (○), anode (◊) and cathode (□)). The arrows indicate correlations to performance data. This analysis was performed using the following samples: (a) after Phase 2 on the planktonic, anodic and cathodic samples of V1C (●,♦,■), V0.5C (●,♦,■), VOC (●,♦,■) and the control reactors C1V, C0.5V, COV and CNV (●,♦,■) and (b) Phase 3 on the planktonic, anodic and cathodic samples of C1V (●,♦,■), C0.5V (●,♦,■), COV and CNV (●,♦,■) and the control reactors V1C, V0.5C and VOC (●,♦,■). The black crosses represent the individual OTUs, and the taxonomy of those contributing most to the variability between samples is given.*

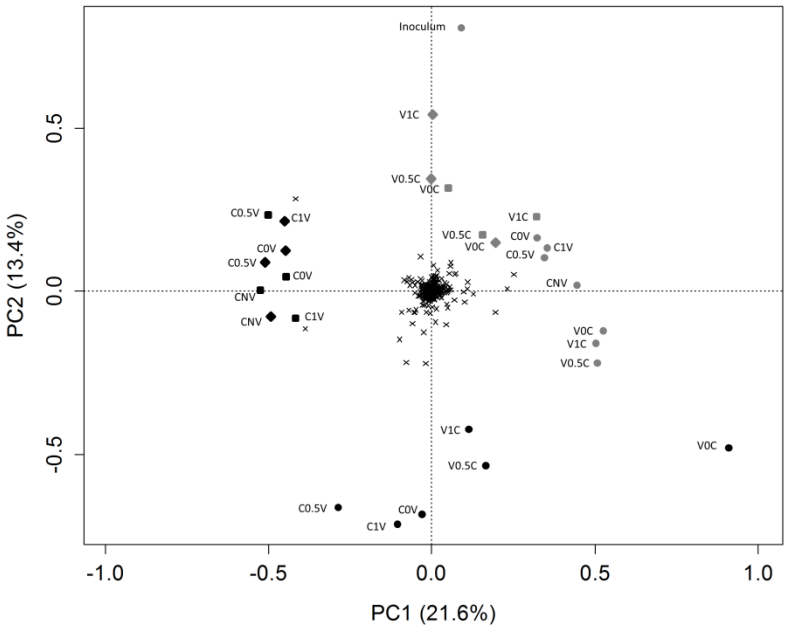

Figure 6.6 *Principal component analysis (PCA) showing the microbial community composition at the OTU level (Hellinger transformed). PCA was carried out on the inoculum sample (●), after Phase 2 on the planktonic (●), anodic (♦) and cathodic (■) samples, and after Phase 3 (●) on the planktonic (●), anodic (♦) and cathodic (■) samples of all reactors.*

3.3.2. Quantitative analysis of the methanogenic community

The real-time PCR results revealed a diverse methanogenic community in the planktonic phase, as well as on the anodes and cathodes (Figure 6.7). The inoculum sludge sample consisted of a diverse methanogenic community, yet, dominated by Methanosaetaceae at a concentration of $4.0 \times 10^9 \pm 2.9\ 10^8$ copies g^{-1} sludge. Hence, acetoclastic methanogenesis in the inoculum sample was most likely already dominated by the Methanosaetaceae. The Methanosaetaceae remained the dominant acetoclastic methanogenic population in all samples in Phase 2 and 3, with the exception of the planktonic sample in V0.5C after 154 days. In contrast to the planktonic acetoclastic methanogens that were dominated by Methanosaetaceae, the planktonic hydrogenotrophic methanogens were represented in both the Methanobacteriales or Methanomicrobiales groups throughout all samples.

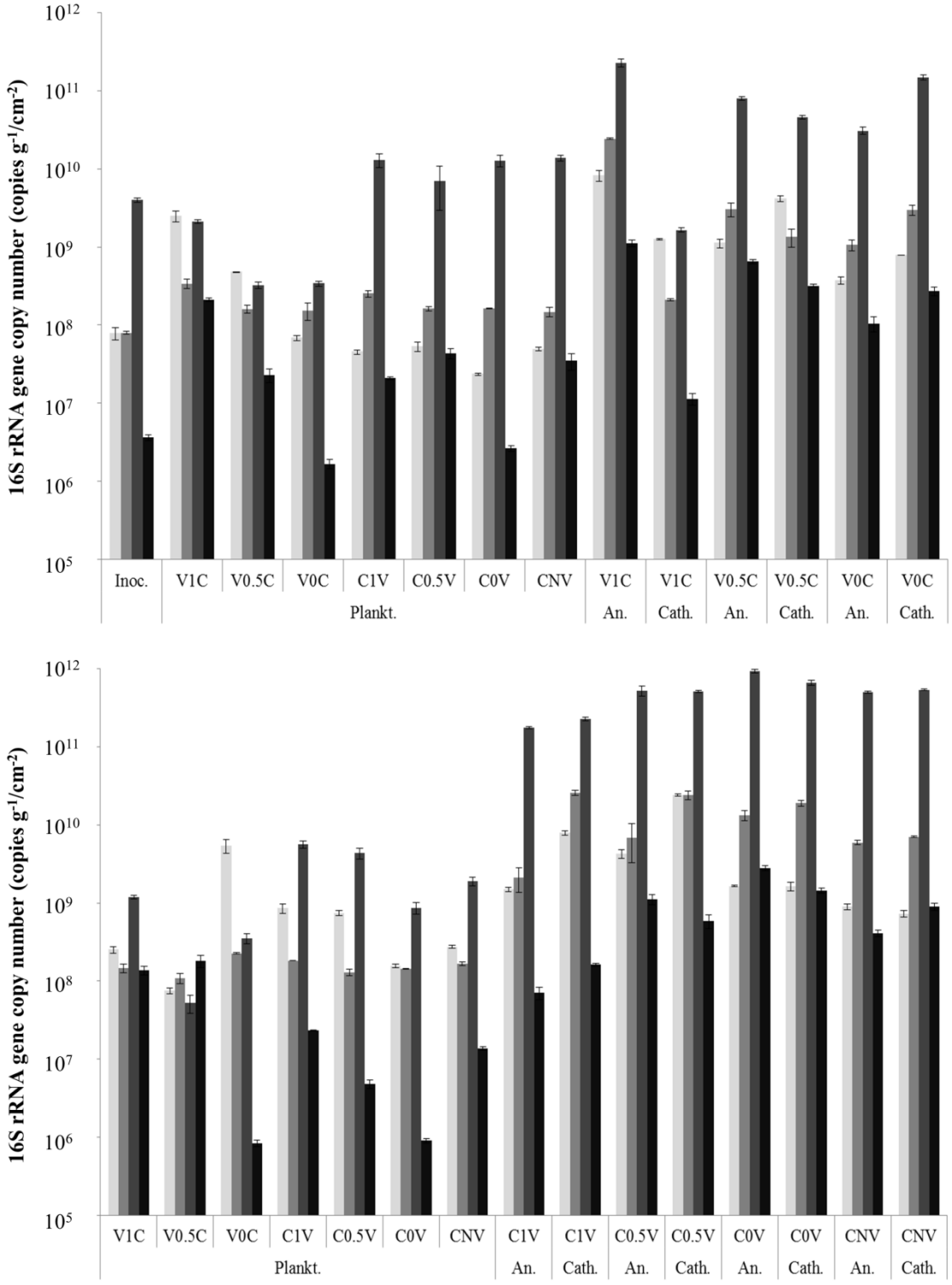

Figure 6.7 *Real-time PCR results of the Methanobacteriales (□), Methanomicrobiales (■), Methanosaetaceae (■) and Methanosarcinaceae (■) after (a) 91 days (Phase 1) and (b) 154 days (Phase 2) of operation. Average values of the triplicate analyses, together with the standard deviations are presented.*

After 91 days of operation, there was a clear increase in Methanobacteriales copy numbers in V1C to a value of $2.5 \times 10^9 \pm 3.9 \times 10^8$ copies g^{-1} sludge in the planktonic phase. This was in

contrast to VOC, which showed a value of only $6.8 \times 10^7 \pm 5.2 \times 10^6$ copies g^{-1} sludge in the planktonic phase. The anode in V1C showed a 100-fold higher concentration of Methanosaetaceae, Methanomicrobiales and Methanosarcinaceae, compared to the cathode, on one hand. A 10-fold higher concentration of Methanosaetaceae, Methanobacteriales, Methanomicrobiales and Methanosarcinaceae, was observed in the anode in V1C compared to the anode in of VOC, on the other hand.

After 154 days, the Methanosarcinaceae copy numbers reached values of $1.4 \times 10^8 \pm 1.7 \times 10^7$, $1.8 \times 10^8 \pm 3.3 \times 10^7$ and $8.4 \times 10^5 \pm 8.2 \times 10^4$ copies g^{-1} sludge in the planktonic phase of V1C, V0.5C and VOC, respectively, thus revealing higher Methanosarcinaceae concentrations in V1C and V0.5C compared to VOC. In contrast, VOC was clearly dominated by the hydrogenotrophic Methanobacteriales, with a value of $2.5 \times 10^8 \pm 2.5 \times 10^7$, $7.5 \times 10^7 \pm 6.7 \times 10^6$ and $5.4 \times 10^9 \pm 1.1 \times 10^9$ copies g^{-1} sludge in V1C, V0.5C and VOC, respectively. There was no difference between the anode and cathode in C1V, C0.5V, COV and CNV after 154 days, as well as between the anodes and cathodes of the different reactors. Overall, all anodes and cathodes were clearly dominated by Methanosaetaceae at similar copy number levels of around 5.0×10^{11} copies cm^{-2}.

4. Discussion

Anaerobic digestion of molasses in the presence of a BES resulted in stable methane production at both a fixed potential and at OCP. The allocation of pre-inoculated electrodes to failing digesters resulted in immediate process remediation, irrespective of the previously applied cell potentials, yet, pre-inoculation of the carbon felt electrodes was crucial to regain stable operation. Overall, it appears that retention of biomass is a critical factor towards the remediation, rather than current.

The decrease in methane production and pH in the four control reactors (C1V, C0.5V, COV and CNV) in Phase 2 indicates that the methanogenic process was disturbed, which could be a consequence of the high concentration of potassium, i.e. 6.90 g L^{-1}, in the molasses feed (Table 6.2) (Ahring et al., 1995). A concentration of 5.85 g L^{-1} is assumed to cause 50% inhibition of acetoclastic methanogens, due to a neutralization of the cell membrane potential from the passive influx of ions (Appels et al., 2008; Chen et al., 2008). Hence, molasses are often diluted prior to their treatment to avoid digester failure (Satyawali & Balakrishnan, 2008).

Methane production remained stable in the reactors with electrodes (V1C, V0.5C and VOC). The presence of the carbon felt electrodes served as carrier material for the formation of a biofilm, which could be detected with SEM (Figure 6.8). Anaerobic digesters with biomass attached on carrier material were reported to show better performance, when digesting difficult substrates, such as cellulose rich organic waste (Held et al., 2002; Yang et al., 2004; Sasaki et al., 2010a). Based on biofilm properties described by Arends and Verstraete (2012) and VS-measurements, the calculated amount of biofilm for the reactors V1C, V0.5C and VOC would account for only 0.22% of the total biomass in the reactor. Indeed, the total surface area of the electrodes was 0.0327 m^2 L^{-1} of reactor. Assuming a uniform biofilm thickness of 20 µm, the total volume of the biofilm would be 6.54 x 10^{-7} m^3 L^{-1}. A biofilm typically has a VS-concentration of 20 kg VS m^{-3} biofilm (Arends & Verstraete, 2012), resulting in 0.0131 g L^{-1} extra biomass in the reactor, while the reactors V1C, V0.5C and VOC had an average VS concentration of 6.0 g VS L^{-1} on day 91 of the test. The biofilm thus accounted for a maximum of 0.22% extra biomass in the reactor.

However, this biomass was protected from potential inhibiting components in the molasses, due to the biofilm organization. A similar concept takes place in sludge granules where micro-organisms in the centre of the granule undergo less negative influence of toxic waste streams (Bae et al., 2002). The presence of the carbon felt electrodes had a positive influence on the process stability in all three experimental reactors, regardless of the applied potential.

The removal of the electrodes from V1C, V0.5C and VOC and subsequent insertion in C1V, C0.5V and COV, respectively (Phase 3), resulted in immediate removal of VFA, showing the importance of the biofilm on the carbon felt electrodes to remediate the digesters. The performance of CNV decreased further during Phase 3, until 15 days after the introduction of the electrodes methane production increased again. This indicates that over time an active methanogenic biofilm developed on the bare carbon felt, allowing protection of sensitive micro-organisms, especially methanogens. This is in accordance with the findings of Lalov et al. (2001), who detected biofilm growth on the carrier material between 10 to 20 days after start-up in a digester treating vinasses.

The reactor COV (operating at OCP after the electrode switch) did not reach stable methane production during Phase 3, in contrast to C1V and C0.5V. This can be due to free ammonia toxicity, since the pH of the reactor increased from 7.87 to 8.12, four days following the electrode switch. The pH in the biofilm was probably higher than in the bulk liquid, due to methanogenic activity, leading to further local increase of the pH. An average ammonium

concentration of 1200 mg TAN L^{-1} was detected in COV in Phase 3, hence a pH of 9 in the biofilm would result in a free ammonia concentration of 500 mg L^{-1}, which can be considered toxic to methanogens (Chen et al., 2008). The increase in methane production in reactors C1V and C0.5V was more gradual and the pH remained lower, decreasing the impact of free ammonia toxicity.

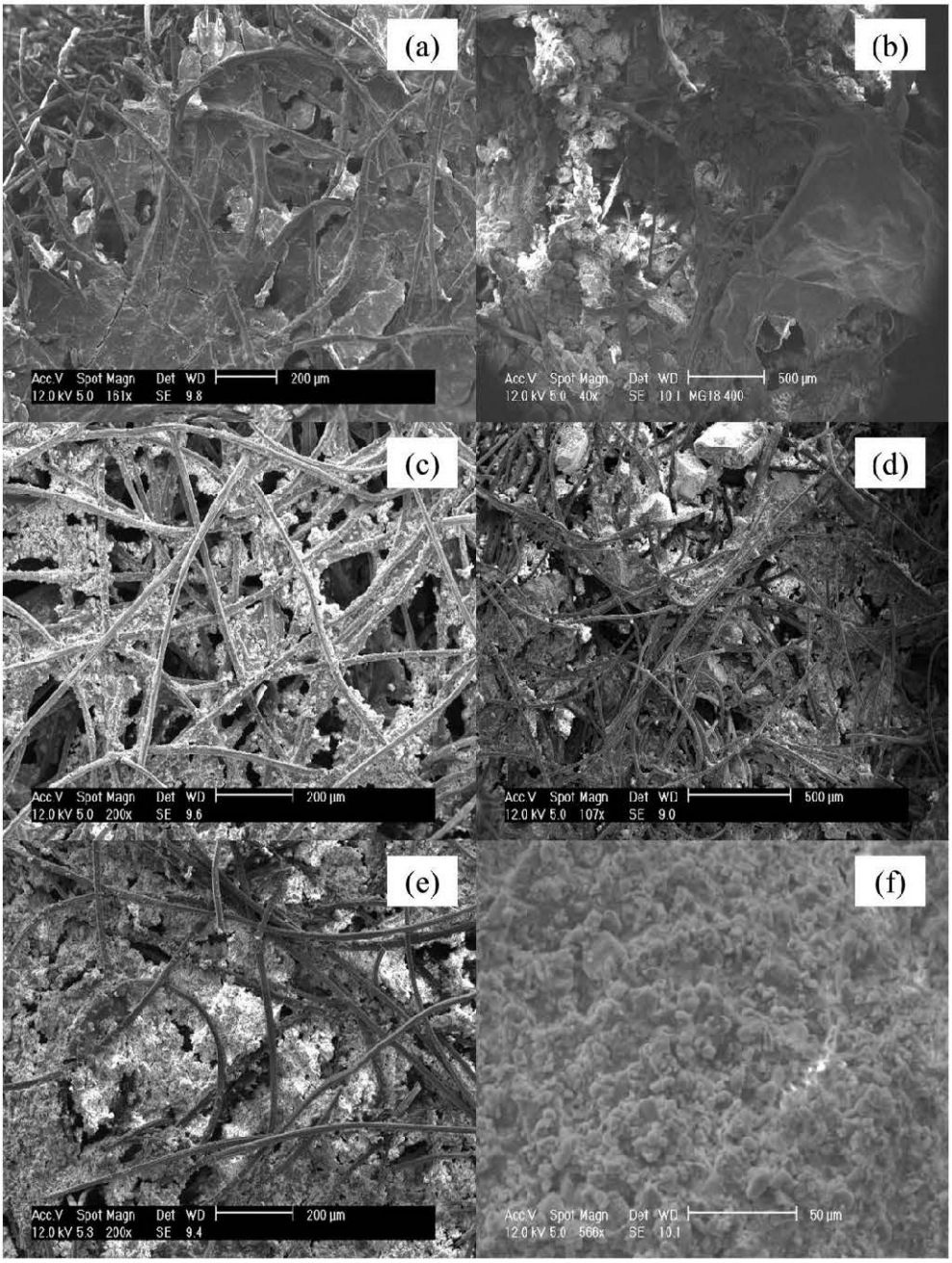

Figure 6.8 *SEM images of (a) the anode of V1C, (b) the cathode of V1C, (c) the anode of V0.5C, (d) the cathode of V0.5C, (e) the anode of VOC and (f) the cathode of VOC at the end of Phase 2.*

In Phase 3, the methane production of VOC decreased to a value below 10 mL L^{-1} d^{-1}, indicating reactor failure. V1C and V0.5C partially recovered, as a stable, yet, lower amount

of methane was produced and residual VFA levels were present at concentrations up to 12.6 and 14.4 g COD L^{-1} in V1C and V0.5C, respectively. This state of 'inhibited steady state', as previously described for free ammonia toxicity (in this work most likely caused by the high potassium concentration in the case of V1C and V0.5C) can therefore be applicable in this case (Angelidaki & Ahring, 1993). The difference in behaviour between the two reactors previously operated at a fixed cell potential and the reactor at OCP suggests a certain impact of the applied potential, however none of the measured physicochemical parameters could account for this. The difference in VFA concentrations between V1C and V0.5C, and VOC, is more likely a consequence of process failure, rather than the immediate cause (Appels et al., 2008).

The measured current densities can be correlated to the occurrence of various processes in the AD-BES configuration. Interestingly, the current density was higher in the system that was operated at 0.5V cell potential (C0.5V) compared to the 1 V system (C1V) in Phase 3. Liquid conductivity was similar in all reactors, so a change in current based on this can be ruled out. Visual observation did not reveal any differences between the various reactors at the end of the experiment, hence, an explanation for this phenomenon is lacking at present. The produced current could have resulted in a theoretical methane production at the cathode of 128 ± 49 and 65± 65 mL L^{-1} d^{-1} for V1C and V0.5C, respectively, in Phase 2 and 72 ± 33 and 111 ± 82 mL L^{-1} d^{-1} for C1V and C0.5V, respectively, in Phase 3 (Table 6.3). These values could amount to maximum 20 % of the measured total methane production in V1C, V0.5C and C1V and C0.5V. The source of the current at the anode remains to be elucidated, as the abundance of known current generating micro-organisms such as *Geobacter sulfurreducens* (Bond & Lovley, 2003) on the anode is relatively low in comparison to other micro-organisms. No H_2 was detected in the biogas, which coincides with the fact that the cathode potentials were not low enough to generate H_2 gas directly. However, biological H_2 production linked to hydrogenotrophic methanogenesis can be an alternative route for enhanced biogas production, as detected in the systems with electrodes. Biologically catalysed H_2 production on cathodes has been shown to yield current densities, during polarization, of up to 3.8 A m^{-2} for mixed culture biocathodes at applied cathode potentials of -0.8 vs. SHE (Rozendal et al., 2008). Direct methanogenesis on electrodes by hydrogenotrophic methanogens has also been suggested, however intermediate H_2 production in the biofilm or at the electrode surface cannot be ruled out in mixed cultures (Villano et al., 2010).

The applied cell potentials and the resulting electrode potentials in this study were lower compared with other work concerning (B)ESs in AD, in which direct (electro)chemical stimulation of the AD process took place. Tartakovsky et al. (2011) observed improved AD performance, based on applied potentials of 2.8-3.5 V with dimensionally stable electrodes in a UASB reactor. The improvement was attributed to enhanced hydrolysis due to micro-aerobic conditions at the anode and additional H_2 input for methanogenesis and improved biogas quality at the cathode. However, the mentioned study was lacking an adequate control, as there was no open circuit system present. The potentials in the current work (Table 6.3) likely do not give rise to micro-aerobic conditions, and would not result in H_2 production at the carbon electrodes. Therefore, any enhancement or stabilization of the AD process in this work that could be attributed to the introduced (bio)electrochemical environment must be due to direct stimulation of the (attached) microbiota or due to a purely electrochemical reactions. Zamalloa et al. (2013) observed the precipitation of various metal salts when operating stainless steel electrodes at a 2V applied potential in an anaerobic septic tank. A higher applied voltage was used, likely leading to higher cathode pH and anodic iron dissolution, causing the precipitation of various salts, as well as sulphide. The possibility of sulphur cycling in BESs has indeed already been demonstrated (Rabaey et al., 2006; Dutta et al., 2009). However, in the current study, species involved in the sulphur cycle could not be detected by means of 16S rRNA gene amplicon sequencing (Figure 6.4), and the potential (bio)electrochemical oxidation of sulphur could not explain the stable operation of V1C, V0.5C after removal of the electrodes (Phase 3).

Sasaki et al. (2010b) placed a carbon electrode (75 cm^2 L^{-1} projected surface) in a methanogenic reactor operated on a complex feed, i.e. artificial garbage slurry. Enhanced methanogenesis was shown at potentials of -0.6 and -0.8 V vs. Ag/AgCl, which is in contrast to the present study, where there was no difference in methane production between the three different cathode potentials. Moreover, Sasaki et al. (2010b) added an artificial electron mediator (0.2 mM Anthraquinone-2,6-disulfonate (AQDS); E_0' = -184 mV), described by Benz et al. (1998), that might have obscured the effect of the biofilm development on the electrodes, by shuttling electrons from low potential cathodes to the bulk solution.

The 16S rRNA gene amplicon sequencing results revealed that community richness, i.e. the number of OTUs, was higher on the anodes and cathodes, compared to the planktonic phase. However, no specific conclusions could be drawn concerning differences in the bacterial community in the reactors, except for the apparent dominance of the Lactobacillales and

Clostridiales. The higher richness in the biofilms that were developed on these electrodes could be explained by the fact that (1) activated carbon fibre is a suitable carrier for microbial biofilm development, and (2) micro-organisms that decreased in abundance in the planktonic phase, because of the changing conditions in the reactor system, maintained stable growth in the biofilm (Fernandez et al., 2008; Gong et al., 2011). The higher richness in the biofilm that had formed on the electrodes is in correlation with the stable methane production in these reactors, as is the case in anaerobic biofilm reactors (Fernandez et al., 2008). The retention of an active methanogenic community in such a biofilm is most often the crucial factor to maintain stable operation. Indeed, amplicon sequencing revealed a high relative abundance of *Methanosaeta* in the biofilm as the main acetoclastic methanogen. The presence of carbon fibres in the electrodes could lead to high methane production efficiencies by protecting the methanogenic community from high levels of residual VFA and salts, which may act as stressors (Sasaki et al., 2009; Sasaki et al., 2011b). The correlation between a higher methane yield and the higher abundance of *Methanosaeta* is to be expected, since an OLR of only 2 g COD L^{-1} d^{-1} was applied, leading to circumstances that favour *Methanosaeta* over *Methanosarcina* (Ribas et al., 2009; De Vrieze et al., 2012). However, as there was no significant difference in community composition between anode and cathode biofilm, nor was there any difference between the planktonic phase in the different reactors, with or without electrodes, it can be confirmed that there was no effect of the applied cell potential and that biofilm development was the crucial factor to obtain stable methane production.

Quantitative real-time PCR analysis confirmed the overall dominance of *Methanosaeta*, seen in the amplicon sequencing. *Methanosaeta* copy numbers were up to a 1000 times higher on the electrodes, compared to the planktonic phase, indicating the important contribution of the biofilm to methane production, in spite of the fact that this accounted for only 0.22% of total biomass. However, *Methanosaeta* copy numbers were a 100-fold higher on the anode compared to the cathode in reactor V1C after 91 days. Not only *Methanosaeta*, but also the other methanogenic groups were more abundant on the anode, compared to the cathode in reactor V1C, indicating that conditions were more favourable for methanogenic growth at the anode, compared to the cathode. The positive charge of the anode, thus attracting the negatively charged bacteria, may be an explanation for this, in contrast to the negatively charged cathode. In the failing reactors, an evolution from a *Methanosaeta* to a *Methanosarcina* dominated methanogenic community was expected in the planktonic phase, because of deteriorating conditions, yet, this shift only took place in V1C and V0.5C after 154

days of operation and not in any of the failing reactors after 91 days (C1V, C0.5V, COV and CNV), nor in VOC after 154 days. These results indicate that a preceding applied cell potential catalyses a transition from a *Methanosaeta* to a *Methanosarcina* dominated methanogenic community. This transition can be directly associated with the partial recovery of methane production in V1C and V0.5C, compared to VOC showing complete failure, yet residual VFA concentrations remained high. The exact mechanism behind this is still unknown. Nonetheless, these results confirm that *Methanosarcina* was responsible for the partial recovery of methane production in V1C and V0.5C, however, at the cost of higher residual VFA concentrations, as stated by Conklin et al. (2006) and De Vrieze et al. (2012).

5. Conclusions

Anaerobic digestion of molasses in a reactor in which a carbon felt electrode pair was introduced, maintained stable methane production, while the control reactors (no electrode pair) failed. There was no direct effect of the applied cell potential on methane production, although a hysteresis effect could be observed after removal of the electrodes. Introduction of pre-inoculated electrodes in failing reactors resulted in immediate process recovery, indicating the remediating capacity of pre-inoculated electrodes. Nonetheless, full-scale application of this concept requires further research concerning potential mixing and clogging problems, related to the application of solid electrode materials in AD. *Methanosaeta* was the dominant acetoclastic methanogen on the electrodes, irrespective of the applied cell potential. This study demonstrated that the main mechanism behind the stabilizing effect of a BES in AD appears to lie in biomass retention, rather than (bio)electrochemical stimulation. This is in contrast to several other studies that, however, lacked a suitable control treatment to distinguish the effect between biomass retention and (bio)electrochemical stimulation.

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CHAPTER 7: ANAEROBIC DIGESTION OF MOLASSES BY MEANS OF A VIBRATING AND NON-VIBRATING SUBMERGED ANAEROBIC MEMBRANE BIOREACTOR

This chapter has been redrafted after:

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Abstract

Bio-refineries produce large volumes of waste streams with high organic content, which are potentially interesting for further processing. Anaerobic digestion can be a key technology for treatment of these sidestreams, such as molasses. However, the high concentration of salts in molasses can cause inhibition of methanogenesis. In this research, concentrated and diluted molasses were subjected to biomethanation in two types of submerged anaerobic membrane bioreactors: one with biogas recirculation and one with a vibrating membrane. Both reactors were compared in terms of methane production and membrane fouling. Biogas recirculation seemed a good way to avoid membrane fouling, while the trans membrane pressure in the vibrating membrane bioreactor increased over time, due to cake layer formation and the absence of a mixing system. Stable methane production, up to 2.05 L L^{-1} d^{-1} and a concomitant COD removal of 94.4%, were obtained, only when diluted molasses were used, since concentrated molasses caused a decrease in methane production and an increase in volatile fatty acids, indicating an inhibiting effect of concentrated molasses on anaerobic digestion. Real-time PCR results revealed a clear dominance of Methanosaetaceae over Methanosarcinaceae as the main acetoclastic methanogens in both anaerobic membrane bioreactors.

1. Introduction

The combination of fossil fuel depletion and detrimental environmental effects caused by their consumption creates an urgent need for alternative resources and processes for both the production of energy and chemicals. Emerging technologies convert bio-based feedstocks through a combination of physical, chemical and biological processes into a range of biofuels and biochemicals. The production of biofuels has reached unprecedented levels, with bioethanol being the uncontested number one on a volume basis, predicted to reach 100 billion litres in 2015 (Sarkar et al., 2012). However, it is becoming clear that the success rate of these so-called 'bio-refineries' depends on the full utilization of all resources present in both the original biomass and the waste streams. This concept of the so-called 'zero waste bio-refinery' considers wastewaters, for example, as sidestreams. In the case of bio-ethanol production, up to 20 litres of wastewater is generated per litre ethanol produced. This water contains a chemical and biological oxygen demand (COD and BOD) in the order of 60-100 g L^{-1} and 35-60 g L^{-1}, respectively (Xinxin et al., 2012). Adequately processing these organics can improve the economics of bio-refineries. Molasses is the most important by-product in cane sugar factories and the production of molasses wastewaters may cause serious environmental problems, due to their high concentration of organic matter, high salt content and low pH (Sirianuntapiboon & Prasertsong, 2008).

One possibility to fully utilize these organics is the production of biogas by means of anaerobic digestion (AD). Indeed, one could produce 1.1 kWh_{elect} at a value of € 0.1 kWh^{-1} starting from 1 kg COD (Desloover et al., 2012). In addition to the value of the bio-ethanol itself (currently € 0.6-0.8 L^{-1}), AD could result in an extra € 0.22 L^{-1} bio-ethanol produced.

Several anaerobic bioreactor designs have been used to treat bio-refinery wastewater. Among these, continuous stirred tank reactors (CSTRs), upflow anaerobic sludge blanket (UASB) reactors and the expanded granular sludge blanket configuration (EGSB) are most commonly described (Harada et al., 1996; Fang et al., 2011b). In the present study, anaerobic membrane bioreactors (AnMBRs) were constructed for the conversion of synthetic bio-refinery streams into biogas. In general, AnMBRs have distinct advantages over other configurations, such as a small footprint, a high effluent quality, a high volumetric loading rate, and a lower sludge production (Skouteris et al., 2012; Smith et al., 2012). The separation of the hydraulic (HRT) and sludge retention time (SRT) can be considered the main advantage in treatment of bio-refinery effluents, given the lower stress on the microbial community. Indeed, these streams typically contain high amounts of sulphate, salts and lipids, which negatively affect the

biofilm and granule formation in UASBs and ESBGs. In AnMBRs, the membrane filtration component can exist in three configurations: external cross-flow, internal submerged or external submerged (Liao et al., 2006a). In an internal submerged membrane configuration, membranes are submerged directly into the suspended biomass in the bioreactor and permeate is produced by exerting a vacuum on the membrane. One of the main challenges for industrial scale applications of this configuration is fouling of the membranes. Fouling is typically controlled by recirculation of biogas to create shear at the membrane surface (Cui & Wright, 1996; Zamalloa et al., 2012). Recently, an innovative system using a magnetically induced membrane vibration system was developed as an alternative shear enhancement device for fouling control in aerobic MBRs (Bilad et al., 2012; Mezohegyi et al., 2012). Aeration was only required to obtain proper mixing of the activated sludge, and the reduced air supply resulted in decreased energy consumption.

The goals of this study were to (1) study the performance of AnMBRs to digest molasses, (2) evaluate a novel vibration membrane filtration system for AD, (3) compare the performance between a scouring configuration and the vibrating membrane configuration and (4) analyse the methanogenic community of both systems. Both concentrated and diluted molasses were used to estimate the possibility to treat highly concentrated bio-refinery sidestreams by means of an AnMBR.

2. Materials and methods

2.1. Experimental set-up

Two different set-ups were constructed to compare the performance of AnMBRs with biogas recirculation and AnMBRs with vibration to control fouling. A schematic representation of both AnMBRs can be found in Figure 7.1. In case of the MBRs with biogas recirculation, two reactors were run in parallel, differing in the applied influent. In the HL-AnMBR (high-load anaerobic membrane bioreactor) concentrated molasses were used in phase 1, after which diluted molasses were used in phase 2. In the NV-AnMBR (low-load non-vibrating anaerobic membrane bioreactor) diluted molasses were used throughout the entire experiment (Table 7.1). All MBRs consisted of a 10 L reservoir with one plate membrane made of chlorinated polyethylene (Kubota, Japan). The pore size of the membranes and the total filtration surface amounted to 0.4 µm and 0.12 m^2, respectively. In the HL-AnMBR and the NV-AnMBR, the headspace of the reactor (2 L) was pulled out continuously by means of a membrane gas

pump, and diffused under the plate membrane. The V-AnMBR (low-load vibrating anaerobic membrane bioreactor) set-up was adapted from the aerobic MBR configuration designed by Bilad et al. (2012). The vibrating device consisted of one Kubota membrane that was attached to a vibrating module, which included a vibration shaft, an amplifier and a vibration engine. The signal was provided by a computer using Test Tone Generator software (Esser Audio, Germany). The vibration was created in the vibration engine by magnetic attraction/repulsion forces in a 'push and pull' mode. The vibration moved the membrane to the left and the right through a sinusoidal pattern. The adjustable vibration parameters were the applied power (determined by combination of vibration amplitude and frequency), the vibration mode and the vibration cycle. However, during the entire operation the vibration amplitude and frequency were fixed at 2 mm and 10 Hz, respectively.

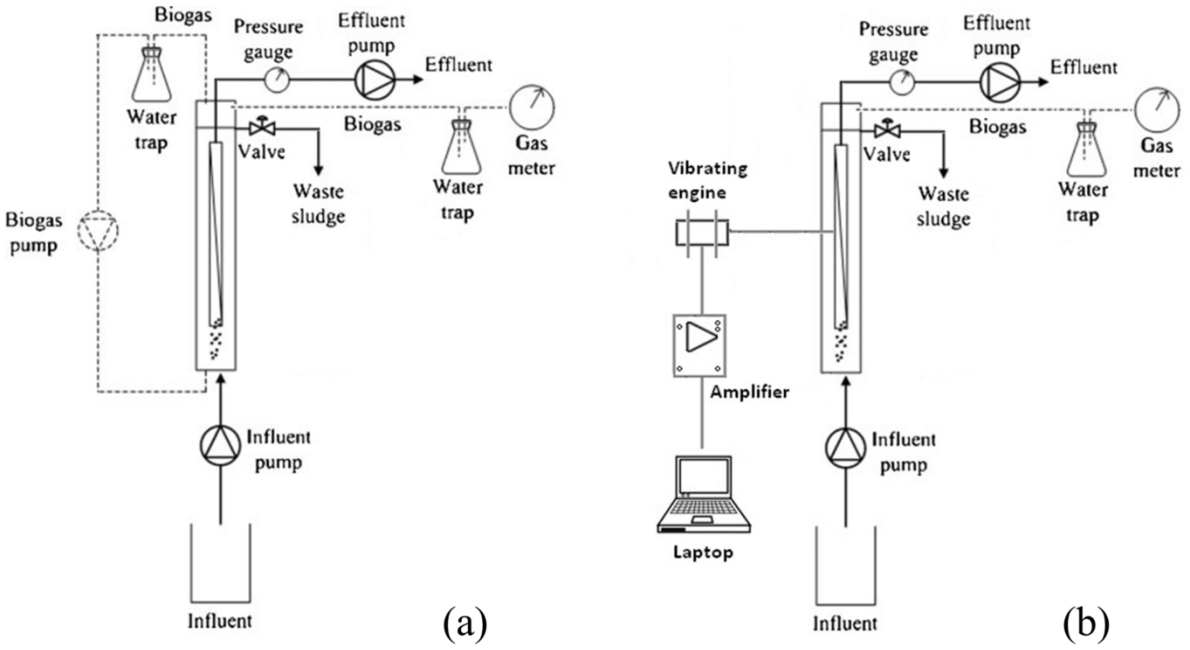

Figure 7.1 *Schematic overview of the lab-scale experimental set-up of (a) the HL-AnMBR and NV-AnMBR and (b) the V-AnMBR.*

2.2. Influent characteristics and operational parameters

All reactors were inoculated with granular anaerobic sludge originating from a full-scale UASB digester treating potato-processing wastewater. The initial volatile suspended solids (VSS) and total suspended solids (TSS) concentrations amounted to 10 g L^{-1} and 18.7 g L^{-1}, respectively. The characteristics of the molasses that originated from potato processing (AVEVE, The Netherlands), which can be considered very similar to sidestreams originating

from bio-refineries, used as influent, are shown in Table 7.1. In case of the HL-AnMBR, the reactor was fed with concentrated molasses, while in the case of the NV-AnMBR and V-AnMBR a 4-40 times dilution was applied. The operational parameters used in the different reactors are described in Table 7.2.

2.3. Critical flux and permeability determinations

The critical flux of the membranes was determined by measuring the trans membrane pressure (TMP) at increasing fluxes. The membrane was placed in a reservoir containing sludge and the flux through the membrane was increased stepwise using a peristaltic pump (Watson Marlow). After a filtration time of 10 min, the TMP was determined, and the flux was increased. The resulting TMPs were plotted against the fluxes and the critical (J_{crit}) and theoretical operational flux (J_{op}) (eq. 7.1 and 7.2).

$$J_{crit}, \frac{\Delta p}{\Delta t} \geq 1 \frac{mbar}{min} \quad (7.1)$$

$$J_{op} = 0.75 * J_{crit} \quad (7.2)$$

The same procedure was performed at the different sludge concentrations: (1) 46.9 g TSS L^{-1} and 27.2 g VSS L^{-1}, (2) 23.5 g TSS L^{-1} and 13.6 g VSS L^{-1} and (3) 11.7 g TSS L^{-1} and 6.8 g VSS L^{-1}.

At the end of the operational phase of each reactor, additional filtration tests were performed to determine the permeance recovery in between a series of subsequent cleanings. The fouled membranes were flushed with tap water, and then soaked in 2 g L^{-1} sodium hypochlorite for 2 h, followed by a more intensive chemical cleaning with 6 g L^{-1} sodium hypochlorite overnight.

2.4. Particle size distribution

The particle size distribution of the sludge in the membrane bioreactor was determined by means of different sieves with varying mesh sizes of 2.0 mm, 1.6 mm, 1.0 mm, 0.50 mm, and 0.25 mm. Particle size distributions were determined for the inoculum sludge and the final sludge in the HL-AnMBR and NV-AnMBR after the experimental period.

Table 7.1 *Characteristics of the influent to the high-load anaerobic membrane bioreactor (HL-AnMBR) during phase 1 and phase 2, the low-load non-vibrating anaerobic membrane bioreactor (NV-AnMBR) and the low-load vibrating anaerobic membrane bioreactor (V-AnMBR) (n.d.= not determined).*

Parameter	HL-AnMBR phase 1	HL-AnMBR phase 2	NV-AnMBR	V-AnMBR
Substrate	Concentrated molasses	Diluted molasses	Diluted molasses	Diluted molasses
pH	5.50 ± 0.12	5.46 ± 0.11	5.36 ± 0.60	5.41 ± 0.66
Conductivity (mS cm^{-1})	35.2 ± 2.4	5.8 ± 0.6	5.3 ± 2.8	3.9 ± 1.6
Total COD (g L^{-1})	110.9 ± 4.4	14.5 ± 0.6	11.4 ± 7.0	8.3 ± 3.9
Soluble COD (g L^{-1})	94.6 ± 2.4	13.3 ± 0.9	n.d.	n.d.
Total suspended solids (g L^{-1})	n.d.	17.1 ± 1.4	12.6 ± 7.5	8.4 ± 3.5
Volatile suspended solids (g L^{-1})	n.d.	12.0 ± 1.3	8.6 ± 5.4	5.6 ± 2.4
Total ammonia nitrogen, TAN (mg N L^{-1})	399.4 ± 161.3	34.0 ± 30.5	39.8 ± 16.7	32.9 ± 8.9
Kjeldahl nitrogen, KjN (mg N L^{-1})	12000 ± 1900	1100 ± 200	680.4 ± 433.0	471.2 ± 234.8
Total phosphorous, TP (mg P L^{-1})	n.d.	151.3 ± 17.7	123.7 ± 73.7	84.1 ± 37.9
COD:N ratio	9.0	12.7	16.8	17.6
COD:P ratio	n.d.	95.8	92.2	98.7
TS:VS ratio	n.d.	1.4	1.5	1.5
COD:VS ratio	n.d.	1.2	1.3	1.5

Table 7.2 *Operational parameters of the high-load anaerobic membrane bioreactor (HL-AnMBR) during phase 1 and phase 2, the low-load non-vibrating anaerobic membrane bioreactor (NV-AnMBR) and the low-load vibrating anaerobic membrane bioreactor (V-AnMBR).*

Parameter	HL-AnMBR phase 1	HL-AnMBR phase 2	NV-AnMBR	V-AnMBR
Substrate	Concentrated molasses	Diluted molasses	Diluted molasses	Diluted molasses
Duration (days)	46	36	87	54
Temperature (°C)	34	34	34	34
Organic loading rate, OLR (g COD L^{-1} d^{-1})	1.1 ± 0.1 – 4.1 ± 0.2	2.8 ± 0.2	1.1 ± 0.0 – 10.1 ± 0.1	1.0 ± 0.1 – 6.0 ± 0.0
Hydraulic retention time, HRT (d)	100.3 ± 10.2 – 26.3 ± 0.7	5.3 ± 0.3	2.5 ± 0.1	2.6 ± 0.2
Solid retention time, SRT (d)	1535	1535	81.8	112.7
Membrane flux, J (L m^{-2} h^{-1})	0.020 ± 0.003 – 0.08 ± 0.01	0.41 ± 0.04	0.86 ± 0.08	0.99 ± 0.11

2.5. Microbial community analysis

Total DNA was extracted from the sludge samples according to the method of Boon et al. (2000). Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 2 g of sample was taken for DNA extraction. The crude extract was then further purified by means of the Wizard DNA Clean-up System (Promega, Madison, Wis), according to the manufacturer's instructions. The DNA concentration in the extracts was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Lifescience, IJsselstein, The Netherlands), by measuring the absorbance ratios at 260 nm and 280 nm. Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Analytical triplicates of a 100-fold dilution of the DNA-samples were analysed for total bacteria, total archaea and the methanogenic families Methanosarcinaceae and Methanosaetaceae. Total archaea can be considered a valid estimation of total methanogens in AD, because of the highly unfavourable conditions of non-methanogenic archaea in AD (Woese et al., 1990; Raskin et al., 1995). To quantify total bacteria, the general bacterial primers P338F (5'-ACTCCTACGGGAGGCAGCAG-3') and P518r (5'-ATTACCGCGGCTGCTGG-3'), as described by Ovreas et al. (1997), were used. The primer sets used for total archaea (ARC) and the methanogenic families Methanosarcinaceae (Msc) and Methanosaetaceae (Mst) were previously described by Yu et al. (2005). The reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, Wis), and consisted of 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure, which consists of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C for total bacteria. The qPCR program for total archaea, Methanosarcinaceae and Methanosaetaceae consisted of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. The qPCR data were represented as copies per gram of wet sludge.

2.6. Analytical methods

Total suspended solids (TSS), VSS, total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (KjN), total ammonia nitrogen (TAN), total phosphorous (TP), total COD (COD_{tot}), and soluble COD (COD_{sol}) were determined according to Standard Methods described by

Greenberg et al. (1992). Biogas composition was analysed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH_4, CO_2 and H_2 were determined using a thermal conductivity detector with a lower detection limit of 1 ppmv for each gas component. The VFA (volatile fatty acids) were extracted with diethyl ether, and measured in a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands), which was equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (dimensions: 25 mm x 0.53 mm, film thickness 1.2 µm; Alltech, Laarne, Belgium), a split injector and a flame ionization detector. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium). Conductivity was measured with a C833 conductivity meter (Consort, Turnhout, Belgium).

3. Results

3.1. Impact of hydraulic retention time on reactor performance

The goal of the first experiment was to determine the effect of the molasses concentration and, hence, of the HRT on the AD process. Bio-refinery waste streams contain high loads of organics and salts, and it is important to define what the lower HRT limits are. The HRT of the reactors was determined by the degree of dilution of the molasses fed to the reactor, i.e. highly concentrated molasses were used during the operation of the HL-AnMBR, whereas diluted molasses were used during the operation of the NV-AnMBR and V-AnMBR. Hence, the resulting hydraulic retention time (HRT) differed between the HL-AnMBR on the one hand and the NV-AnMBR and V-AnMBR on the other hand (Table 7.2). During phase 1, the OLR in the HL-AnMBR was increased by decreasing the HRT, keeping the molasses concentration in the feed constant, whereas in phase 2 the HRT and OLR were kept constant. During operation of the NV-AnMBR and V-AnMBR, the HRT was kept constant and the OLR was increased by increasing the molasses concentration in the feed. The results of the methane production and COD removal efficiencies of these three reactors are given in Figure 7.2 and 7.3, respectively.

During operation of the HL-AnMBR, the organic loading rate (OLR) was stepwise increased from 1.1 g COD L^{-1} d^{-1} on day 0 to 4.1 g COD L^{-1} d^{-1} on day 24 (Figure 7.2). At that point, the methane production and COD removal efficiency amounted to 0.50 L L^{-1} d^{-1} and 92.2%, respectively. This value was approximately only one third of the theoretically maximum yield of 1.43 L L^{-1} d^{-1}. The discrepancy between COD removal and methane conversion efficiency

can be attributed to the retention of the non-degraded solid fraction of the molasses in the reactor, due to the presence of the membrane. In addition, the total VFA effluent concentration amounted to 3.0 g COD L^{-1} (Fig.7.4), with propionate and acetate as the most important components, with 52 and 30%, respectively. Therefore, the OLR was lowered to 2.9 COD L^{-1} d^{-1} on day 32. However, the performance of the reactor further decreased, resulting in a methane production, COD removal and VFA concentration of 0.16 L L^{-1} d^{-1}, 71.4% and 42.8 g COD L^{-1}, respectively. Acetate and propionate remained the most important components with 58 and 26%, respectively, on day 46 (Figure 7.2, 7.3 and 7.4). During this entire period (phase 1), the HRT was kept at a value between 100 and 26 days (Table 7.2). After day 46 (phase 2), the HRT was lowered to a value of 5.3 days, and diluted molasses were used, to regain stable methane production and COD removal, because of the lower resulting salt concentrations in the reactor. However, methane production, COD removal efficiency and VFA concentrations reached values of 0.10 L L^{-1} d^{-1}, 26.3% and 10.7 g COD L^{-1}, respectively, containing 59% acetate and 20% propionate, respectively, on day 82 (Figure 7.2, 7.3 and 7.4). This observation suggested severe limitations of the AD process. Concomitantly, the pH dropped to 6.45 at the end of the experiment. The TAN concentration reached a maximum value of 3307 mg N L^{-1} at the end of phase 1 (day 46), after which a decrease to values below 1000 mg N L^{-1} were observed at the end of phase 2 (Figure 7.5).

In contrast to the HL-AnMBR configuration, the HRT was kept constant at a value of 2.5 and 2.6 days for the NV-AnMBR and the V-AnMBR, respectively (Table 7.2). In case of the NV-AnMBR, the OLR was gradually increased from 1.1 g COD L^{-1} d^{-1} on day 0 to 10.1 g COD L^{-1} d^{-1} at the end of the experiment (day 80). A maximum methane production of 2.05 L L^{-1} d^{-1} and a concomitant COD removal of 94.4% were observed at an OLR of 7.2 g COD L^{-1} d^{-1}. Subsequently, a sudden drop in methane production to 0.65 L L^{-1} d^{-1} was detected. However, the COD removal remained constantly higher than 90% and no significant amounts of VFA were detected. Therefore, the OLR was further increased until 10.1 g COD L^{-1} d^{-1} was reached. At this point, the methane production and COD removal efficiency amounted to 1.54 L L^{-1} d^{-1} and 92.6%, respectively. The TAN concentration remained below 800 and 500 mg N L^{-1}, in the NV-AnMBR and V-AnMBR, respectively, during the entire experimental period.

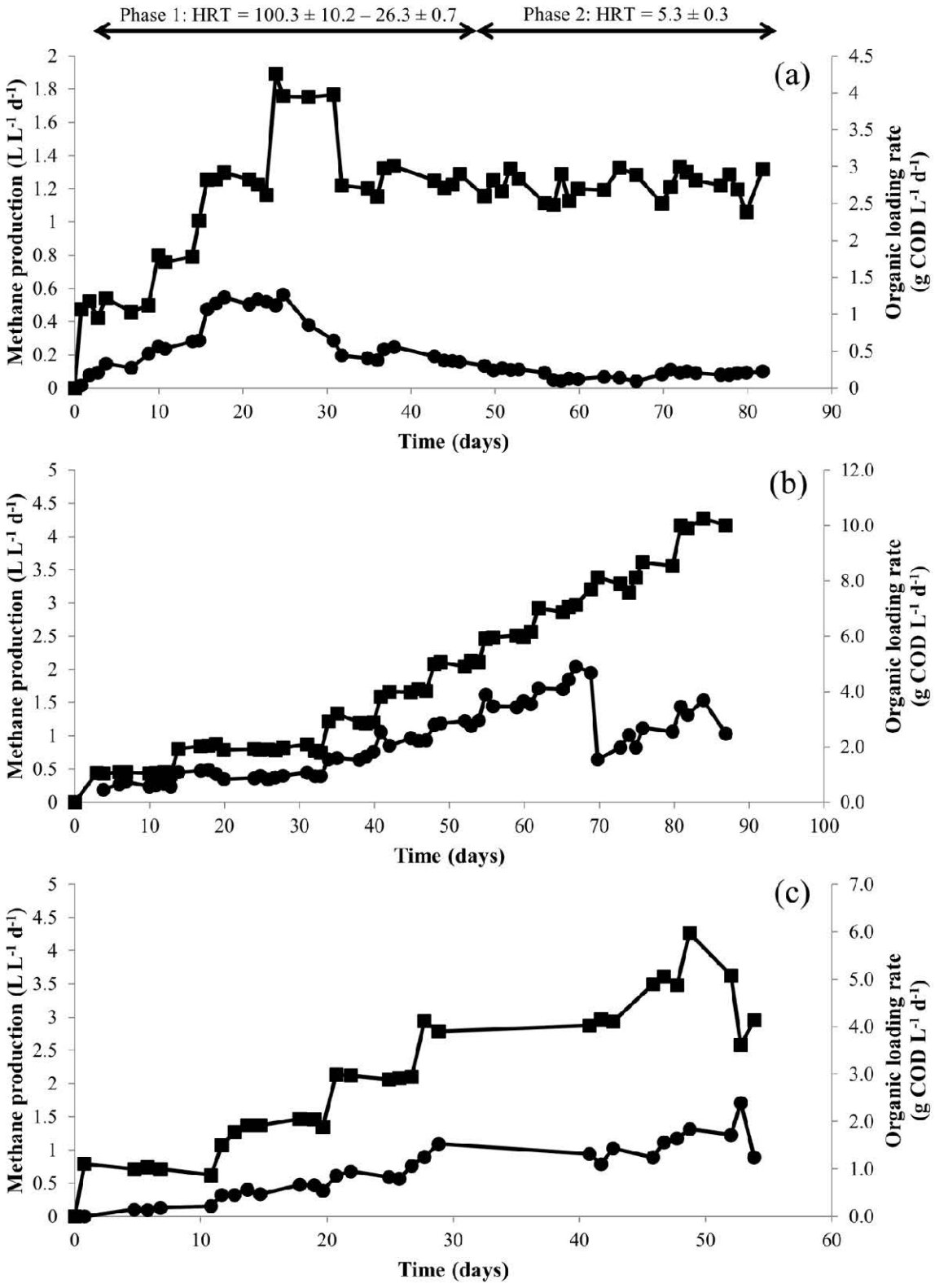

Figure 7.2 *Organic loading rate (OLR) (■) and methane production (●) in the HL-AnMBR (a), NV-AnMBR (b) and V-AnMBR (c).*

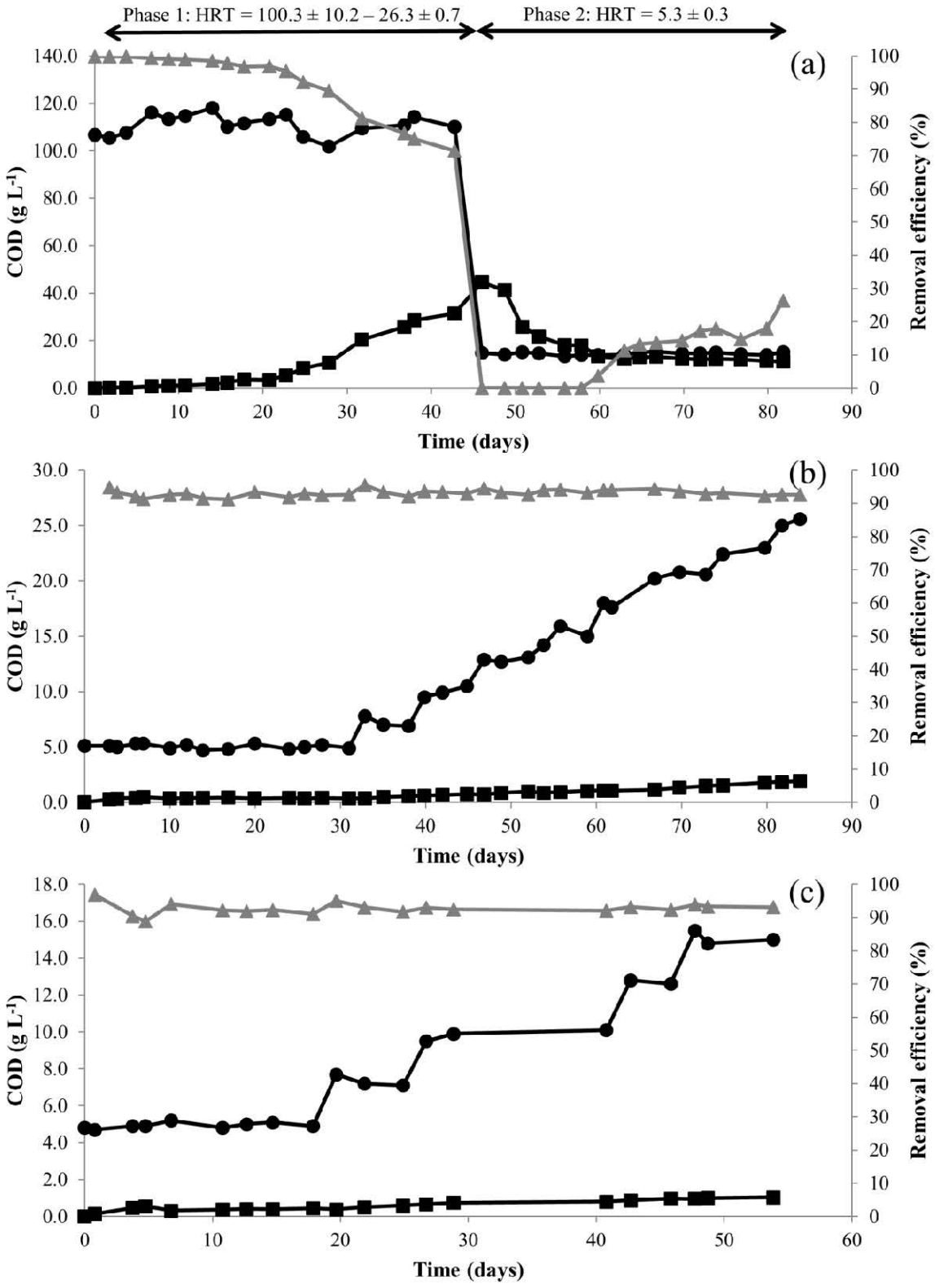

Figure 7.3 *Influent (●) and effluent (■) total COD and removal efficiency (%) (▲) of the HL-AnMBR (a), NV-AnMBR (b) and V-AnMBR (c).*

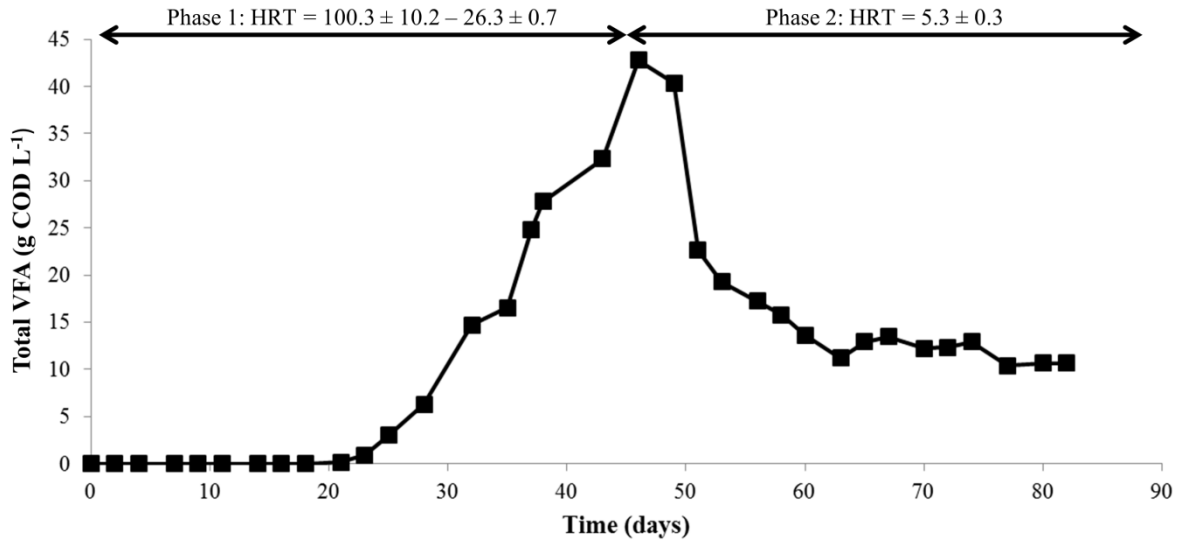

Figure 7.4 *Total VFA concentration in the HL-AnMBR. VFA concentrations remained below the detection limit in the NV-AnMBR and V-AnMBR during the entire experiment.*

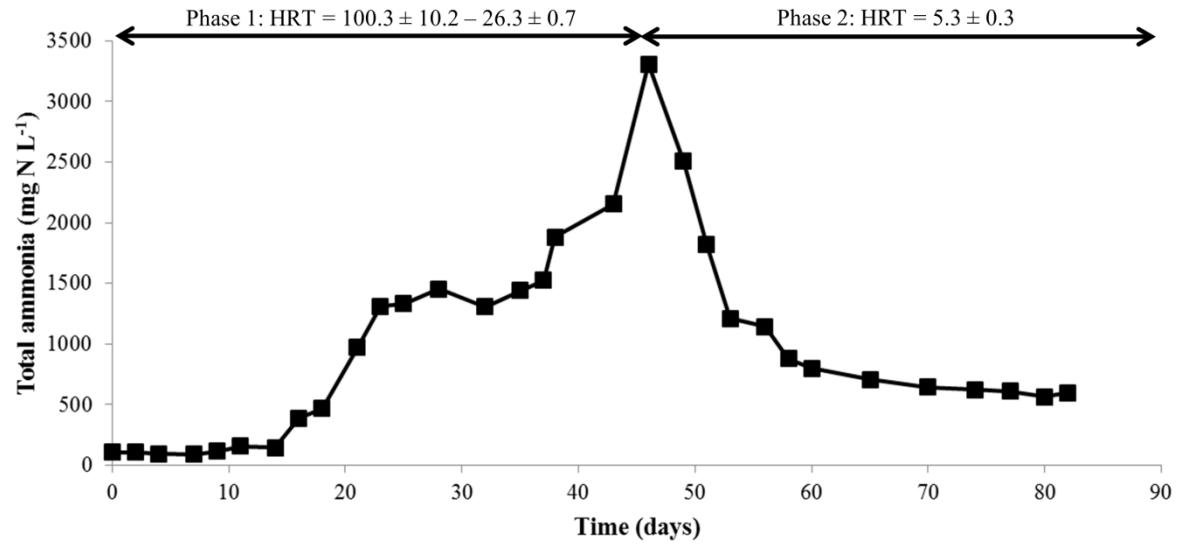

Figure 7.5 *Total ammonia concentration (mg N L^{-1}) in the HL-AnMBR. Total ammonia concentrations remained below 800 and 500 mg N L^{-1} in the NV-AnMBR and V-AnMBR, respectively, during the entire experiment.*

The substantial difference in HRT, determined by the dilution degree of the molasses, is clearly reflected in the degree of methane production, COD removal and VFA accumulation. That is, a higher degree of molasses dilution leads to higher methane production and reactor stability, even at higher organic loading rates. The conductivity measurements, which greatly differed between the HL-AnMBR and the NV-AnMBR and V-AnMBR, (i.e. values of 40.3

mS cm^{-1} on day 46 in the HL-AnMBR and 14.8 and 8.7 mS cm^{-1} in the NV-AnMBR and V-AnMBR, respectively) may have contributed to this discrepancy.

3.2. Impact of AnMBR configuration on reactor performance

In the second part of the research, the goal was to explore the influence of different fouling control systems on the AD and filtration processes. Therefore, a set-up with scouring via biogas recirculation (NV-AnMBR) and a set-up with a vibrating membrane (V-AnMBR) were run in parallel. The performance of the NV-AnMBR was described in the previous paragraph. In case of the V-AnMBR, the OLR was ramped up from 1.0 g COD L^{-1} d^{-1} to 6.0 g COD L^{-1} d^{-1} at the end of the experiment. The reactor was stopped due to increasing TMPs, which had a significant effect on the effluent flow rates. Increased methane production values up to 1.71 L L^{-1} d^{-1} were obtained. During the entire experiment, the COD removal efficiencies were never lower than 88.9% and amounted to 93.1% at the end of the experiment (Figure 7.2 and 7.3). The pH remained stable throughout the experiment, with final values of 7.60 and 7.47 in the NV-AnMBR and the V-AnMBR, respectively. No significant VFA could be detected in the effluents throughout the entire experiment.

To compare the different devices for fouling control, i.e. biogas recirculation vs. membrane vibration, the filtration capacities of the membrane was studied. The critical flux was 6 L m^{-2} h^{-1} at a sludge concentration of 27.2 g VSS L^{-1} and 9 L m^{-2} h^{-1} at a sludge concentration of 13.6 g VSS L^{-1} and 6.8 g VSS $L^{-1.}$ The mean sludge concentration during operation of the different reactors was kept around 14 g VSS L^{-1}. Hence, a critical and theoretical operational flux for this specific experiment amounted to 9 L m^{-2} h^{-1} and 6.75 L m^{-2} h^{-1}, respectively. The HL-AnMBR, NV-AnMBR and V-AnMBR were operated at maximum fluxes of 0.41 ± 0.04, 0.86 ± 0.08 and 0.99 ± 0.11 L m^{-2} h^{-1}, respectively (Table 7.2). These are more than a factor 5 lower compared to the experimentally determined theoretical operational flux. For the reactors with biogas recirculation, the TMP never exceeded 10 mbar throughout the entire experiment. In contrast, the TMP in the V-AnMBR increased stepwise to 400 mbar on day 18 and further to 560 mbar on day 53 at the end of the experiment. Since the operational flux in this experiment was almost 10 times lower than the theoretical flux, filtration experiments were performed to further study the fouling. The Kubota membranes were removed and the TMP and permeability were determined at a flux of 9 L m^{-2} h^{-1} (Table 7.3). The TMP amounted to 98 mbar and 94 mbar for the NV-AnMBR and V-AnMBR, respectively, with corresponding permeabilities of 92.3 L m^{-2} h^{-1} bar^{-1} and 96.3 L m^{-2} h^{-1} bar^{-1}. Cleaning with

water did not result in decreased TMPs or increased permeabilities, but after intense cleaning with hypochlorite, the TMPs decreased to 77 mbar and 88 mbar for the NV-AnMBR and V-AnMBR. The difference in membrane permeability between the NV-AnMBR (117 L m^{-2} h^{-1} bar^{-1}) and the V-AnMBR (102 L m^{-2} h^{-1} bar^{-1}) was relatively small. These results suggest that the TMP increase in the V-AnMBR was due to cake layer formation. Since no mixing was provided for the V-AnMBR, the liquid adjacent to the membrane surface was relatively stagnant, allowing the build-up of a cake layer.

Table 7.3 *Permeability recovery of the NV-AnMBR and V-AnMBR at the end of the experiment, following several cleaning treatments. Filtration was carried out with tap water at the flux of 9 L m^{-2} h^{-1}. The permeability of the pristine Kubota membrane is 1110 L m^{-2} h^{-1} bar^{-1} (manufacturer data).*

	Cleaning technique	TMP (mbar)		Permeability (L m^{-2} h^{-1} bar^{-1})	
		NV-AnMBR	V-AnMBR	NV-AnMBR	V-AnMBR
1	No	98	94	92	96
2	Flushing	98	106	92	85
3	Chemical (NaOCl)	129	168	70	54
4	Intense chemical (NaOCl)	77	88	117	102

3.3. Macroscopic sludge property evolution

The reactors were inoculated with granular sludge from an UASB digester. The influence of the operational parameters (varying HRT and molasses concentration) on the granular structure was evaluated by means of the particle size distribution (PSD), as presented in Figure 7.6. The PSD of the inoculum sludge for experiment 1 was more or less equally divided between the different fractions (Figure 7.6a). In contrast, the fraction smaller than 0.25 mm of the sludge in the HL-AnMBR, after 82 days of operation, contained 90.83% of total VS, which indicated that the sludge experienced severe fragmentation during operation in experiment 1. The PSD of the inoculum sludge for experiment 2 showed a similar equally divided particle size contribution, when compared to the sludge inoculum of experiment 1 (Figure 7.6b). The fragmentation of the sludge in experiment 2 seemed to be limited, with an increase to only 54.92% in the fraction < 0.25 mm. These results are in contrast to the PSD of the HL-AnMBR, in which almost complete fragmentation of the sludge took place.

3.4. Microbial community analysis

The microbial community in the membrane bioreactors was analysed by means of real-time PCR, i.e. total bacteria, total archaea and Methanosarcinaceae and Methanosaetaceae were quantified in the inoculum sludge and in each reactor on weekly basis (Figure 7.7). These results indicate that there was already a remarkable difference in copy numbers between Methanosarcinaceae ($6.5 \times 10^6 \pm 2.5 \times 10^6$ copies g^{-1}) and Methanosaetaceae ($3.8 \times 10^9 \pm 5.1 \times 10^8$ copies g^{-1}) in the inoculum sludge. The total bacteria and total archaea copy numbers in the inoculum sludge were almost equal, i.e. $2.2 \times 10^{10} \pm 2.3 \times 10^9$ copies g^{-1} and $3.4 \times 10^{10} \pm 3.5 \times 10^9$ copies g^{-1}, respectively.

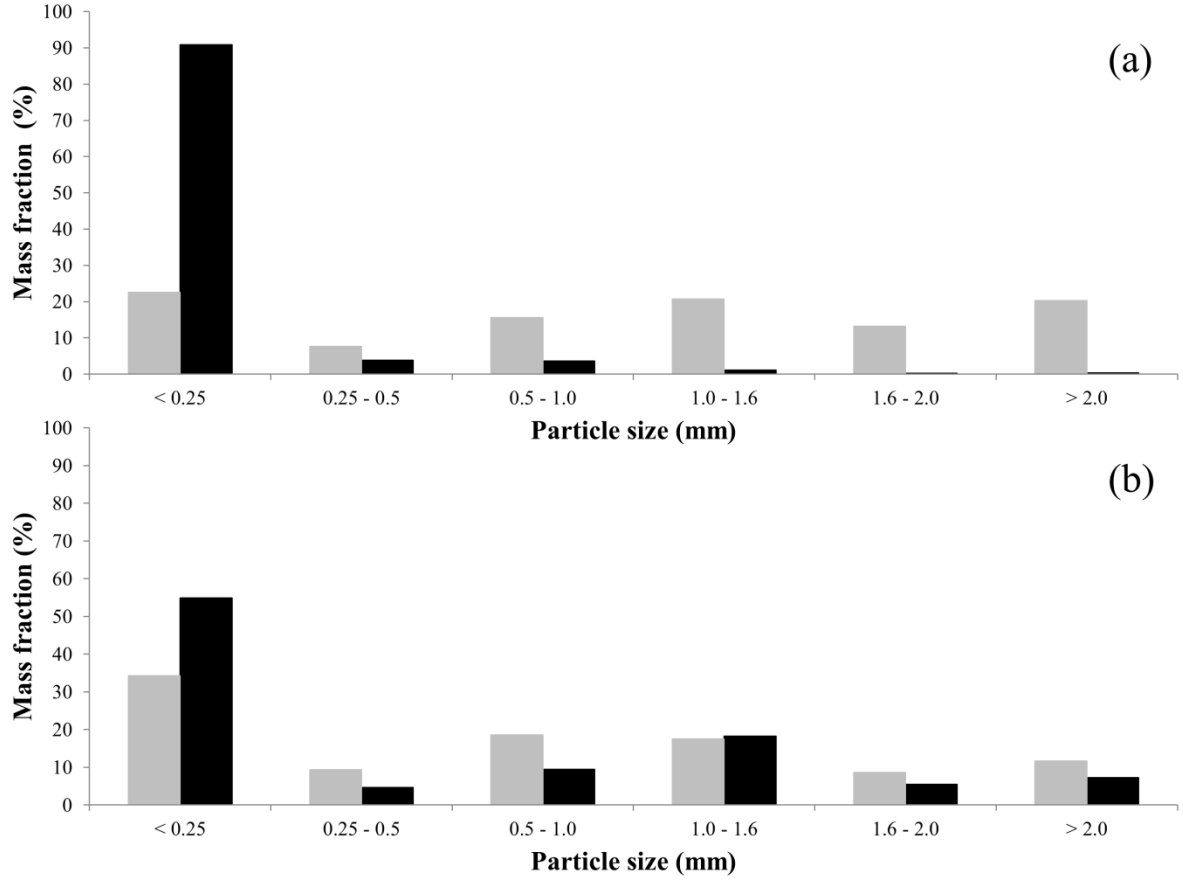

Figure 7.6 *Particle size distribution (PSD) of the sludge in (a) the HL-AnMBR and (b) the NV-AnMBR. The inoculum sludge (■) was compared to the sludge at the end of the experiment (■).*

Total bacteria copy numbers showed a uniform increasing trend in all three reactors towards the end of the experiment to values of $2.2 \times 10^{11} \pm 9.9 \times 10^{10}$, $3.0 \times 10^{11} \pm 4.0 \times 10^{10}$ and $1.8 \times 10^{11} \pm 1.8 \times 10^{10}$ copies g^{-1} in the HL-AnMBR, NV-AnMBR and V-AnMBR, respectively, which corresponds to an almost 10-fold increase in all three reactors. This is in contrast to the total archaea, which showed no remarkable increase in copy numbers, that is $4.2 \times 10^{10} \pm 9.0 \times 10^9$, $6.4 \times 10^{10} \pm 1.4 \times 10^{10}$ and $7.0 \times 10^{10} \pm 3.2 \times 10^9$ copies g^{-1} in the HL-AnMBR, NV-

AnMBR and V-AnMBR, respectively, at the end of the experiment. This indicates that during operation of the three reactors, there was a definite expansion of the bacterial community, whereas the archaeal community only managed to maintain its numbers. In all three reactors Methanosarcinaceae copy numbers remained at least a factor 100 lower than Methanosaetaceae copy numbers, which indicates that acetoclastic methanogenesis was probably dominated by Methanosaetaceae species throughout the entire experiment. The Methanosarcinaceae copy numbers did, however, show a slight increase in the HL-AnMBR, with a maximum of $2.0 \times 10^{7} \pm 1.3 \times 10^{7}$ copies g^{-1}, but after this maximum the copy numbers decreased again to a value of $9.8 \times 10^{5} \pm 8.7 \times 10^{5}$ copies g^{-1}, which was even lower than in the inoculum sample. This trend is not observed in the Methanosaetaceae copy numbers, which remained stable throughout the entire experiment, indicating that at a certain point the Methanosarcinaceae species obtained better growing conditions than the Methanosaetaceae species. Yet, these conditions were apparently not maintained, because a decrease in Methanosarcinaceae copy numbers was observed. In contrast to the HL-AnMBR, copy numbers of both Methanosarcinaceae and Methanosaetaceae showed a 10-fold increase towards the end of the experiment in the NV-AnMBR and V-AnMBR. This indicates favourable growth conditions for both families in the two reactors.

4. Discussion

The production of highly loaded waste streams, combined with more stringent environmental regulations, is forcing bio-refineries to treat their wastewaters more effectively. At the same time, given the high organic loads, more and more bio-refineries are making efforts to fully utilize these streams. Several treatments, such as the upgrade to secondary fermentation products, have been suggested (Agler et al., 2011; Agler et al., 2012). In this study, AD was applied to exploit the energy present in bio-refinery streams. Although the value of fermentation products is higher, the biggest advantage of AD is that the product (biogas) can be separated from the broth easily and without additional costs. Anaerobic digestion of bio-refinery waste streams has been applied in several reactor systems. The use of an UASB allows for the operation at high SRT, which can be favourable when treating waste streams containing substances that may cause inhibition of the methanogenic community. However, under specific conditions, such as the high salinity and high lipid content, encountered in bio-refinery waste streams, biofilm and granule formation are negatively affected. The adverse effect of monovalent cations is especially severe in this regard (Liu et al., 2002; Liu et al.,

2003). The presence of lipids can result in the adsorption of a light lipid layer around biomass particles causing biomass flotation, as well as washout and acute toxicity of LCFA (long chain fatty acids), especially unsaturated ones, to both methanogens and acetogens, the two main trophic groups involved in LCFA degradation (Alves et al., 2001). In this study, HRT and SRT were uncoupled using plate membranes, which allowed for operation at a high SRT, and elimination of the need for granule or biofilm formation.

Anaerobic digestion of molasses in an AnMBR with biogas recirculation, operated at low HRT, treating diluted molasses (NV-AnMBR), resulted in stable methane production. This was in contrast to an AnMBR at high HRT, treating concentrated molasses (HL-AnMBR), which resulted in process failure, indicated by severe acidification. Indeed, the VFA reached values up to 42.8 g COD L^{-1} at the end of the experiment. Conversely, the reactor treating diluted molasses (low HRT), showed no residual VFA throughout the entire experiment. The failure of the AnMBR at high HRT could not be attributed to overloading, since the maximum OLR (10.1 g COD L^{-1} d^{-1}) in the NV-AnMBR was much higher, compared to the maximum OLR (4 g COD L^{-1} d^{-1}) in the HL-AnMBR. Moreover, stable methanation has been obtained at OLR values around 25 g COD L^{-1} d^{-1} in anaerobic digesters using submerged membranes (Jeison & van Lier, 2008; Van Zyl et al., 2008; Stuckey, 2012). However, these studies used no bio-refinery type wastes, but mixtures of VFAs as feedstock. The failure of the HL-AnMBR could be attributed to the high concentrations of salt in the concentrated molasses (Table 7.1), indicated by the high conductivity of 40.3 mS cm^{-1} on day 46 in the reactor. Indeed, methanogens are especially susceptible to conductivity > 30 mS cm^{-1} (Chen et al., 2008; De Vrieze et al., 2012). Molasses have been shown to contain high levels of salt, especially potassium, which can be inhibitive towards AD (Satyawali & Balakrishnan, 2008; Fang et al., 2011b). A decrease of the HRT to 5.3 days on day 46 resulted in a sharp decrease in the conductivity to 17.4 mS cm^{-1} on day 82. This conductivity value can be considered non-inhibiting towards methanogens (De Vrieze et al., 2012). However, no recovery in methane production could be detected, despite the constant loading rate. This indicates that the methanogenic activity was irreversibly inhibited by the high salinity of the reactor. The concentrated molasses, as described in Table 7.1, contained high concentrations of potassium and sulphate (16.3 g L^{-1} and 2.44 g L^{-1}, respectively), and sulphate reduction might have resulted in toxic sulphide levels.

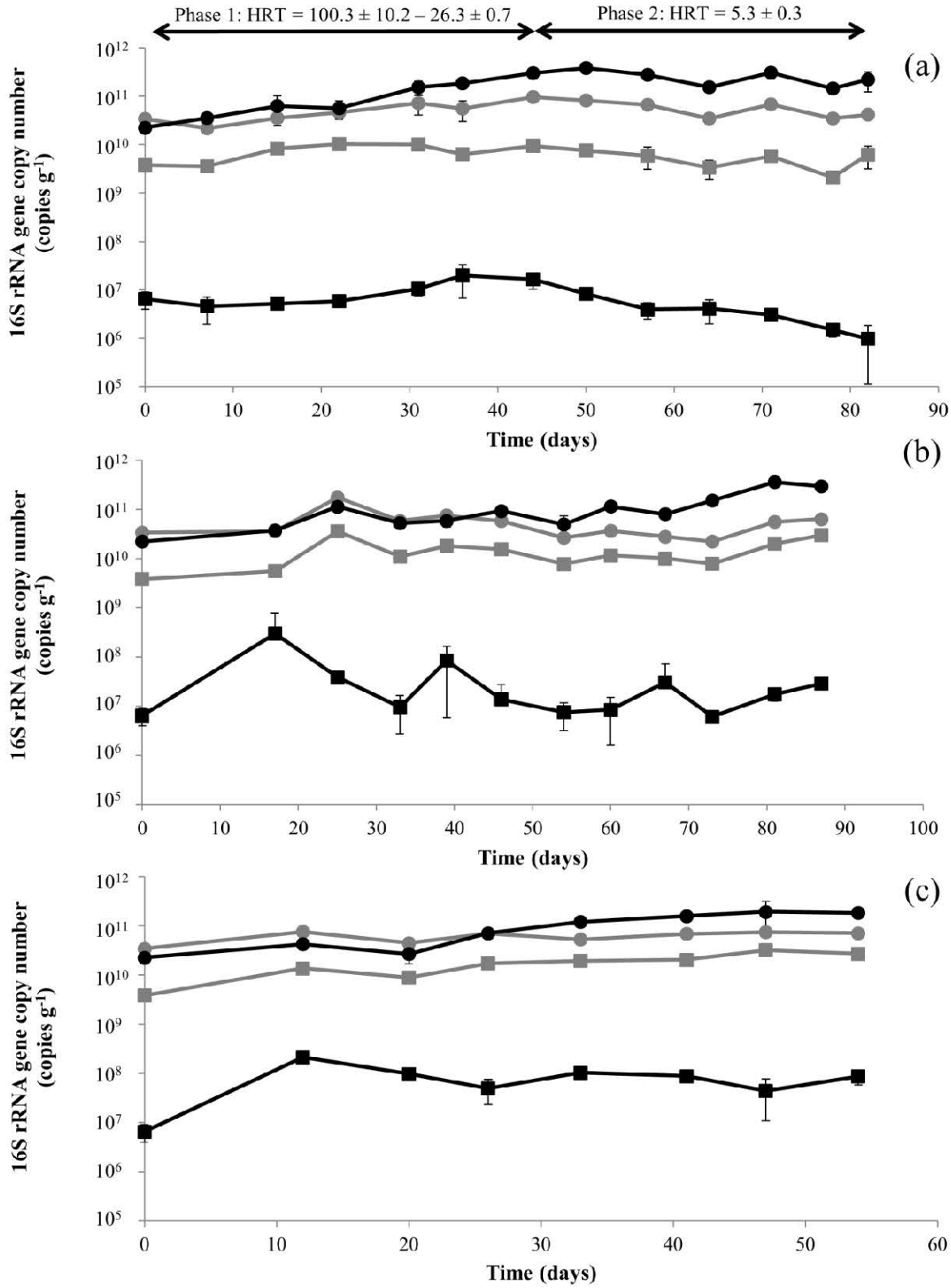

Figure 7.7 *Real-time PCR results of the DNA samples of the HL-AnMBR (a), NV-AnMBR (b) and V-AnMBR (c). 16S rRNA gene copy numbers were determined specifically for total bacteria (●), total archaea (●), Methanosaetaceae (■), and Methanosarcinaceae (■). Average values of the triplicate analyses, together with the standard deviations are presented.*

The high TAN concentration might also be one of the causes of failure of the HL-AnMBR, as a maximum TAN concentration of 3307 mg N L^{-1} was observed, which may have (partially) inhibited methanogenesis (Chen et al., 2008; De Vrieze et al., 2012). Another possible explanation for the inhibition at high HRT could be the presence of toxic organics in the molasses feed. It has been shown that the main organic components in raw molasses wastewater were theanone, phenylethyl alcohol, benzoic acid, and phenol derivatives with methoxy or other substituents (Satyawali & Balakrishnan, 2008). Many phenol derivatives have been shown to be toxic and interfere with the activity of methanogens (Olguin-Lora et al., 2003). Negative effects of feeding highly concentrated molasses were also observed in UASB digesters (Zhang et al., 2009). Towards practical applications, it could be concluded that high strength molasses wastewaters are difficult to treat and that they have to be diluted prior to digestion. The necessary dilution factor will have to be established on a case-by-case basis (Sanchez Riera et al., 1985), but amounts, to our knowledge, to at least a factor 2 at high OLRs. Furthermore, the dilution factor should be minimized to limit (an)aerobic membrane surface requirements and wastewater production.

In this study, an innovative system using a magnetically induced membrane vibration system was used as an alternative shear enhancement device for fouling control. This system was previously developed for aerobic MBRs, in which consumption of scouring air can be considered as one of the main costs (Bilad et al., 2012; Mezohegyi et al., 2012). In the case of AnMBRs, energy consumption would not be the limiting factor, as the biogas could be recirculated to avoid fouling. This was shown to be very effective in this study and elsewhere (Zamalloa et al., 2012). Indeed, no significant increase in TMP could be observed throughout the experiment. Moreover, permeability measurements at the critical flux demonstrated that the permeability could only be increased by 21% after intense cleaning with hypochlorite. This means there was an excessive residual fouling remaining on the membranes. In contrast to the energy consumption, the collection of biogas at the top of reactors and the subsequent compression and re-injection at the bottom can pose many practical problems for full-scale applications. Indeed, safety considerations concerning biogas compression, methane losses and the presence of sulphides in the biogas, which will create corrosion in piping, might limit further development of submerged plate membrane digesters. Moreover, continuous scouring might result in varying gas equilibriums, methane oversaturation and changing pH as function of the CO_2/HCO_3^- ratios. These parameters could affect both the microbial community and the microbial metabolism and, hence, result in a decrease in methane production. In this study, no

significant difference in the CH_4/CO_2 ratio of the biogas produced by both reactors could be observed (1.90 and 1.88 for the V-AnMBR and the NV-AnMBR, respectively). Moreover, the microbial community in both reactors did not differ throughout the experiment. These results suggest that the AD process was not affected by biogas recirculation. On the other hand, the improved mixing obtained by biogas circulation did not seem strictly required for the anaerobic processes. However, the TMPs of the V-AnMBR increased throughout the experiment, while no increase in TMP could be observed in the NV-AnMBR. Permeability measurements at critical flux values after the experimental runs elucidated that there was no significant difference in TMP and permeability between the membranes from the NV-AnMBR and V-AnMBR. Moreover, after intense cleaning with hypochlorite, the permeability of the V-AnMBR membrane could only be increased with 5.6%. These results suggest that the increase in TMP during the AD process is dominated by cake layer formation, and is not due to other types of membrane fouling. However, the limited positive effect of chemical cleaning on membrane permeability points to a high degree of irreversible fouling of the membrane, both in the NV-AnMBR and V-AnMBR. The cake layer formation can be readily explained by the absence of sludge mixing in the V-AnMBR. In case of the NV-AnMBR, the biogas recirculation not only resulted in fouling prevention, but also in continuous mixing of the sludge. Since the absence of mixing in the V-AnMBR did not negatively affect the biogas production, a conventional mixing device would only be required to obtain equal VSS concentration in the reactor, and avoid cake layer build up at the membrane, which would not mean that there is no market for the vibrating membrane system. Indeed, the magnetic shear control system in the V-AnMBR allowed for changing the required vibration parameters (frequency and amplitude) during the filtration operation. Operational parameters, such as MLVSS (mixed liquor volatile suspended solids) and flux rates, might differ during the process, and such a system could result in significant power savings.

Particle size distribution results demonstrated that there was a clear fragmentation of the granular sludge particles during operation of both the HL-AnMBR and NV-AnMBR. This fragmentation was most likely caused by the recirculation of the biogas to obtain sufficient mixing. It was already demonstrated in aerobic processes that excess shear stress could lead to fragmentation of granules (Shin et al., 1992; Dulekgurgen et al., 2008), which is apparently also the case for anaerobic granules. However, degranulation cannot be contributed to shear stress alone, because unlike in upflow anaerobic sludge blanket (UASB) reactors, there is no driving force for granulation in a membrane bioreactor (Liu et al., 2003; Hulshoff Pol et al.,

2004). The HL-AnMBR revealed a much higher degree of degranulation, compared to the NV-AnMBR, i.e. 90.83% of total VS was contained within the fraction < 0.25 mm in the HL-AnMBR, while this was only 54.92% in the NV-AnMBR at the end of the experiment. This discrepancy can be attributed to the difference in reactor performance between the HL-AnMBR and NV-AnMBR. Indeed, the severe decrease in methane production in the HL-AnMBR was attributed to the high conductivity in the reactor, caused by the high salt concentration, which resulted in severe sludge fragmentation and process failure. This effect was not observed in the NV-AnMBR, due to the much lower salt concentration (Satyawali & Balakrishnan, 2008; Fang et al., 2011b; De Vrieze et al., 2012).

Real-time PCR results revealed a dominance of Methanosaetaceae over Methanosarcinaceae as the acetoclastic methanogens in the inoculum sludge, as well as in the reactors throughout the entire experiment (Figure 7.7). Methanosaetaceae and Methanosarcinaceae families are considered the only acetoclastic methanogens (Conklin et al., 2006; Zamalloa et al., 2012). *Methanosaeta* sp. dominate at low residual acetate concentrations, because of a low K_S value, whereas *Methanosarcina* sp. are more likely to be dominant when residual acetate concentrations are high (Conklin et al., 2006; De Vrieze et al., 2012). The inoculum sludge sample was dominated by Methanosaetaceae, which was to be expected, since residual VFA concentrations were below detection limit in the UASB reactor from which the sludge originated. Despite the differences in Methanosaetaceae and Methanosarcinaceae between the different reactors, there appeared to be a similar trend in total archaea and total bacteria in the three reactors. The limited degree of change in total archaea copy numbers was also reflected in the low variability in Methanosaetaceae and Methanosarcinaceae copy numbers in all three reactors. Methanosarcinaceae copy numbers were a factor 100 lower than Methanosaetaceae copy numbers in the three reactors. This was to be expected in the NV-AnMBR and V-AnMBR, which showed no residual VFA, and achieved stable operation until the tests were finished. The endurance of the Methanosaetaceae in the HL-AnMBR was rather unexpected, since VFA accumulation occurred rapidly, reaching a maximum value of 42.8 g COD L^{-1} on day 46, hence, a shift to a Methanosarcinaceae dominated community was expected (Gujer & Zehnder, 1983; Conklin et al., 2006; De Vrieze et al., 2012). However, methane production quickly declined to very low levels, indicating that the expected shift from a Methanosaetaceae to a Methanosarcinaceae dominated methanogenic community did not take place (Chen et al., 2012). Indeed, Methanosarcinaceae copy numbers did show a slight increase during VFA accumulation in the HL-AnMBR, followed yet again by a decrease after

day 46. The sharp increase in conductivity, followed by a very fast accumulation of VFA to very high values most likely did not provide sufficient time to obtain a sustainable shift from a Methanosaetaceae to a Methanosarcinaceae dominated methanogenic community.

5. Conclusions

In this research, it was demonstrated that anaerobic digestion of molasses wastewater in an anaerobic membrane bioreactor resulted in stable methane production and high COD removal efficiencies, yet, only when diluted molasses were used. The application of a novel strategy for fouling prevention, i.e. a vibrating membrane, resulted in a strong increase in trans membrane pressure and cake layer formation, which demonstrates that an anaerobic membrane bioreactor with biogas recirculation is the best choice for anaerobic molasses wastewater treatment. Acetoclastic methanogenesis was dominated by Methanosaetaceae in all treatments, despite high salt concentrations. This research, hence, demonstrated the suitability of anaerobic membrane reactor systems with biogas recirculation to treat bio-refinery sidestreams.

Acknowledgments

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CHAPTER 8: AMMONIA AND TEMPERATURE DETERMINE POTENTIAL CLUSTERING IN THE ANAEROBIC DIGESTION MICROBIOME

This chapter has been redrafted after:

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Abstract

Anaerobic digestion is regarded as a key environmental technology in the present and future bio-based economy. The microbial community completing the anaerobic digestion process is considered complex, and several attempts already have been carried out to determine the key microbial populations. However, the key differences in the anaerobic digestion microbiomes, and the environmental/process parameters that drive these differences, remain poorly understood. In this research, we hypothesized that differences in operational parameters lead to a particular composition and organization of microbial communities in full-scale installations. A total of 38 samples were collected from 29 different full-scale anaerobic digestion installations, showing constant biogas production in function of time. The bacterial community in all samples was dominated by representatives of the Firmicutes, Bacteroidetes and Proteobacteria, covering 86.1 ± 10.7% of the total bacterial community. Acetoclastic methanogenesis was dominated by Methanosaetaceae, yet, only the hydrogenotrophic Methanobacteriales correlated with biogas production, confirming their importance in high-rate anaerobic digestion systems. In-depth analysis of operational and environmental parameters and bacterial community structure indicated the presence of three potential clusters in anaerobic digestion. These clusters were determined by total ammonia concentration, free ammonia concentration and temperature, and characterized by an increased relative abundance of Bacteroidales, Clostridiales and Lactobacillales, respectively. None of the methanogenic populations, however, could be significantly attributed to any of the three clusters. Nonetheless, further experimental research will be required to validate the existence of these different clusters, and to which extent the presence of these clusters relates to stable or sub-optimal anaerobic digestion.

1. Introduction

Anaerobic digestion (AD) can be considered one of the most prominent technologies in the field of renewable energy production. This microbial technology has been applied at full scale for the treatment of organic waste for several decades (Angenent et al., 2004a; Verstraete et al., 2005; Holm-Nielsen et al., 2009; Appels et al., 2011). The amount of organic waste that is treated by means of AD still increases every year with almost 25%, as new industrial organic waste streams are constantly being generated in the emerging bio-refineries (Ryan et al., 2009; Verstraete, 2010; Appels et al., 2011; Menardo & Balsari, 2012). Unlike energy consuming aerobic treatment technologies, AD leads to the formation of biogas that can be used as a renewable energy source, and a nutrient-rich digestate, that can be used as a fertilizer (Holm-Nielsen et al., 2009; Appels et al., 2011).

The microbial community completing the AD processes has a high complexity in terms of functionality and community diversity, and several attempts already have been carried out to determine the key microbial populations (Vanwonterghem et al., 2014). This resulted in the well-known AD food web, consisting of 4 steps, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis, with each step taken care of by specific micro-organisms (Angenent et al., 2004a; Vanwonterghem et al., 2014). The first three steps are performed by bacteria, while the methanogenesis is completed by a specific branch of archaea. Methanogenesis can take place via the hydrogenotrophic or acetoclastic pathway. Stable and continuous methane production, starting from complex organic substrates, requires an accurate and close interaction between the micro-organisms carrying out the different steps (McInerney et al., 2009; Stams & Plugge, 2009). These interactions can take place through successive metabolite or (in)direct electron transfer (McInerney et al., 2009; Stams & Plugge, 2009; Rotaru et al., 2014). The specific case, in which the partnership between two micro-organisms becomes necessary to maintain the metabolic activity that they are unable to achieve on their own, due to energy limitations, is called syntrophy (Schink, 1997; Schink & Stams, 2013).

Syntrophic interactions, however, require specific partners to perform particular processes. For instance, syntrophic propionate oxidation, one of the crucial steps in AD to avoid acidification, can be carried out by specific species of the Syntrophobacterales order and the Peptococcaceae family, in close interaction with hydrogenotrophic methanogens (de Bok et al., 2004; Gallert & Winter, 2008; McInerney et al., 2008; Stams & Plugge, 2009; Muller et al., 2010). Syntrophic acetate oxidation also requires partnership of specific bacteria, of which most are representatives of the Clostridia class, with hydrogenotrophic methanogens

(Schnurer et al., 1996; Hattori et al., 2000; Westerholm et al., 2011b). The presence or absence of specific microbial taxa, as well as the occurrence of certain (syntrophic) pathways, depends on several factors. First, the substrate composition determines to a great extent the microbial community composition and organization. Indeed, it not only defines reactor conditions, but also provides the introduction of new species that are present in the substrate matrix, as, for instance, is the case for manure and waste activated sludge (Sundberg et al., 2013; Zhang et al., 2014b). For example, nitrogen-rich substrates lead to high total ammonia concentrations, which in several cases initiated a shift from acetoclastic methanogenesis to syntrophic acetate oxidation, coupled to hydrogenotrophic methanogenesis (Schnurer et al., 1999; Karakashev et al., 2006; Schnurer & Nordberg, 2008; Sundberg et al., 2013). Second, the feeding pattern of the substrate may also influence the microbial community (Xing et al., 1997; Conklin et al., 2006; De Vrieze et al., 2013b). Finally, other operational parameters of the digester, such as temperature, organic loading rate, sludge retention time, and reactor configuration, also determine the microbial community to a large extent (Leitao et al., 2005; Carballa et al., 2011).

This high degree of potential variables makes it difficult to determine the main selecting factors for microbial community composition and organization. Evaluation of the microbial community of in total 51 full-scale AD plants, and 28 full-scale aerobic wastewater treatment plants led to the identification of a core microbial community in both cases. However, no clear significant correlation with operational parameters or plant design could be determined (Leclerc et al., 2004; Riviere et al., 2009; Mielczarek et al., 2012; Mielczarek et al., 2013).

In this study, an extensive molecular analysis by means of 16S rRNA amplicon sequencing and real-time PCR was carried out on the microbial community of 38 samples from 29 full-scale AD plants. It was hypothesized that differences in operational parameters might lead to particular configurations of microbial communities in full-scale AD installations. Potential clustering of the samples was investigated, and environmental and operational parameters driving the overall microbial community composition and organization were identified.

2. Materials and methods

2.1. Sample and data collection

In total, 38 samples were collected from 29 different full-scale AD installations. Samples of at least 1 litre and up to 10 litres were taken directly from the reactor suspension, and transferred

to the laboratory in air-tight recipients, upon which a direct measurement of the pH was carried out. An aliquot of 50 mL was taken for total ammonia, conductivity, volatile solids and total solids analysis, and stored at 4 °C until further analysis. An aliquot of 10 mL was taken for volatile fatty acids and microbial community analysis and stored directly at -20 °C, prior to analysis. Samples were shaken manually before the aliquots were taken. Information concerning the organic loading rate, sludge retention time, biogas production and composition, temperature, reactor type and volume, and influent stream composition of the different digesters was obtained directly from the plant operator.

2.2. 16S rRNA gene amplicon sequencing

Total DNA extraction from the digestate samples was carried out by means of the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), following the manufacturer's instructions. Prior to extraction, the samples were thawed, and homogenized by means of a Vortex-Genie® 2T (Scientiis International, Baltimore, MD, USA) at maximum speed for 1 minute, after which 200 mg of sample was taken for DNA extraction. Total DNA concentration was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands). The quality of the extracted DNA was evaluated by electrophoresis on a 1% agarose gel, and by measuring the absorbance ratios at 260 nm and 280 nm by means of the Nanodrop.

PCR amplification of the variable region 4 (V4) of the universal 16S rRNA genes was carried out, following the protocol of Caporaso et al. (2012). The PCR reaction mixture (25 µL) contained 10 ng of template DNA, 2.5 µL 10x buffer Platinum® High Fidelity, 2 µL dNTP mix (5 µM each), 0.75 µL 50 mM $MgCl_2$, 0.1 µL Platinum® Taq DNA Polymerase High Fidelity (0.5 U μL^{-1}), 0.5 µL BSA (0.2 mg mL^{-1}) and 0.5 µL (10 µM) of each of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') modified on the 5' end with the sequences complementary to the Illumina specific adaptors. The reverse primer was modified with a 12 nucleotide (nt) Golay unique error-correcting barcode. The first PCR run with non-modified primers consisted of an initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 20 sec, annealing at 50 °C for 30 sec, and extension at 68 °C for 30 sec, followed by a final extension at 68 °C for 5 min. Each sample was run in triplicate, after which the triplicates were pooled and quality checked on a 2% agarose gel. In the second PCR run 2.5 µL of the first PCR run served as template. The same PCR protocol was used, yet,

with only 7 cycles, using PCR primers with attached barcodes and sequencing adaptors. The PCR products were purified with the MinElute PCR Purification Kit (Qiagen) and quantified with the PicoGreen dsDNA reagent and kit (Invitrogen). The amplicons were sequenced in an Illumina HiSeq 2000, producing 2 x 150 bp paired-end reads.

2.3. Bioinformatic analysis of amplicons

The paired-end reads were merged using PANDAseq v.2.0 (Masella et al., 2012), and sequencing noise was removed by discarding unique reads observed less than three times in all samples. Sequences were then reformatted for QIIME, using the custom script pandaseq.to.qiime.pl. QIIME v1.5.0 (Caporaso et al., 2010) was used for clustering (uclust), reference sequence picking, chimera removal (ChimeraSlayer), and finally taxonomic assignment (RDP Classifier) against a manually curated version of the GreenGenes taxonomy (midasfieldguide.org). A table containing the abundance of different operational taxonomic units (OTUs) and their taxonomic assignments in each sample was generated.

To estimate whether the number of samples per population was sufficient, species accumulation curves using species observed and the Chao 1 species richness estimator, were used (Chao, 1984; Chao, 1987). Alpha rarefaction was performed using the Chao1 and observed species metrics. Beta diversity was investigated using principal coordinates analysis (PCoA), which was applied (in Mothur) to reduce the dimensionality of the weighted and unweighted UniFrac distances matrices implemented in QIIME (Caporaso et al., 2010). Analysis of molecular variance (AMOVA) was applied to test if the spatial separation of the defined groups visualized in the PCoA plot was statistically significant. Statistical differences of specific operational parameters and bacterial orders between the groups visualized in the PCoA plot were evaluated by means of the Kruskal-Wallis Rank Sum test, followed by a Mann-Whitney U test with Bonferroni-Holm correction, both carried out by means of TIBCO Spotfire S+ 8.2 (TIBCO Software Inc., Palo Alto, CA, USA). Differences were considered significant at $P < 0.05$. Relative abundances of OTUs were used to generate dendrograms and a heatmap, relating the similarity in community structure, and were generated using R packages phyloseq and vegan (Oksanen et al., 2012; McMurdie & Holmes, 2013). Correlations between bacterial groups and between methanogenic groups and functional data were determined by means of the two-tailed Spearman's Rank Order Correlation test, for which the statistical software SPSS Statistics (IBM SPSS Statistics, Version 22, IBM Corp., Armonk, NY, USA) was used.

2.4. Real-time PCR analysis

A StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used for real-time PCR (qPCR) triplicate analysis of samples of a 100-fold dilution of the DNA-samples for the methanogenic populations Methanobacteriales, Methanomicrobiales, Methanosarcinaceae, and Methanosaetaceae. The primer sets used were previously described (Yu et al., 2005). The reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, WIS, USA), and consisted of 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water, 0.75 µL of each primer (final concentration of 375 nM), and 5µL of template DNA. A two-step thermal cycling procedure, which consisted of a predenaturation step of 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C was used to quantify the Methanobacteriales, Methanosarcinaceae and Methanosaetaceae. An identical program was used for the Methanomicrobiales, yet with an annealing temperature of 63 °C. The qPCR results were presented as copies per gram of wet sludge.

2.5. Analytical techniques

Analysis of total ammonia (NH_4^+ + NH_3), volatile solids and total solids was carried following the Standard Methods (Greenberg et al., 1992). Free ammonia (NH_3) was calculated based on total ammonia, pH and temperature values. The volatile fatty acid concentrations were analysed by means of gas chromatography (GC-2014, Shimadzu®, The Netherlands), equipped with a DB-FFAP 123-3232 column (30 m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector. Digestate samples were conditioned with sulphuric acid and sodium chloride, and as internal standard 2-methyl hexanoic acid was used for quantification of further extraction with diethyl ether. The ether extract (1 µL) was injected at 200 ºC with a split ratio of 60 and a purge flow of 3 mL min^{-1}. The oven temperature increased by 6 ºC min^{-1} from 110 ºC to 165 ºC, where it was kept for 2 min. The flame ionization detector had a temperature of 220 ºC. Nitrogen gas was used as carrier gas at a flow rate of 2.49 mL min^{-1}. The pH was measured with a C532 pH meter (Consort, Turnhout, Belgium), and conductivity was determined using a C833 conductivity meter (Consort, Turnhout, Belgium).

2.6. Data deposition

The reported sequences have been deposited in the GenBank database (study no. PRJEB6324).

3. Results

3.1. Performance and operating condition data

In total, 38 samples were collected from 29 full-scale operational AD plants, during a steady state period of operation with constant biogas production through time. Despite their stable operation, a wide range in operational parameters could be observed between the different installations, with pH values ranging between 7.10 and 8.52, TAN (total ammonia nitrogen) between 128 and 6427 mg N L^{-1}, FA (free ammonia) between 2 and 1460 mg N L^{-1}, total VFA (volatile fatty acids) between 0 and 36.8 g COD L^{-1} (chemical oxygen demand), conductivity between 6 and 62 mS cm^{-1}, and biogas production between 1.1 and 12.0 m^3 m^{-3} d^{-1} (Table 8.1). Additionally, parameters that were not directly influenced by the substrate, i.e. parameters that were controlled by the operator, greatly varied between the different installations, with an organic loading rate between 1.5 and 11.0 kg COD m^{-3} d^{-1} and sludge retention time values between 18 and 124 days. Samples covered the temperature range from mesophilic (33 °C) to thermophilic (55 °C) conditions.

Table 8.1 *Overview of the operational parameters in the samples of the different full-scale anaerobic digestion plants. Samples with the same name and a different number originate from the same anaerobic digestion plant at a different time point. TAN = total ammonia, VFA = volatile fatty acids, OLR = organic loading rate, SRT = sludge retention time, FA = free ammonia, VS = volatile solids, TS = total solids, CSTR = continuous stirred tank reactor, UASB = upflow anaerobic sludge blanket, OBW = organic biological waste, OFMSW = organic fraction municipal solid waste, MSW = municipal solid waste, n.a. = data not available.*

Name	Type	Capacity	Substrate	pH	TAN	Biogas	Total VFA
		m^3			mg N L^{-1}	m^3 m^{-3} d^{-1}	mg COD L^{-1}
RESa	CSTR	n.a.	MSW	8.30	1150	< 2.5	429
RESb	CSTR	n.a.	MSW	8.50	2150	4-5.5	502
VCE1	CSTR	1500	Maize, manure	8.52	4647	5.6	5735
VCE2	CSTR	1500	Maize, manure	8.24	4282	5.6	0

DRZ1	CSTR	1000	Slaughterhouse waste	8.03	2428	7.5	11110
DRZ2	CSTR	1000	Slaughterhouse waste	8.03	2428	7.5	11110
WEE	CSTR	2500	Maize, manure	8.12	4032	4.8	557
BBy1	CSTR	3600	Manure, OBW, energy crops, slaughterhouse waste	7.52	3288	1.5	6610
BBy2	CSTR	3600	Manure, OBW, energy crops, slaughterhouse waste	7.52	3197	2.0	7609
GFTa	CSTR	n.a.	OFMSW	8.50	1690	5.5-7.0	130
GFTb	CSTR	n.a.	OFMSW	8.30	1450	2.5-4	3347
GFTc	CSTR	n.a.	OFMSW	8.20	2730	2.5-4	0
AGRa	CSTR	n.a.	Maize, manure	n.d.	1430	10-12	2369
AGRb	CSTR	n.a.	Maize, manure	n.d.	1430	10-12	49
AGRc	CSTR	n.a.	Maize, manure	n.d.	1430	10-12	n.d.
AGRd	CSTR	n.a.	Maize, manure	n.d.	2120	< 2.5	5693
Agri	CSTR	1000	Maize, fats, fruit waste	8.19	2904	5.7	912
Den	CSTR	3255	Sludge, manure	7.35	508	2.7	0
Oss1	CSTR	4000	Wastewater sludge	7.35	1077	2.0	0
Oss2	CSTR	4000	Wastewater sludge	7.48	953	2.0	0
Oss3	CSTR	4000	Wastewater sludge	7.43	950	2.0	0
SEH1	CSTR	1200	Maize, manure	8.00	3522	1.1	4688
SEH2	CSTR	1200	Maize, manure	8.06	3497	1.1	4467
BIF	CSTR	1250	Manure	8.05	4982	4.1	713
BIE1	CSTR	2000	Maize, manure	7.92	3123	7.4	8114
BIE2	CSTR	2000	Maize, manure	7.86	3280	7.4	7894
CAZ	CSTR	3000	Maize, manure	7.76	4986	5.7	36760
SMA	CSTR	3200	Maize, manure	8.25	6427	2.1	434
BCI1	CSTR	1500	OBW	8.02	4019	2.8	5593
BCI2	CSTR	1500	OBW	8.02	2684	2.8	22601
BCI3	CSTR	1500	OBW	8.02	4169	2.8	830
EcP	CSTR	n.a.	n.a.	8.30	3091	n.a.	0
SHA	CSTR	1500	OBW	8.10	3896	6.4	242
BAT	CSTR	n.d.	Manure, OBW	8.35	4639	n.d.	1096
Myd	UASB	n.a.	Potato wastewater	7.12	836	n.a.	325
Vst	UASB	274	Brewery wastewater	7.14	253	1.5	0

VPK	UASB	1210	Paper mill wastewater	7.19	222	1.2	251
Clar	UASB	n.a.	Potato wastewater	7.10	128	n.a.	0

Name	**OLR**	**SRT**	**Temperature**	**FA**	**Conductivity**	**VS**	**TS**
	kg COD m^{-3} d^{-1}	d	°C	mg N L^{-1}	mS cm^{-1}	g L^{-1}	g L^{-1}
RESa	< 5	23	47	378	n.a.	131	372
RESb	7.5 - 10	45	47	940	n.a.	157	623
VCE1	1.5	100	38	1460	29	77	127
VCE2	1.5	100	34	666	37	86	159
DRZ1	11.0	20	54	696	31	17	24
DRZ2	11.0	20	54	696	25	35	51
WEE	3.0	60	34	495	31	68	102
BBy1	4.5	40	54	363	32	28	42
BBy2	5.6	40	54	353	33	29	45
GFTa	12.5 - 15	22	50	816	n.a.	131	153
GFTb	7.5 - 10	25	52	579	n.a.	87	109
GFTc	4 5 - 7.5	38	50	870	n.a.	146	309
AGRa	12.5 - 15	124	55	n.a.	n.a.	138	319
AGRb	12.5 - 15	33	55	n.a.	n.a.	170	389
AGRc	12.5 - 15	23	55	n.a.	n.a.	119	418
AGRd	5 - 7.5	23	42	n.a.	n.a.	154	349
Agri	n.a.	40	34	410	38	48	133
Den	3.0	20	33	11	8	18	36
Oss1	3.0	18	34	25	8	21	44
Oss2	3.0	18	34	30	7	24	46
Oss3	3.0	18	34	26	6	23	43
SEH1	4.0	40	34	338	40	33	58
SEH2	4.0	40	34	380	40	33	59
BIF	2.5	60	34	530	42	68	103
BIE1	4.0	30	54	743	39	67	109
BIE2	4.0	30	54	701	38	68	109
CAZ	5.0	40	34	287	25	50	74
SMA	4.0	40	34	1020	62	63	112
BCI1	3.0	80	34	402	32	16	77
BCI2	3.0	80	34	268	32	24	44

BCI3	3.0	80	34	417	32	23	36
EcP	n.a.	n.a.	34	540	39	13	21
SHA	2.5	60	34	459	32	79	124
BAT	n.a.	n.a.	34	890	62	55	95
Myd	n.a.	n.a.	34	12	8	20	30
Vst	3.3	n.a.	34	4	6	48	57
VPK	5.6	n.a.	35	4	7	85	165
Clar	n.a.	n.a.	34	2	9	43	54

3.2. Bacterial community composition

An average of 39682 ± 17441 reads was obtained per sample (in total about 1.5 million reads), resulting in a total of 3640 OTUs. This led to the identification of 15 different phyla, 23 classes and 36 orders, present at > 1% in at least one of the samples (Figure 8.1, 8.2 and 8.3). Rarefaction curves were generated to estimate the coverage of the microbial community in the samples by the created dataset (Figure 8.4). An average species richness value of 1711 ± 538 was observed, with values ranging between 686 and 3250 (Figure 8.4 and 8.5).

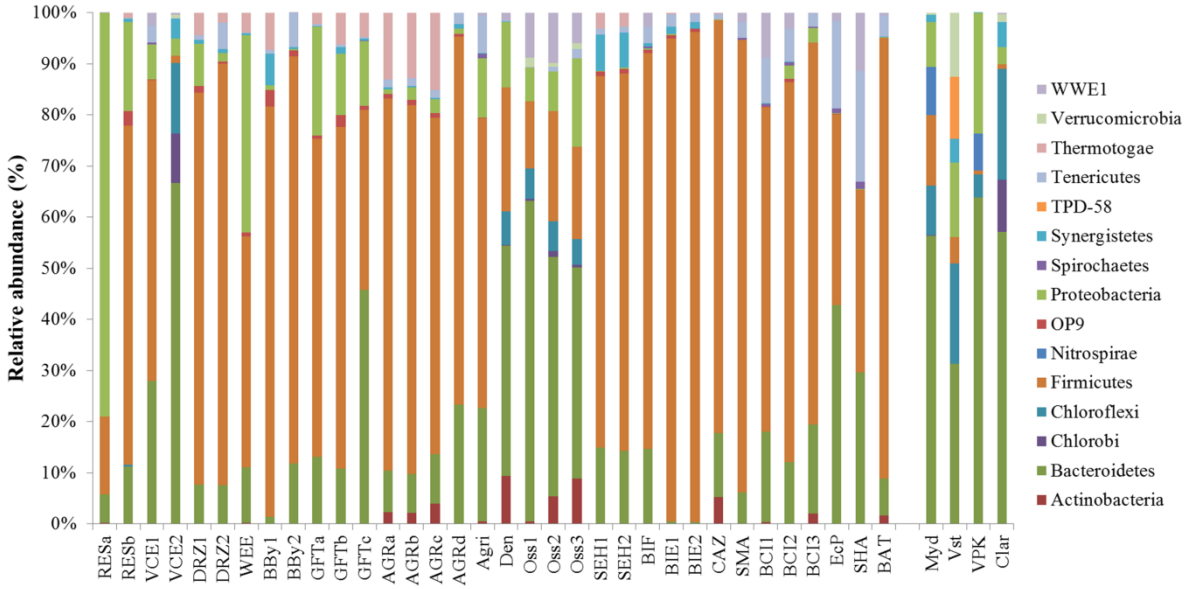

Figure 8.1 *Relative abundances of the bacterial community in the different samples at phylum level, normalized to 100%. Only those phyla that were present at an abundance > 1% in at least one sample were considered.*

The dominant bacterial populations belonged to the Firmicutes, Bacteroidetes and Proteobacteria phyla (Figure 8.1 and 8.6). In total, averaged over all samples, 86.1 ± 10.7% of all sequences belonged to these 3 phyla. In all samples, one of these phyla was the most

abundant, with the Firmicutes phylum being the dominant phylum in most cases (26 samples), followed by the Bacteroidetes (11 samples) and Proteobacteria (1 sample). Despite the overall dominance of these three phyla, a high degree of variation can be considered in relative abundance of these three phyla between the different samples. Samples from the same plant at different time points (at least two months between sampling) were more similar than samples from other plants, with the exception of the VCE plant, indicating a stable community over time during steady state operation.

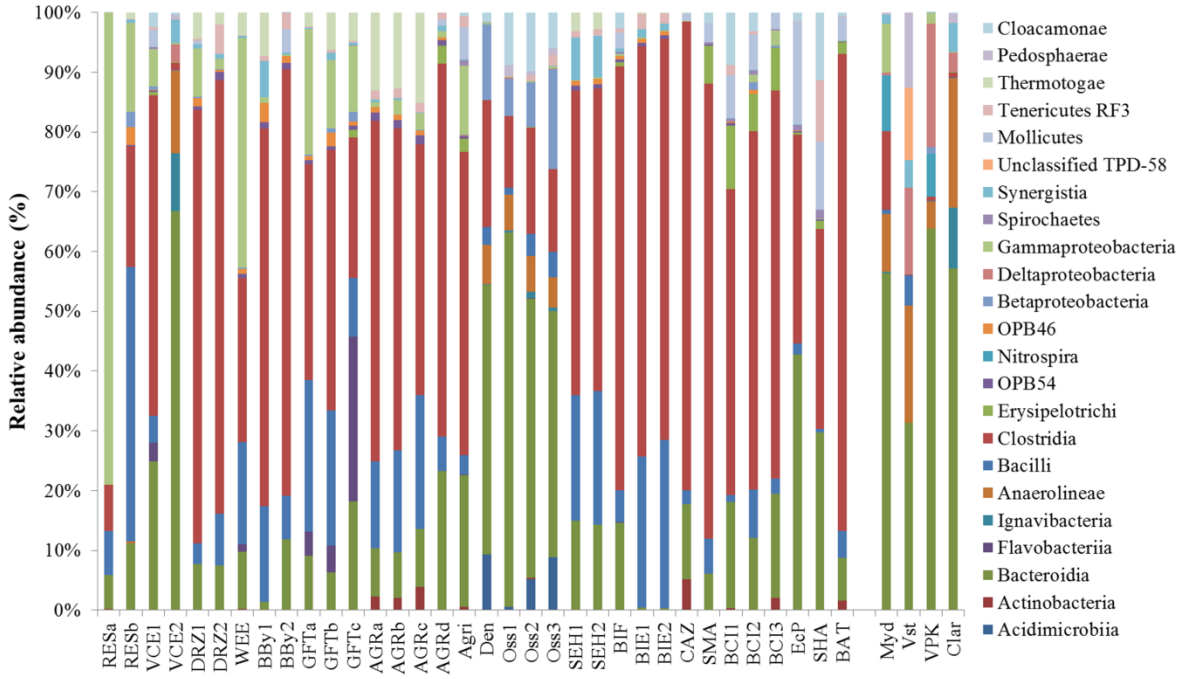

Figure 8.2 *Relative abundances of the bacterial community in the different samples at class level, normalized to 100%. Only those classes that were present at an abundance > 1% in at least one sample were considered.*

The observed taxa were also conserved at lower taxonomic levels. Within the Firmicutes phylum, the Bacilli and Clostridia were the main classes that contained on average 97.5 ± 3.6% of all sequences. A similar dominance of the Bacteroidia class was observed in the Bacteroidetes phylum (95.9 ± 12.7%) (Figure 8.2). The Clostridiales, Bacillales, Lactobacillales and MBA08 orders covered on average 92.2 ± 7.2% of all sequences in the Firmicutes phylum, while 96.0 ± 12.7% of the sequences from Bacteroidetes phylum belonged to the Bacteroidales order (Figure 8.3 and 8.6). No class or order consistently dominated the sequences from the Proteobacteria phylum, as there was a high level of variation between the different samples (Figure 8.2 and 8.3). The Clostridiales and Bacteroidales order were detected in every sample, with a relative abundance between 75.9 and 0.1% for the Clostridiales, and between 66.8 and 0.3% of all sequences for the

Bacteroidales (Figure 8.6). Hence, these orders can be considered as part of the core microbiome, as they are present ≥ 0.1% relative abundance in each sample. Additionally, a number of taxa were non-core, but frequently observed. The Lactobacillales order was present in all samples, with the exception of the VPK plant, at relative abundances between 27.7 and 0.1%. The Bacillales, MBA08, Pseudomonadales, Synergistales and ML615J-28 orders were detected in at least 75% of all samples.

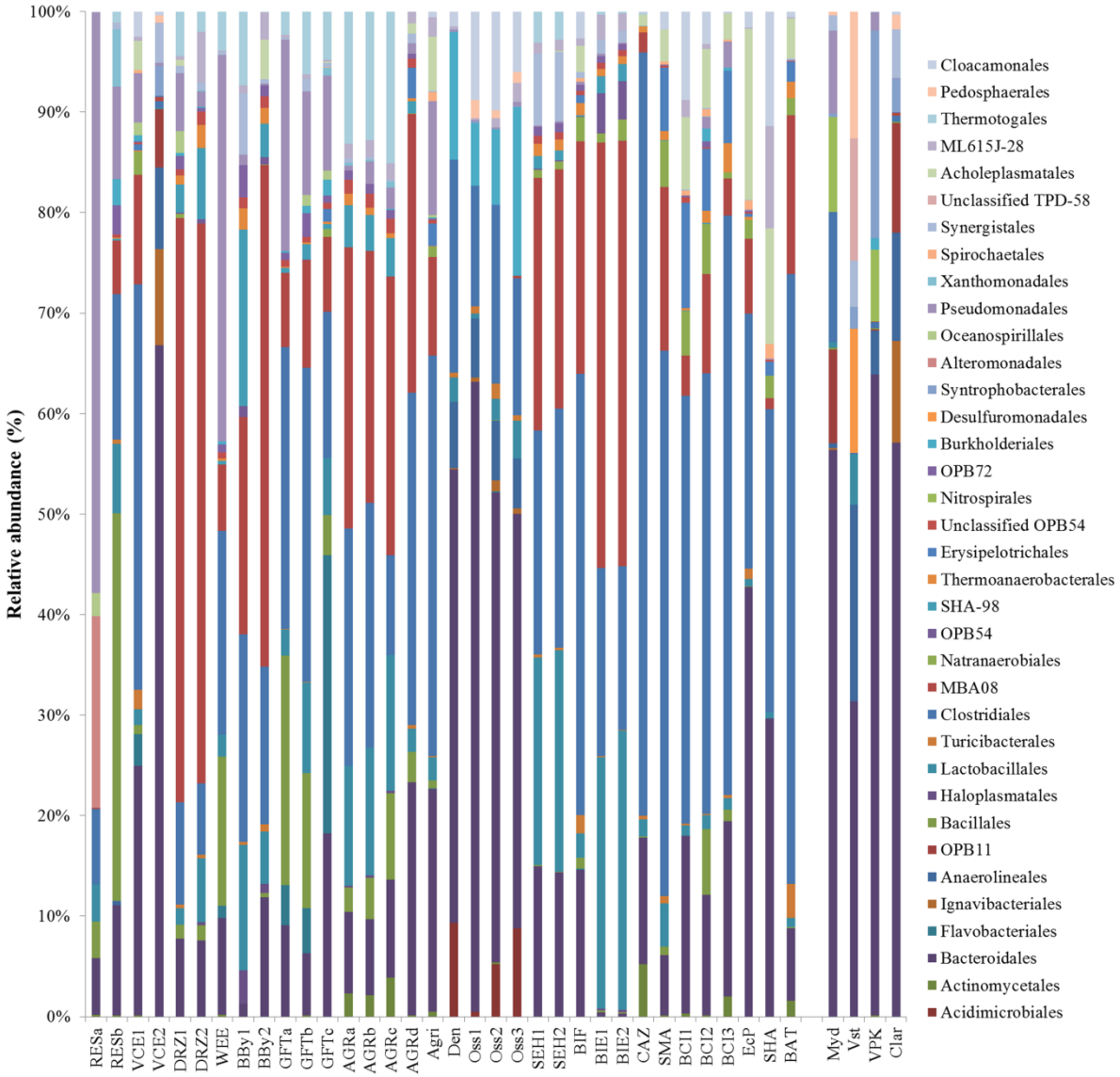

Figure 8.3 *Relative abundances of the bacterial community in the different samples at order level, normalized to 100%. Only those orders that were present at an abundance > 1% in at least one sample were considered.*

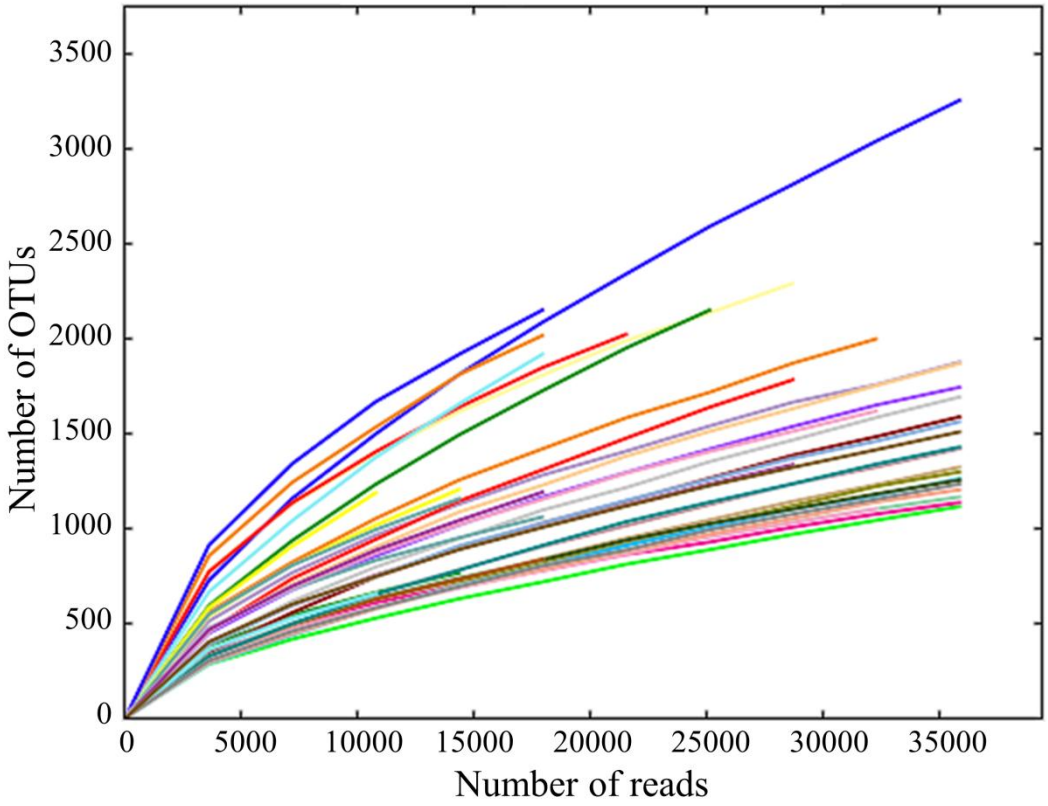

Figure 8.4 *Rarefaction curves portraying the number of OTUs against sampling depth of each of the 38 samples.*

3.3. Archaeal community composition

The methanogenic community was not covered by the primers used for amplicon sequencing, and the populations were evaluated by means of real-time PCR targeting the 16S rRNA genes of the Methanosaetaceae, Methanosarcinaceae, Methanobacteriales and Methanomicrobiales (Figure 8.7 and 8.8). Methanosaetaceae were present in all samples, with values ranging from $2.6 \times 10^{6} \pm 2.2 \times 10^{5}$ to $1.5 \times 10^{10} \pm 3.3 \times 10^{9}$ copies g^{-1} sludge. In contrast, the Methanosarcinaceae were only detected in 21 samples (Figure 8.7). Both the hydrogenotrophic methanogenic orders Methanobacteriales and Methanomicrobiales were present in all samples, with the exception of the RESb sample, in which no Methanomicrobiales were detected (Figure 8.8). Total abundances between $1.0 \times 10^{7} \pm 2.3 \times 10^{6}$ and $1.5 \times 10^{11} \pm 2.4 \times 10^{8}$ copies g^{-1} for the Methanobacteriales and $7.8 \times 10^{5} \pm 2.0 \times 10^{5}$ and $1.8 \times 10^{10} \pm 2.1 \times 10^{9}$ copies g^{-1} for the Methanomicrobiales, were observed. Only limited variation was observed between samples from the same AD plant, with the exception of the

VCE plant, indicating that the methanogenic community in a given plant is stable through time.

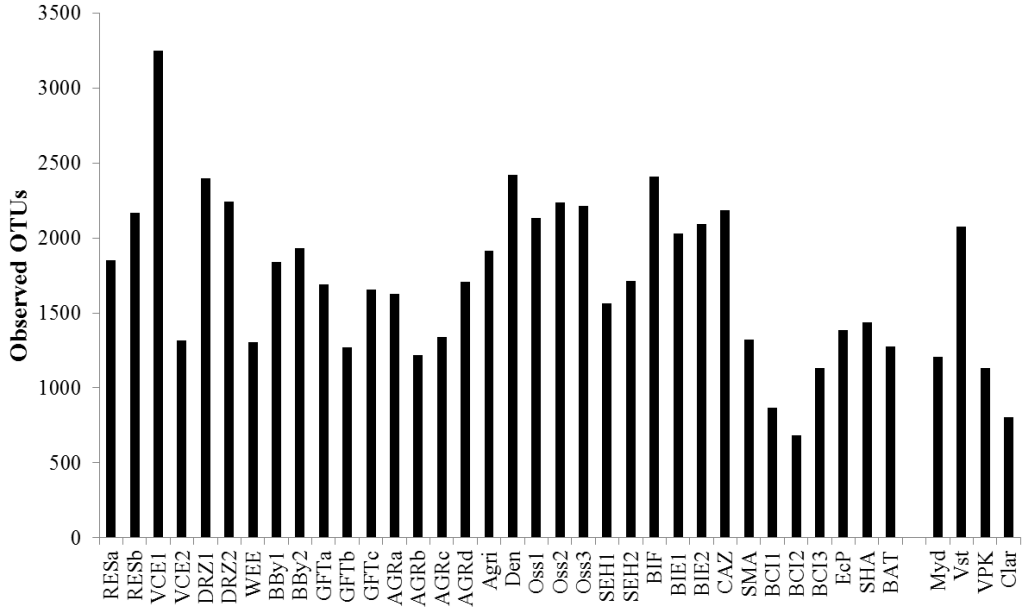

Figure 8.5 *Observed species richness.*

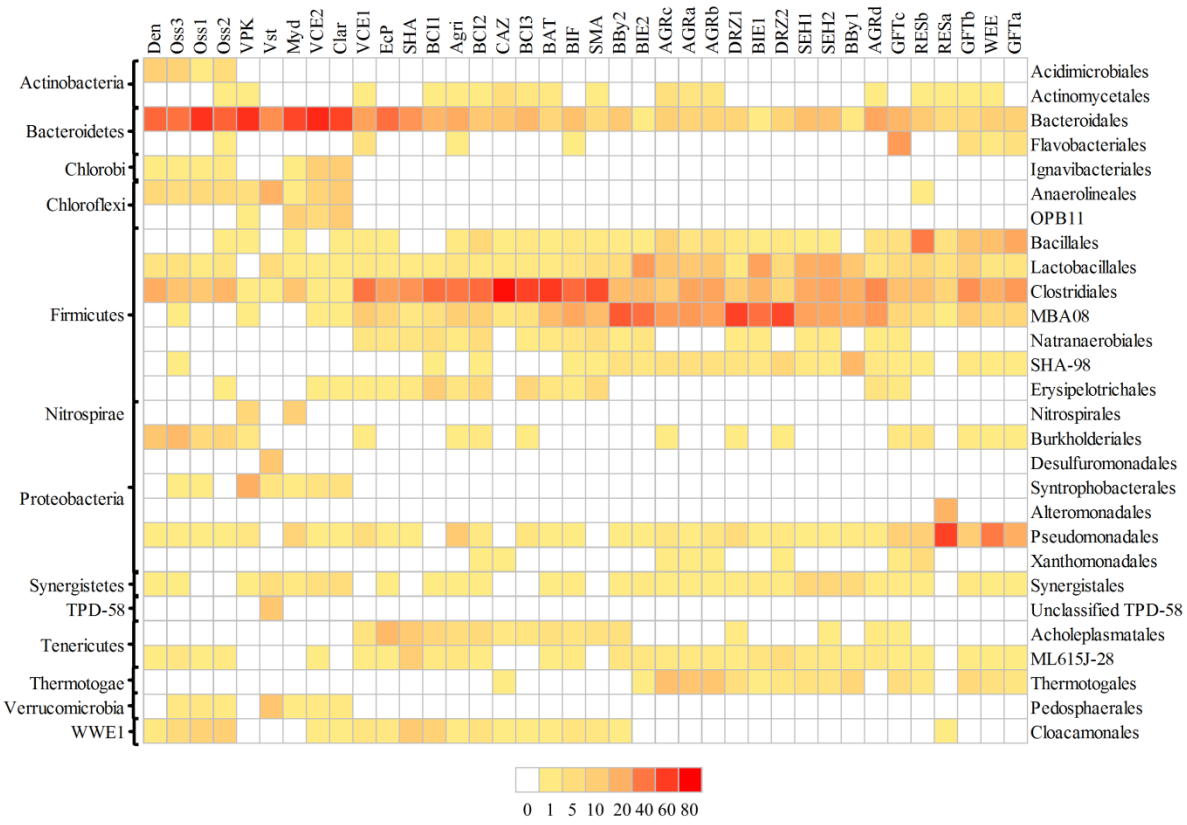

Figure 8.6 *Heatmap representing all orders present at a relative abundance > 5% of total reads in at least one of the samples. The colour scale ranges from 0 to 80% relative abundance. Taxonomy is shown at the phylum level (left column) and at the order level (right column).*

3.4. Correlation within the microbial community and between the microbial community and operational data

Correlation analysis of the amplicon sequencing data revealed both positive and negative correlations between the 36 orders that were present in the different samples (Figure 8.9). An overall negative correlation was observed between the Bacteroidales order and the orders belonging to the Firmicutes phylum, with a strong negative correlation ($P < 0.01$) between the Bacteroidales and the Lactobacillales. The Acidimicrobiales, Ignavibacteriales, Anaerolineales, Syntrophobacterales and Pedosphaerales orders were negatively correlated to most Firmicutes orders. Within the Firmicutes phylum most orders were positively correlated to each other.

The acetoclastic methanogenic families Methanosaetaceae and Methanosarcinaceae, that were strongly positively correlated ($P < 0.01$), showed a similar trend in their correlation to operational data, despite their considerable difference in absolute abundance (Figure 8.10). Significant negative correlations were observed, for both families, with total ammonia ($P < 0.01$), volatile fatty acids and conductivity ($P < 0.01$ for Methanosaetaceae and $P < 0.05$ for Methanosarcinaceae). The Methanosaetaceae were also negatively correlated to the sludge retention time ($P < 0.01$) and free ammonia concentration ($P < 0.05$). In relation to the hydrogenotrophic methanogenic orders, Methanosaetaceae ($P < 0.01$) and Methanosarcinaceae ($P < 0.05$) were positively correlated to the Methanobacteriales, whereas only Methanosarcinaceae were negatively correlated ($P < 0.05$) to the Methanomicrobiales. The Methanobacteriales order was the only methanogenic group significantly positively correlated to biogas production ($P < 0.05$), organic loading rate ($P < 0.01$) and temperature ($P < 0.01$). The Methanomicrobiales showed an overall negative correlation pattern to most operational data.

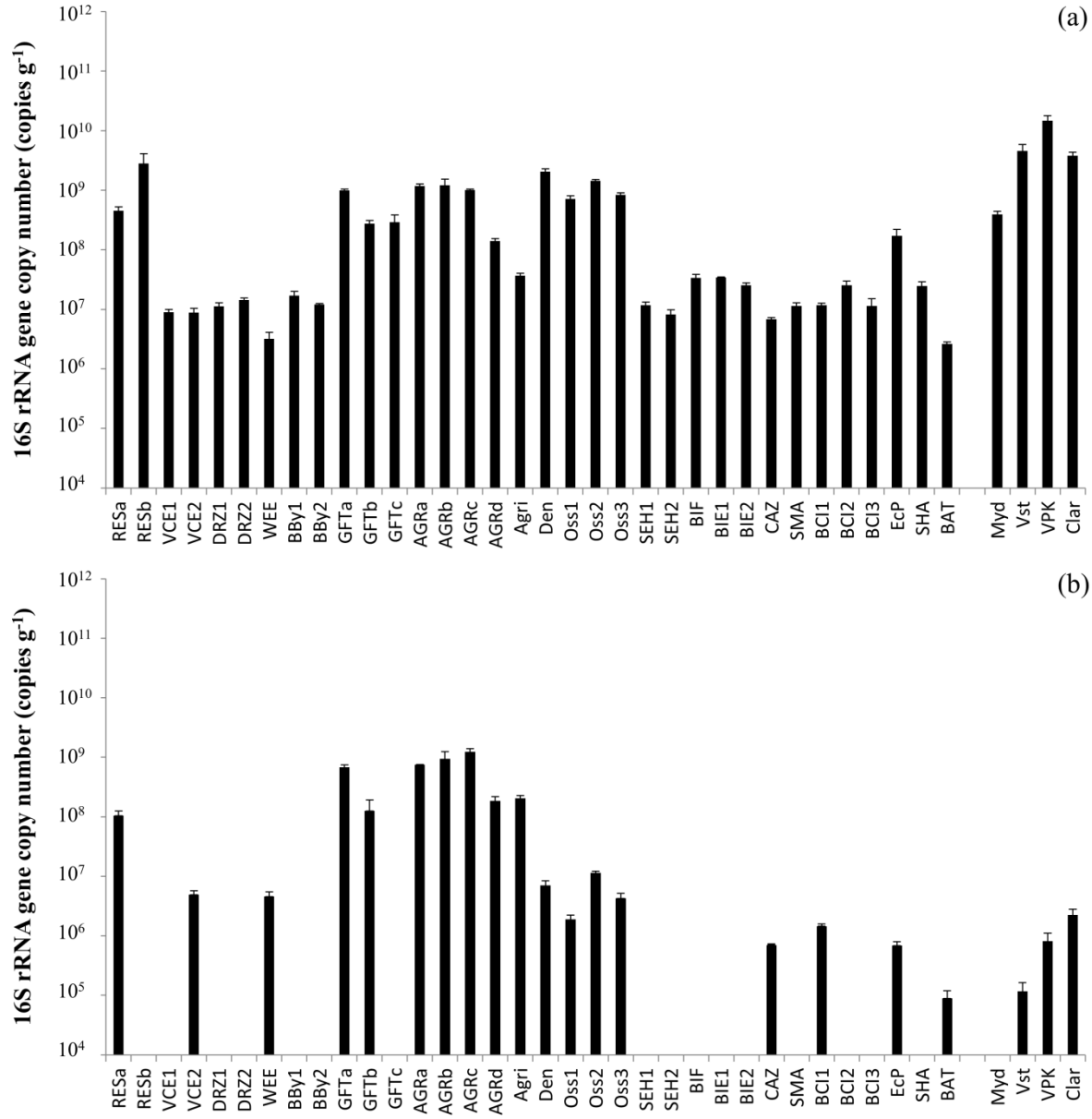

Figure 8.7 *Real-time PCR results of the Methanosaetaceae (a) and Methanosarcinaceae (b) in all 38 samples, expressed as copies of the target 16S rRNA gene per gram of wet sludge. Average values of the triplicate analyses, together with the standard deviations are presented.*

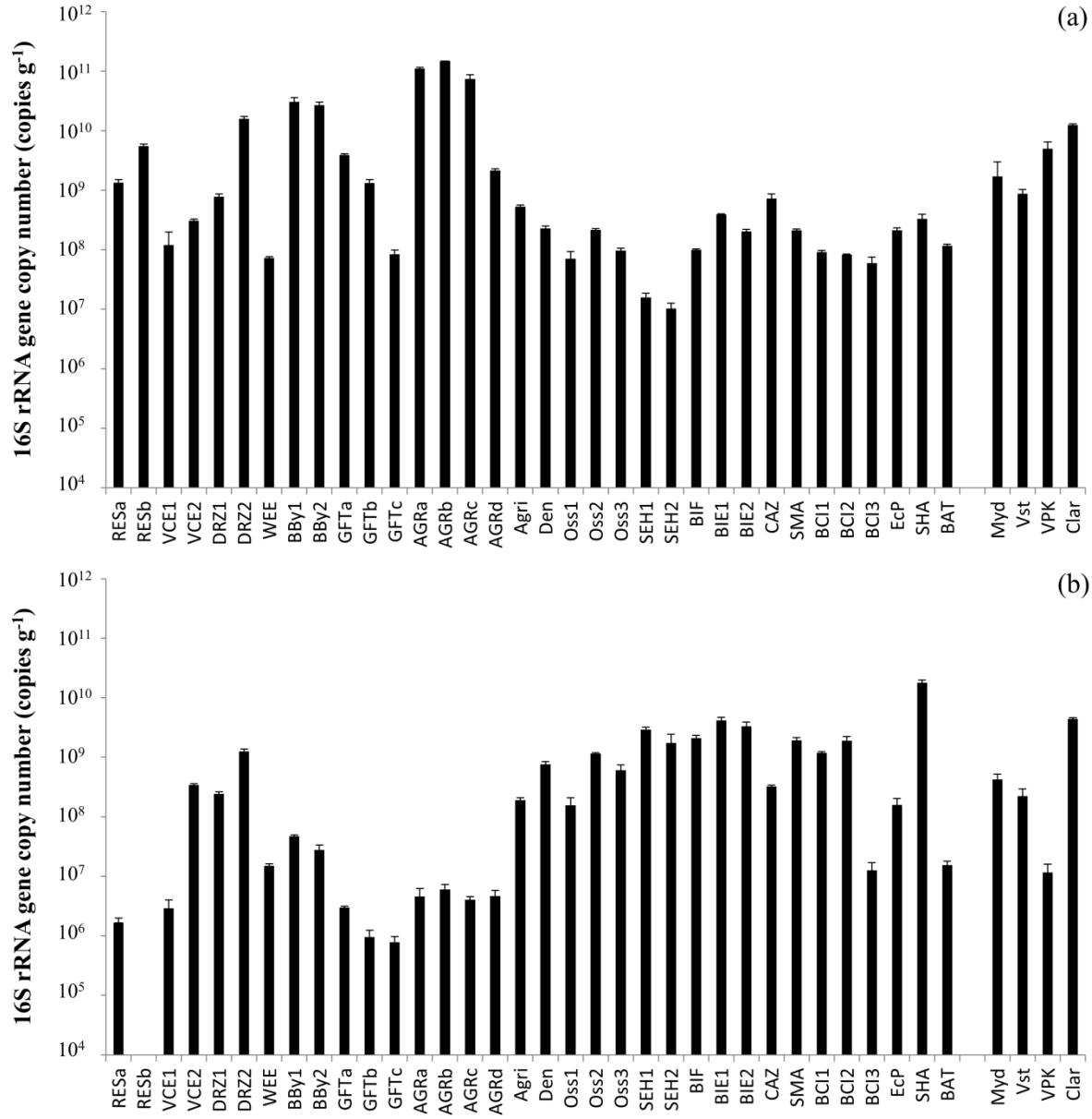

Figure 8.8 *Real-time PCR results of the Methanobacteriales (a) and Methanomicrobiales (b) in all 38 samples, expressed as copies of the target 16S rRNA gene per gram of wet sludge. Average values of the triplicate analyses, together with the standard deviations are presented.*

3.5. Bacterial clustering analysis

Three potential clusters were visualized by means of principal coordinates analysis (PCoA) analysis (Figure 8.11). The statistical significance of this clustering was verified with AMOVA, which confirmed a significant difference in composition between the three groups (PC1 scores: $P < 0.001$, PC2 scores: $P < 0.001$). Cluster 1 contained 8 samples, mainly from mesophilic sludge digesters and UASB (upflow anaerobic sludge bed) reactors, with the VCE2 sample as exception. Cluster 2 consisted of 11 samples, exclusively originating from

mesophilic digesters, whereas Cluster 3 comprised thermophilic samples, yet, the mesophilic WEE and SHE plants also belonged to this cluster. All samples from the same digester at a different time point could be found in the same group, with the exception of the VCE plant.

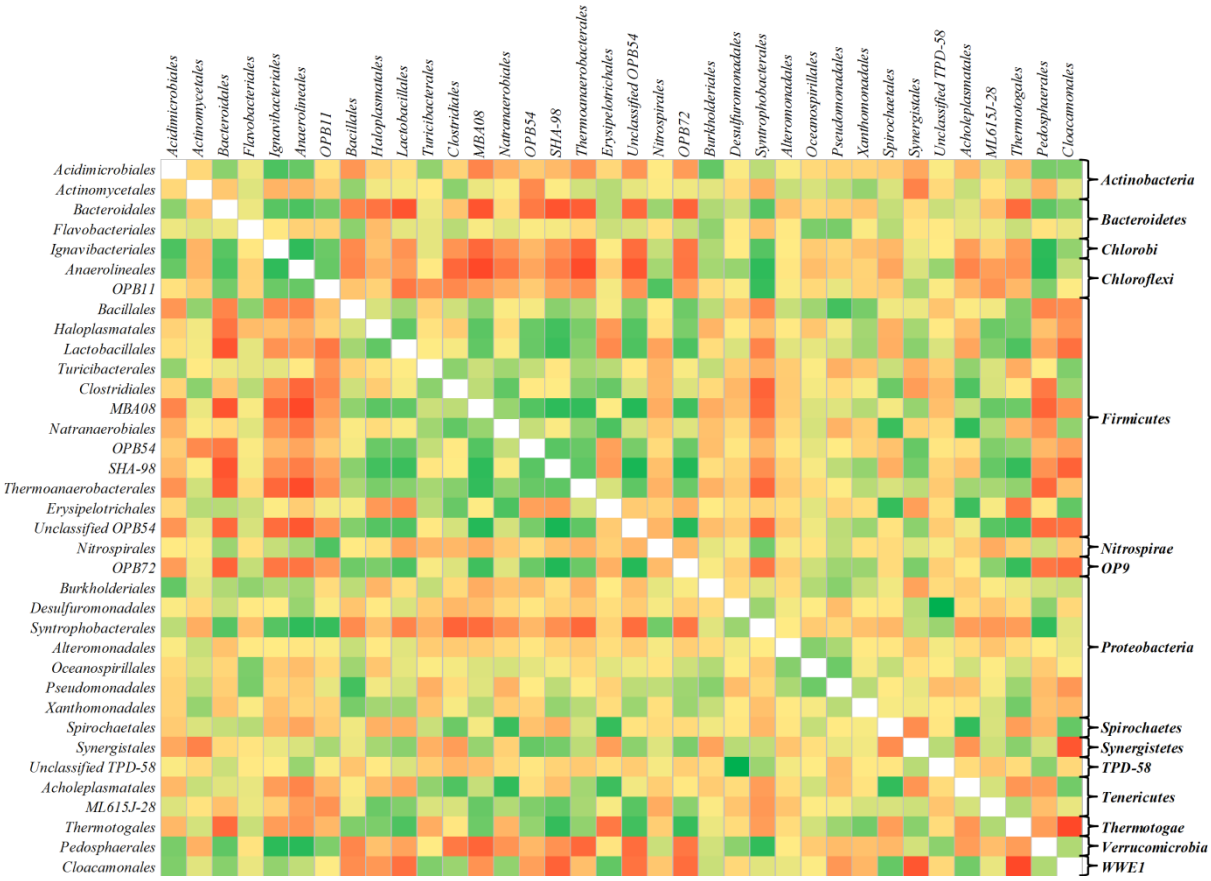

Figure 8.9 *Correlation matrix between the different bacterial orders. Both positive correlations (green) and negative correlations (red) are represented. Correlations were determined by means of the two-tailed Spearman's Rank Order Correlation test. Taxonomy is shown at the order level (left column) and at the phylum level (right column).*

These 3 potential clusters were characterized by the variation in relative abundance of three specific highly abundant bacterial orders (Figure 8.12). The relative abundance of the Bacteroidales order was significantly higher in cluster 1 ($P < 0.001$) compared to cluster 2 and 3. The Clostridiales order reached significant higher relative abundance values in cluster 2 ($P < 0.001$), whereas the Lactobacillales order was the determining factor in cluster 3, compared to cluster 1 and 2 ($P < 0.01$).

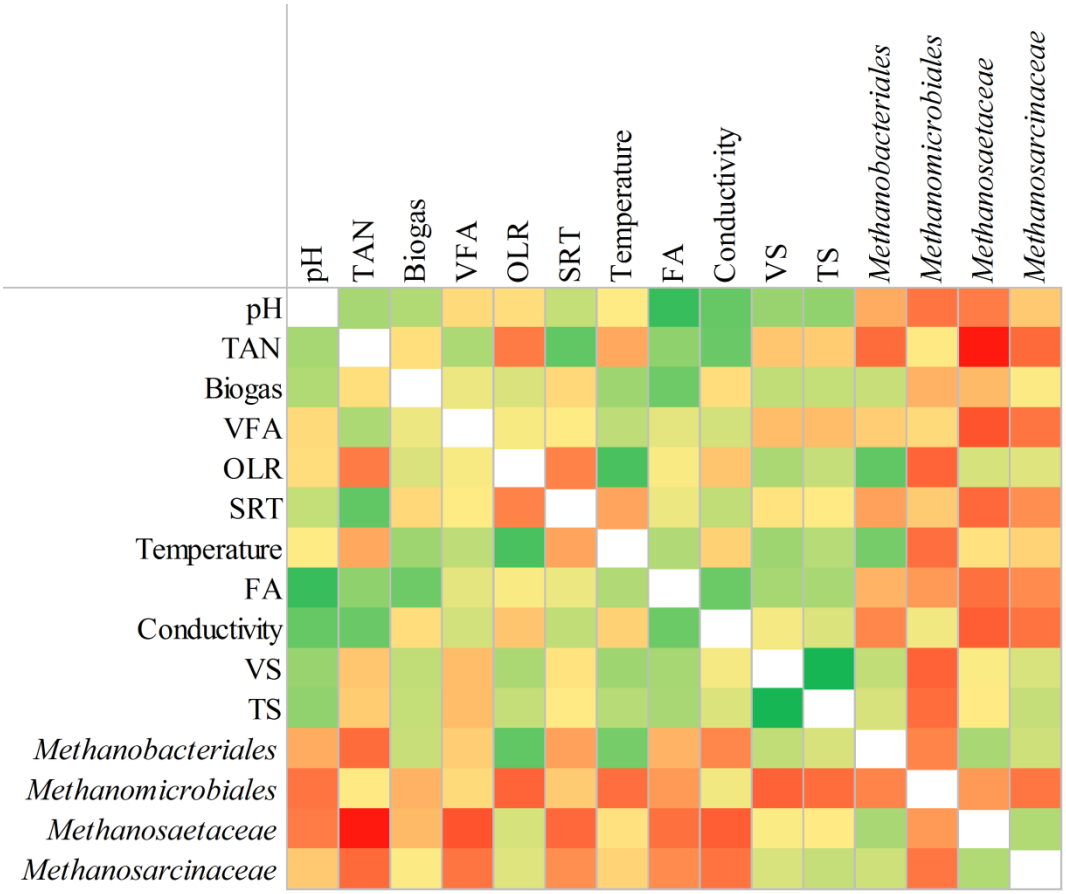

Figure 8.10 *Correlation matrix between operational data and the different methanogenic groups, expressed as log copies g-1 wet sludge. Both positive correlations (green) and negative correlations (red) are represented. Correlations were determined by means of the two-tailed Spearman's Rank Order Correlation statistic.*

The operational parameters determining the different clusters were identified. Total ammonia concentration was the only operational parameter that was significantly different ($P < 0.01$) between the 3 clusters (Figure 8.13). The highest total ammonia concentrations were present in the samples from cluster 2, whereas the lowest concentrations were observed in the samples belonging to AD-type 1. cluster 1 could be distinguished from the 2 other clusters, based on the parameters pH, volatile fatty acids, conductivity and free ammonia, with significantly ($P < 0.01$) higher values in the samples belonging to cluster 2 and 3, compared to cluster 1 (Figure 8.14). Cluster 3 showed significantly higher ($P < 0.01$) values in comparison to cluster 1 and 2 in terms of the organic loading rate and reactor temperature. Surprisingly, no significant difference in biogas production could be observed between the different clusters. No significant differences in methanogenic groups could be observed between the different clusters, with the exception of the Methanosaetaceae family that reached significant ($P < 0.01$) higher values in cluster 1 compared to cluster 2.

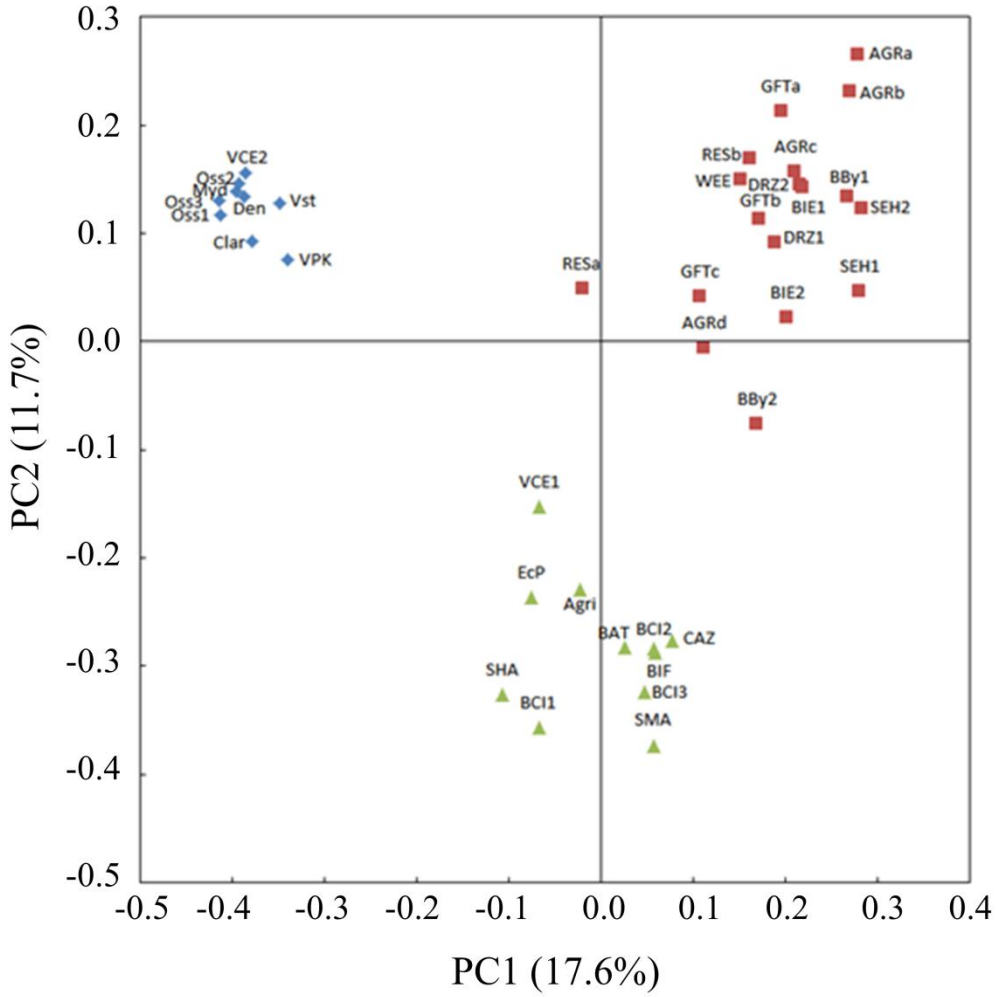

Figure 8.11 *Unweighted UniFrac principal coordinates analysis (PCoA) showing the microbial community composition at the OTU level. PCoA was carried out on all samples, resulting in three clusters: Cluster 1 (♦), Cluster 2 (▲) and Cluster 3 (■).*

4. Discussion

In this research an in-depth evaluation of the microbial community of 38 samples from 29 full-scale AD installations was carried out to correlate microbial community composition and organization to operational data. Correlations were observed between specific microbial populations and operational parameters. Three potential clusters were distinguished and characterized.

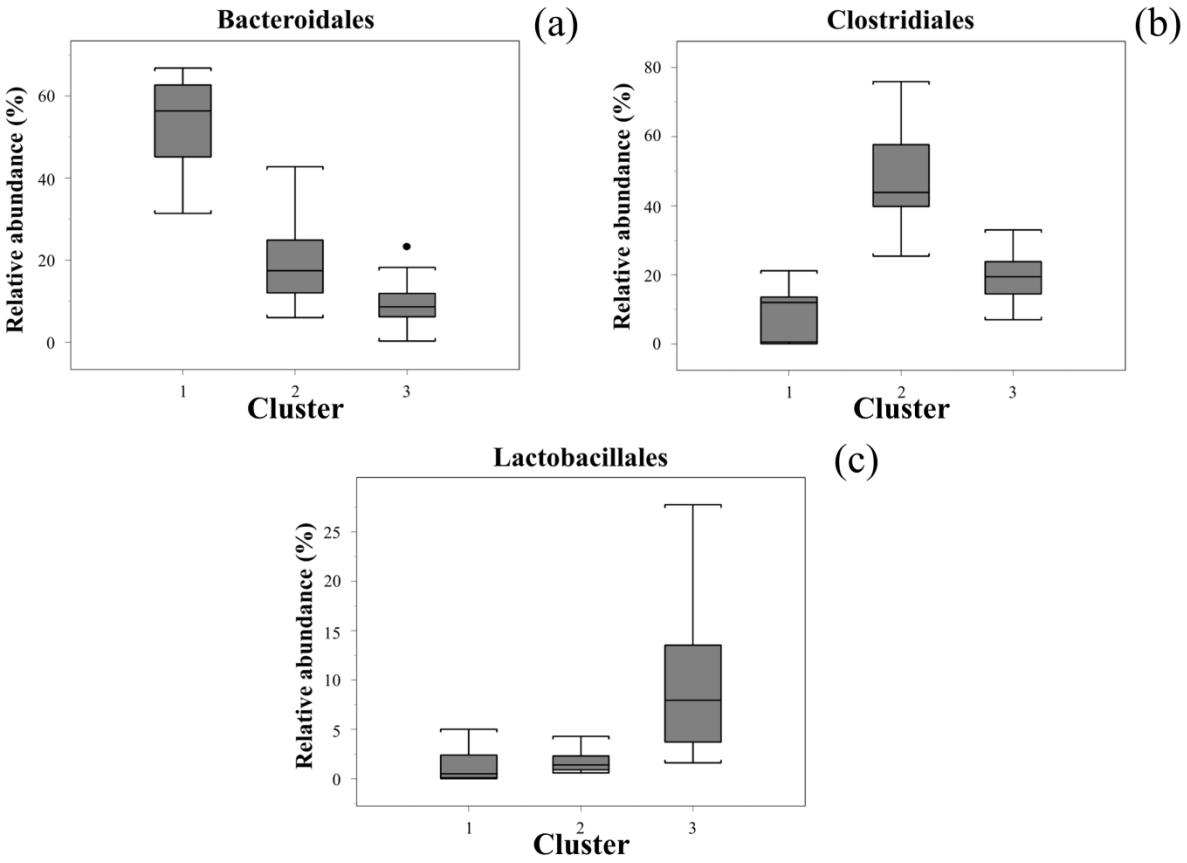

Figure 8.12 *Relative abundances of the main contributors of each* ***cluster*** *based on the 16S rRNA gene sequencing results.* ***Cluster*** *1 was characterized by an increased abundance of the Bacteroidales (a),* ***Cluster*** *2 by the Clostridiales order (b), and* ***Cluster*** *3 by the Lactobacillales order (c).*

The Firmicutes, Bacteroidetes and Proteobacteria phyla dominated the bacterial community in all full-scale AD plants, yet a high degree of variation in the relative abundance of each phylum was observed between the samples. At a first glance, this could be attributed to the difference in substrate, as it has been reported that substrate composition determines the AD microbiome (Neumann & Scherer, 2011; Zhang et al., 2014b). However, in-depth evaluation of the microbial community in full-scale plants treating manure and brewery wastewater, revealed an equal high level of community diversity between the different reactors, despite a similar substrate, thus indicating that other aspects also contribute to microbiome diversity in AD (Werner et al., 2011; St-Pierre & Wright, 2014). The dominance of the Firmicutes phylum in 26 samples was likely due to these organisms holding a crucial position in several steps of the AD process. Several Firmicutes species possess hydrolytic activity for lipids, proteins and polymeric carbohydrates, while other species are able to perform syntrophic propionate and butyrate oxidation (Lynd et al., 2002; Riviere et al., 2009; Nelson et al., 2011; Sundberg et al., 2013; Vanwonterghem et al., 2014). The Bacteroidetes phylum dominated

over the other phyla in 11 samples, mainly originating from waste activated sludge and UASB digesters, which corresponds with the results of other studies on similar full-scale AD plants (Chouari et al., 2005; Riviere et al., 2009; Werner et al., 2011). Their overall high hydrolytic activity explained the high abundance of Bacteroidetes representatives in waste activated sludge digesters (Cardinali-Rezende et al., 2012; Regueiro et al., 2012). Their high presence in UASB systems lies in their ability to ferment sugars to acetate and propionate (Nelson et al., 2011; Cardinali-Rezende et al., 2012). The Proteobacteria phylum only dominated in 1 sample, yet, was present in all but one sample, especially in those originating from a UASB reactor, which is confirmed by several studies (Chouari et al., 2005; Sundberg et al., 2013; St-Pierre & Wright, 2014). However, other studies reported an overall dominance of Proteobacteria (Riviere et al., 2009; Lee et al., 2012). The Proteobacteria phylum contains several members able to convert glucose, butyrate, propionate and acetate, thus explaining their increased abundance in UASB digesters (Ariesyady et al., 2007; Nelson et al., 2011; Werner et al., 2011).

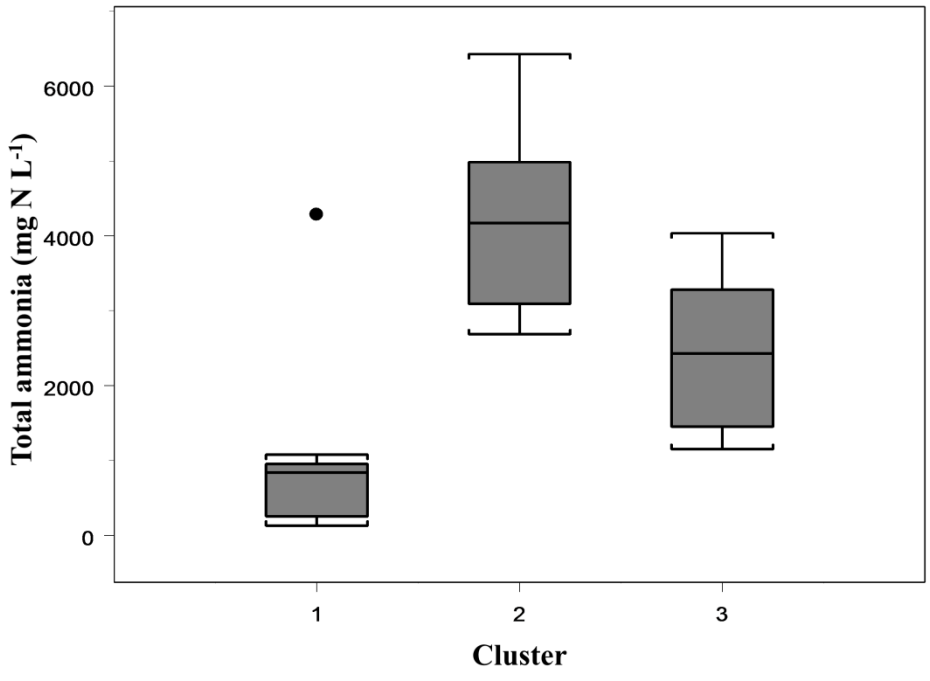

Figure 8.13 *The influence of the total ammonia concentration on the formation of three potential clusters. Total ammonia concentration was significantly different (P < 0.01) between the three clusters.*

In general, Methanosaetaceae dominated over Methanosarcinaceae, with the exception of 4 samples, as the main acetoclastic methanogens, despite high total ammonia concentrations

and conductivity values in several of these plants. These results were unexpected, as previous studies of lab-scale reactors concluded that Methanosarcinaceae were the main acetoclastic methanogens at elevated total ammonia, salt and/or volatile fatty acid concentrations (McMahon et al., 2001; McMahon et al., 2004; Conklin et al., 2006; Vavilin et al., 2008b; De Vrieze et al., 2012). However, investigations of full-scale plants reported an overall dominance of Methanosaetaceae over Methanosarcinaceae, consistent with the results obtained in our study (Leclerc et al., 2004; Ariesyady et al., 2007; Sundberg et al., 2013). Nonetheless, other studies did report a shift to a Methanosarcinaceae dominated methanogenic community (Karakashev et al., 2005; Williams et al., 2013). In our study a significant positive correlation was determined between the Methanosaetaceae and Methanosarcinaceae, together with a negative correlation of both families with total ammonia, volatile fatty acids and conductivity (a measurement of total salt concentration), despite their morphological and physiological differences (Conklin et al., 2006; De Vrieze et al., 2012). This demonstrates that Methanosaetaceae and Methanosarcinaceae favour similar conditions in these full-scale plants. Hence, the exact role of Methanosarcinaceae and the factors determining its dominance in full-scale plants remain unclear, yet, the crucial role of Methanosaetaceae, especially at low total ammonia, salt and volatile fatty acid concentrations is uncontested.

The hydrogenotrophic Methanobacteriales were positively correlated to biogas production, organic loading rate and temperature, in contrast to the other methanogenic populations, indicating that these methanogens play a crucial role in the so-called high-rate AD systems (Bialek et al., 2011). An increased (relative) abundance of Methanobacteriales has been observed in AD reactors with high residual volatile fatty acid concentrations (Delbes et al., 2001; McMahon et al., 2004; Steinberg & Regan, 2011). However, Methanobacteriales are also related to syntrophic acetate oxidation, a process that can become of crucial importance to maintain acetate removal when acetoclastic methanogenesis fails, due to changing conditions (Horn et al., 2003; Karakashev et al., 2006; Hattori, 2008; Hao et al., 2011; Hao et al., 2013). Indeed, an increased abundance of Methanobacteriales, with a parallel decrease in abundance of acetoclastic methanogens, has been reported at elevated volatile fatty acid concentrations, which indicates a transition from acetoclastic methanogenesis to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (Horn et al., 2003; Karakashev et al., 2006; Hao et al., 2013; Sun et al., 2014). Hence, Methanobacteriales, rather than Methanosaetaceae, can be considered as the ‘drivers’ of methanogenesis at elevated

volatile fatty acid concentrations, due to high total ammonia and/or salt concentrations in AD (Niu et al., 2013).

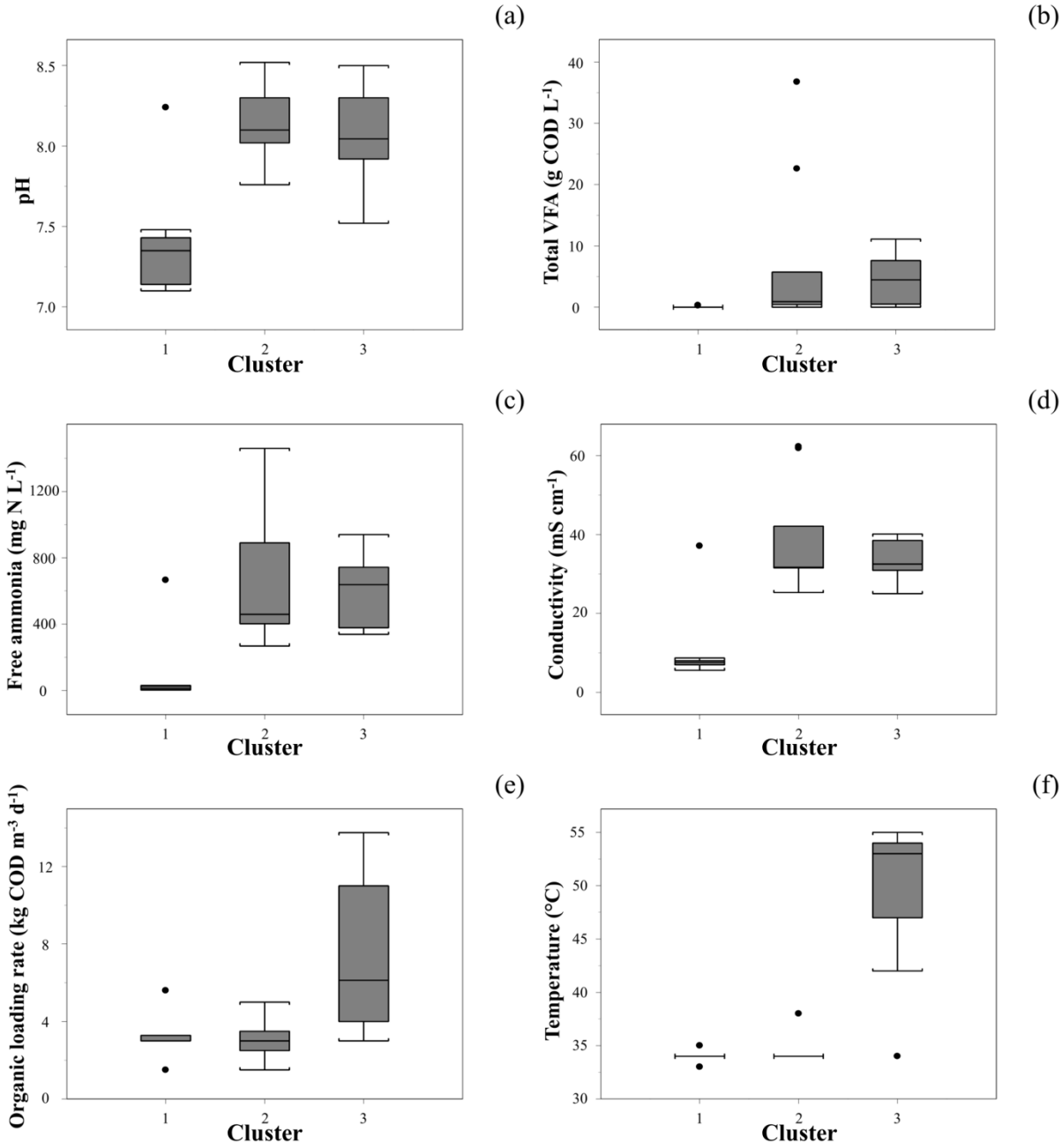

Figure 8.14 *Overview of the main operational parameters that determine the* ***clusters****: (a) pH, (b) total VFA, (c) FA, (d) conductivity, (e) OLR and (f) temperature.*

The observed clustering of the samples from different full-scale digesters was determined by the total ammonia concentration, as this was the only parameter significantly different in the three clusters. The main influence of total ammonia on the AD process is however attributed to the free ammonia fraction of total ammonia, as this is the most toxic to the methanogenic community (Hashimoto, 1986; Chen et al., 2008). However, the free ammonia concentration

could distinguish cluster 1 from cluster 2 and 3, but could not significantly separate cluster 2 and 3. This may be explained by cluster 2 containing samples with the highest total ammonia concentration, yet, cluster 3 included mostly samples from thermophilic full-scale digesters. The free ammonia concentration is determined by total ammonia, temperature and pH, and this resulted in similar average free ammonia concentrations in cluster 2 and 3 (Anthonisen et al., 1976). Despite similar free ammonia concentrations, a clustering of mesophilic and thermophilic samples was observed, which confirms that the clustering of AD samples is the result of a combined effect of total ammonia and free ammonia concentration and temperature.

Cluster 1 could be distinguished by a significantly higher abundance of the Bacteroidales order, compared to cluster 2 and 3, and was characterized by low values of pH, total ammonia, free ammonia, volatile fatty acids, conductivity and mesophilic conditions. Bacteroidales, which are in most cases the main representatives of the Bacteroidetes phylum, appeared to dominate in AD systems that operate at 'easy' mesophilic conditions, i.e. low volatile fatty acids, total ammonia and salt concentrations (Riviere et al., 2009; Nelson et al., 2011). This relates to their overall dominance in mesophilic waste activated sludge digesters and UASB reactors, from which the samples in cluster 1 originated (Chouari et al., 2005; Werner et al., 2011; Lee et al., 2012).

A significantly higher relative abundance of Clostridiales was observed in cluster 2, compared to cluster 1 and 3. The Clostridiales order is considered one of the most abundant in AD, which can be attributed to their involvement in hydrolysis, acidogenesis and acetogenesis stages (Krause et al., 2008; Krober et al., 2009; Wirth et al., 2012; Hanreich et al., 2013). Several species belonging to the Clostridiales order are reported to be involved in syntrophic acetate oxidation, which is the main pathway for acetate removal at elevated total ammonia concentrations (Schnurer et al., 1996; Karakashev et al., 2006; Schnurer & Nordberg, 2008; Westerholm et al., 2011b; Lu et al., 2013a). This is consistent with previous reports of acetate oxidizing Clostridiales species being tolerant to high total ammonia concentrations (Kelly et al., 2012).

The last cluster (cluster 3) was characterized by an increased abundance of Lactobacillales, and mainly contains samples from thermophilic AD installations. Lactobacillales, hosting the main genera *Lactobacillus* and *Enterococcus*, are often reported to be present in AD, but despite their major role in lactic acid production in the acidogenesis stage, their in situ function in AD is unknown (Krause et al., 2008; Nelson et al., 2011; Bengelsdorf et al., 2013;

De Vrieze et al., 2014). In several cases they are detected at higher abundance in thermophilic digesters than mesophilic systems, which is consistent with our results (Krause et al., 2008; Weiss et al., 2008; Bengelsdorf et al., 2013). However, given the fact that they characterize one of the three clusters, their role in AD appears to be more important than anticipated, and warrants further in-depth research.

The identification and characterization of three clusters was similar to the proposed enterotypes in the human gut and two separate clusters in the human axillary region (Arumugam et al., 2011; Callewaert et al., 2013). Hence, these three clusters in AD could be defined as 'AD-types'. However, it has been shown that the degree of clustering can depend on several factors, such as the 16S region that was targeted, the taxonomic level at which the evaluation was carried out, and the methods used for distance matrix and clustering analysis (Koren et al., 2013). Therefore, it may be possible that adding more samples to the dataset could lead to the appearance of a fourth cluster or the merging of two clusters, depending on the clustering method (Koren et al., 2013). Moreover, the presence of the clusters 2 and 3 (both at high free and total ammonia concentration) could correspond with a microbial community at unstable or sub-optimal conditions. Hence, a decrease in the ammonia concentration could therefore again lead to a shift in the microbial community from the clusters 2 and 3 to cluster 1. Indeed, high (free and total) ammonia concentrations have been shown to cause a shift in the microbial communities, in several cases leading to an unstable anaerobic digestion process, as also observed in Chapter 5 (Calli et al., 2005a; Schnurer & Nordberg, 2008; Westerholm et al., 2011a; Niu et al., 2013; Werner et al., 2014; Zhang et al., 2014a). Hence, the full-scale AD clusters 2 and 3 could be present in an inhibited steady-state, operating below their optimal potential (Angelidaki & Ahring, 1993). Furthermore, the sampling method may also have influenced the results, as samples were taken from full-scale anaerobic digesters that are sometimes heterogeneous, despite continuous mixing (Holm-Nielsen et al., 2006). The homogeneity of the 10 mL aliquot can be guaranteed, since a standardised DNA extraction method was applied. However, since only 1-10 litre of sample was taken from a full-scale reactor, it cannot be ruled out that the potential heterogeneity in these full-scale plants had an influence on the final molecular results. Hence, this will require further research. Nonetheless, a strong correlation with the environmental parameters total ammonia, free ammonia and temperature was found, while a similar strong correlation has not (yet) been detected in the human gut microbiome or human axillary region (with the exception of a gender effect), nor in any other study of microbial ecosystems similar to AD

(Leclerc et al., 2004; Riviere et al., 2009; Arumugam et al., 2011; Mielczarek et al., 2012; Callewaert et al., 2013; Mielczarek et al., 2013).

5. Conclusions

This study demonstrated the presence of three potential clusters in anaerobic digestion. These clusters were distinguished based on bacterial composition and operational factors, using data from 29 full-scale AD plants. Total ammonia concentration, together with the free ammonia concentration and digester temperature, were identified as the main contributing factors to cluster formation, which were characterized by an increased abundance of Bacteroidales, Clostridiales and Lactobacillales, respectively. Although Methanosaetaceae and Methanobacteriales could not be directly correlated to one of the clusters, their overall importance in AD remains uncontested. These three clusters could be defined as 'AD-types', however, their validity in terms the actual number of AD-types and their relation to operation at unstable or sub-optimal conditions needs to be investigated. Hence, further in-depth research will be required to determine the exact role of the core micro-organisms in each cluster, in order to promote AD and direct product formation optimization.

Acknowledgments

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CHAPTER 9: GENERAL DISCUSSION

1. Introduction

Anaerobic digestion (AD) is a leading edge microbial technology for the treatment of and energy recovery from organic side-stream products and waste streams. This technology has been applied at full scale for several decades, and its central role in wastewater treatment and the emerging bio-based economy is uncontested (Figure 9.1).

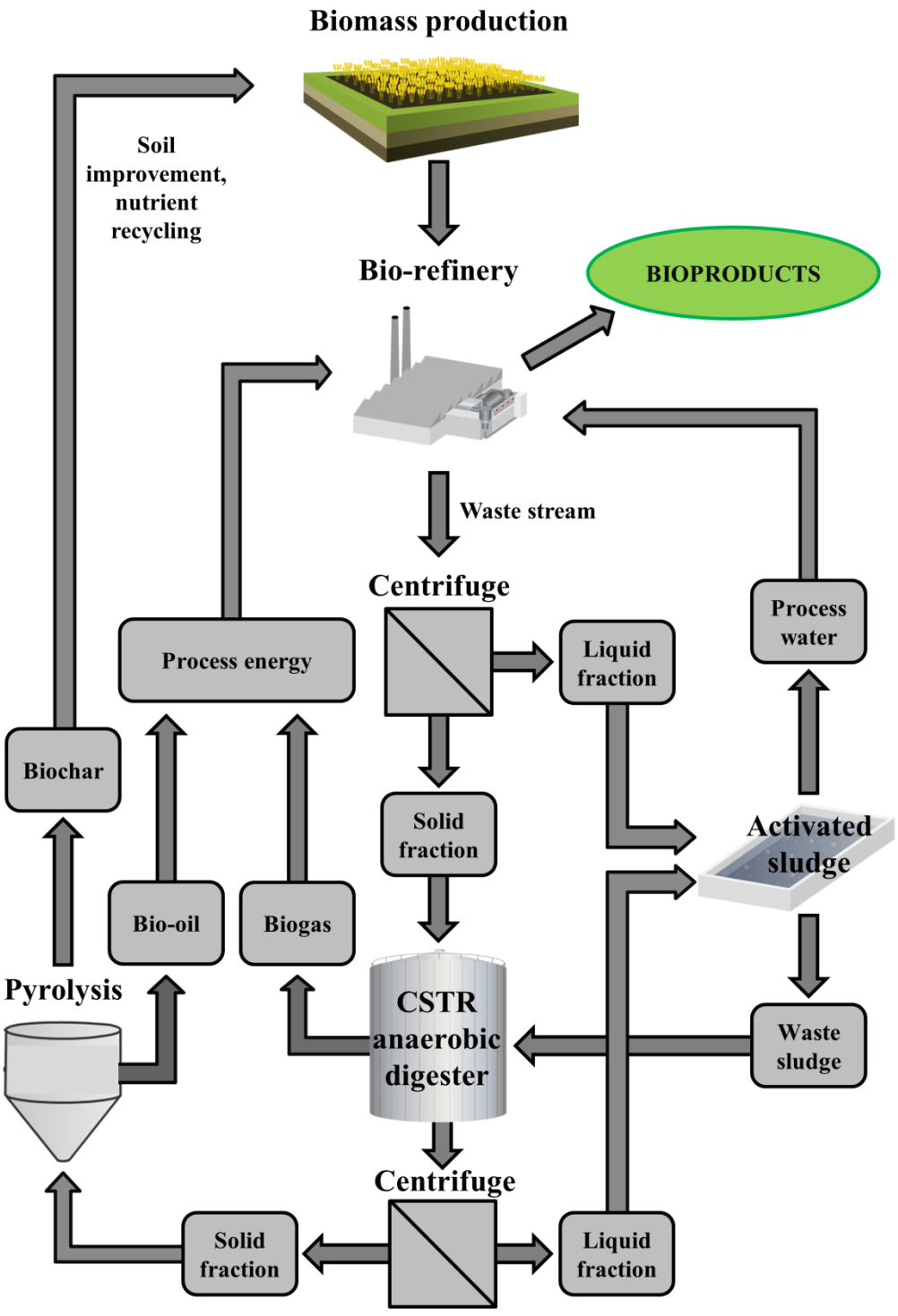

Figure 9.1 *Central role of anaerobic digestion in the bio-refinery concept.*

However, despite its widespread application on full-scale, AD still poses several problems, such as low methane production and process instabilities. This relates to the fact that the microbial community in AD remains a 'black box', that is, the composition and function of the microbial community is still largely unknown, despite several attempts.

In this research several strategies were applied to improve stability, and to increase methane production in AD through (in)direct steering of the microbial community. In **Chapter 2 and 3** high-rate iron-rich sludge from the Adsorptions-Belebungsverfahren' or A/B activated sludge system (A-sludge) for municipal wastewater treatment was used as co-substrate for AD of kitchen waste and molasses, resulting in both cases in stable methane production, while mono-digestion of kitchen waste and molasses always resulted in process failure. Depending on the co-substrate and corresponding operational conditions, a differentiating response was observed in both the bacterial and methanogenic (archaeal) community. The application of a different feeding pattern in **Chapter 4** led to a higher level of bacterial dynamics in the stronger pulse fed reactor, with a correlating higher degree of tolerance to common stressors in AD, such as organic overloading and increased TAN concentrations. The methanogenic community, however, was not affected by the variation in the feeding pattern. The role of the selected inoculum in terms of start-up efficiency and tolerance to stress, in this case increased TAN concentrations, was investigated in **Chapter 5**. A different response was observed for the different inocula, indicating the importance of inoculum selection to ensure stable methane production, yet towards the end of the experiment, a similar microbial community composition was observed, despite the large initial differences. Methanosaetaceae were the dominant acetoclastic methanogens, while Methanosarcinaceae increased in abundance at elevated ammonium concentrations.

The application of operational management strategies, as described in Chapter 2 to 5, resulted in increased methane production rates and/or a higher degree of process stability in AD. Next to these operational strategies, two technological management strategies were applied, as described in Chapter 6 and 7, to optimize the AD process. The introduction of a (bio)electrochemical system in AD in **Chapter 6** caused the stabilization of molasses digestion. Both Methanosaetaceae and Methanosarcinaceae abundances increased, yet, the key impact of the bioelectrochemical system was attributed to biomass retention, rather than electrochemical stimulation, due to the absence of a direct effect of the applied cell potential or resulting current. The application of an anaerobic membrane bioreactor for bio-refinery wastewater treatment in **Chapter 7** resulted in stable methane production, yet, only when

diluted wastewaters were used. Methanosaetaceae dominated the (acetoclastic) methanogenesis, irrespective of the membrane fouling control strategy, yet biogas recirculation proved to be a better fouling prevention mechanism than a membrane with vibrational fouling control.

Several operational strategies were successfully applied to increase biogas production in AD. However, the extrapolation of these lab-scale experimental results to application in full-scale installations requires in-depth knowledge of the microbial communities in these installations to allow application of the correct control strategy. In **Chapter 8**, an extensive survey of 38 samples from 29 full-scale AD installations was performed. This led to the discovery of three clusters in AD, also called AD-types, that were distinguished based on the relative abundance of the Bacteroidales, Clostridiales and Lactobacillales orders. Methanosaetaceae were the dominant acetoclastic methanogens, yet only Methanobacteriales positively correlated to biogas production, indicating the importance of both acetoclastic and hydrogenotrophic methanogenesis in full-scale AD.

2. The microbial community in anaerobic digestion: who is there?

2.1. Microbial community organization and dynamics: bacteria vs. archaea

Anaerobic digestion is a microbial technology that allows the conversion of a wide range of organic waste streams to biogas by means of a succession of different pathways, hence different microbial populations are required to carry out the AD process (Fernandez et al., 1999; Briones & Raskin, 2003). Consequently, microbial richness and diversity are important, not only to maintain an extensive metabolic capacity, but also to guarantee the resilience of the AD ecosystem (Fernandez et al., 1999; Briones & Raskin, 2003; Bell et al., 2005; Baho et al., 2012). In Chapter 4, it was demonstrated that range-weighted bacterial richness (Rr), based on DGGE (denaturing gradient gel electrophoresis) analysis, can be high in AD, compared to other (natural) ecosystems, despite the fact that a well-characterized substrate was used (Marzorati et al., 2008). A similar result was obtained in Chapter 5, as irrespective of the selected inoculum, a microbial species richness of 623 ± 23 OTUs was observed, averaged over the different reactors in Phase 1, 2 and 3. An extensive survey of several full-scale plants (Chapter 8) even led to an average microbial species richness of 1711 ± 538 OTUs. These results are in agreement with other studies estimating the microbial richness in AD by means of amplicon sequencing or metagenomics methods (Krober et al., 2009; Nelson

et al., 2011; Lee et al., 2012; Hanreich et al., 2013; Sundberg et al., 2013; St-Pierre & Wright, 2014; Town et al., 2014).

However, in AD, a determination of the overall microbial richness and diversity is insufficient to estimate the metabolic potential, as both bacteria and archaea play a crucial role in the conversion of the organic substrate to biogas. As bacteria are responsible for the first three steps, and archaea only carry out the final step, it is generally assumed that bacterial diversity or richness and absolute or relative abundance should roughly be three times higher than for archaea (Fernandez et al., 1999; Briones & Raskin, 2003).

Real-time PCR analysis of total archaea and total bacteria in three different anaerobic membrane bioreactors (AnMBR) (Chapter 7) resulted in a bacteria:archaea ratio of 3.2 ± 1.5 in the HL-AnMBR, 2.8 ± 2.3 in the NV-AnMBR and 1.7 ± 0.9 in the V-AnMBR, which closely relates to the 3:1 ratio that was set forth. A similar result was obtained for the carbon felt carrier material in the AD-BES reactors (Chapter 6), showing an average bacteria:archaea ratio of 6.7 ± 3.4, although slightly higher than the 3:1 ratio. Screening of four full-scale UASB (upflow anaerobic sludge bed) plants (Chapter 8) showed bacteria:archaea ratios of 2.5, 5.0, 9.2 and 21.8, which, with the exception of the last value, only slightly deviate from the 3:1 ratio. These results, hence, indicate that activate retention of anaerobic biomass by means of carrier material, membrane separation systems or in anaerobic granules leads to higher relative archaeal abundances, which correlates to their overall lower growth rates compared to bacteria (Gujer & Zehnder, 1983; Ince et al., 1997; Diaz et al., 2003; Sasaki et al., 2006; Satoh et al., 2007; Sasaki et al., 2010a; Gong et al., 2011; Skouteris et al., 2012). In stable lab-scale CSTR (continuous stirred tank reactor) systems, however, higher bacteria:archaea ratios were observed, ranging from average values of 10.9 ± 1.9 (Chapter 4) and 14.8 ± 6.7 (Chapter 2) to 40.1 ± 21.5 (Chapter 6) and 41.0 ± 26.2 (Chapter 5), based on real-time PCR and amplicon sequencing results. In full-scale CSTR plants, the bacteria:archaea ratio showed a high level of variation, with values between 19.1 and 1304.4 (Chapter 8), however, clearly surpassing the 3:1 ratio, in most cases with at least a factor 10. Based on these results, it can be assumed that the high relative abundance of bacteria compared to archaea in anaerobic CSTR systems relates to an equal high richness or diversity of bacteria compared to archaea, which is confirmed by several studies (Table 9.1).

In 17 of the 21 studies a bacteria:archaea ratio higher than the ~~of~~ 3:1 ratio is observed, irrespective of the molecular technique that was used, e.g. real-time PCR, clone libraries, ARDRA (Amplified Ribosomal DNA Restriction Analysis), amplicon sequencing or

metagenomics. This indicates that, in general, archaeal richness is lower than estimated by its actual role in AD, although in several cases this does not correlate to the relative bacteria:archaea abundance (Table 9.1). The main reason for the low archaeal richness in AD, compared to the bacterial richness, can be attributed to the limited substrate diversity for the methanogenic community, as only acetate and CO_2 and H_2, and in some cases also formate, methanol, methylamines, methylsulphide, dimethylsulphide, and CO may serve as precursor for methane production (Ferguson et al., 1996; Ferry, 1999; Rother & Metcalf, 2004; Liu & Whitman, 2008; Bizukojc et al., 2010; Ferry, 2011; Kumar et al., 2011). A much wider gamma of substrates is available for the bacterial community, thus allowing a higher degree of community richness (Li et al., 2013; Lu et al., 2013b; Regueiro et al., 2014; Zhang et al., 2014b). Hence, the limited substrate diversity appears to be the main cause for the low archaeal richness in AD.

Richness or diversity as such are, however, not sufficient to support a stable and resilient community as these do not directly relate to the presence of stress resistant species, nor do they provide information on the adaptive potential or 'elasticity' of the microbial community (McCann, 2000; Briones & Raskin, 2003; Dearman et al., 2006). In some cases high levels of microbial diversity or richness may even provoke antagonistic interactions, thus, lowering ecosystem functioning (Becker et al., 2012). Community dynamics are therefore of greater importance to allow a rapid adaptation of the microbial community in AD to changing and/or stressful conditions, such as organic overloading, increasing salt or TAN concentrations or temperature fluctuations (McCann, 2000; Delbes et al., 2001; McMahon et al., 2004; Dearman et al., 2006; Yu et al., 2006; Boon et al., 2011; Hao et al., 2013; Niu et al., 2013; Williams et al., 2013).

Table 9.1 *Overview of the bacteria:archaea ratio, based on total abundance and richness measurements, in different lab- and full-scale anaerobic digestion systems, using different methods. n.a. = data not available.*

			Bacteria:archaea			
Reactor type	Scale	Reactors	Abundance	Richness	Method	Reference
CSTR	Lab	2	n.a.	1.55	T-RFLP	Carballa et al., 2011
CSTR	Full	1	1.61	2.76	Clone library	Liu et al., 2009
CSTR	Lab	1	n.a.	2.82	Clone library	Cardinali-Rezende et al., 2009
CSTR	Full	1	5.67	3.00	Clone library	Cardinali-Rezende et al., 2012
CSTR	Lab	4	0.59	3.90	454 sequencing	Ritari et al., 2012
CSTR	Lab	1	2.38	5.00	Clone library	Kobayashi et al., 2008
CSTR	Lab	2	n.a.	5.50	DGGE	Carballa et al., 2011
CSTR	Lab	4	65.67	6.53	Clone library	Hanreich et al., 2013
CSTR	Full	1	3.07	6.92	Clone library	Ariesyady et al., 2007
CSTR	Full	1	10.87	10.30	Clone library	Chouari et al., 2005
UASB	Full	12	1.78	11.94	Clone library	Narihiro et al., 2009
CSTR	Full	21	19.00	12.75	454 sequencing	Sundberg et al., 2013
CSTR	Lab	1	15.33	14.00	ARDRA	Klocke et al., 2007
Fixed bed	Full	1	0.78	14.50	Clone library	Kobayashi et al., 2014
CSTR	Full	1	n.a.	16.67	Clone library	Goberna et al., 2009
CSTR/UASB	Lab/full	n.a.	5.76	20.02	Clone library	Nelson et al., 2011
Fluidized bed	Lab	1	4.79	22.17	Clone library	Godon et al., 1997
CSTR	Full	9	5.03	25.98	Clone library	Riviere et al., 2009
CSTR	Lab	1	16.67	59.42	qPCR/454 sequencing	Town et al., 2014
CSTR	Lab	1	10.14	n.a.	Metagenomics	Wirth et al., 2012
CSTR	Full	1	4.88	n.a.	Clone library	Nettmann et al., 2008

In Chapter 4, a different feeding pattern was applied in two different reactors, which resulted in a higher degree of bacterial community dynamics in the pulse feeding reactor. However, in both reactors no changes were observed in the methanogenic community, despite the high bacterial community dynamics, up to 50%. The stability of the archaeal/methanogenic community in the two reactors in Chapter 4 can be attributed to the fact that no VFA (volatile fatty acids) were observed, and pH remained constant and within the optimal range for AD. Similar results were observed in other studies, yielding no or only very low dynamics in the methanogenic community, despite changing operational conditions, such as an increase in OLR, yet, with no or limited increase in VFA or decrease in pH (Gomez et al., 2011; Krakat et al., 2011; Ritari et al., 2012; Regueiro et al., 2014; Town et al., 2014). In contrast, in Chapter 5 and 6, increasing TAN or salt concentrations, as well as an increase in the OLR, resulting in higher VFA levels, did cause a shift in the methanogenic community, which relates to other studies (Delbes et al., 2001; Lee et al., 2009; Niu et al., 2013; Williams et al., 2013; Regueiro et al., 2014).

The bacterial community, in contrast to the methanogenic community, always showed a certain degree of dynamics. In Chapter 4, a different feeding pattern resulted in a different degree of community dynamics, yet, even though this feeding pattern was constantly maintained, even after 49 days a rate of change of 8.3 and 24.7% was still observed in the stable and pulse feeding reactor, respectively. Hence, (high) levels of bacterial dynamics do not directly relate to changing process conditions or failure, as even during functional stable operation high bacterial community dynamics can be observed (Krakat et al., 2011; Regueiro et al., 2014; Town et al., 2014). In addition, our results in Chapter 4 demonstrated that a higher degree of bacterial community dynamics leads to a higher tolerance to stressors, such as high TAN concentrations and organic overloading (Fernandez et al., 1999; Fernandez et al., 2000). In Chapter 3, (co-)digestion of different substrates resulted in a distinct bacterial community. In Chapter 5, the application of the same feed, starting from different inocula, resulted in a similar bacterial community in four of the five reactors throughout the entire experiment, despite a different response to high TAN concentrations. Eventually, the fifth reactor also evolved to a similar bacterial community. Finally, the application of a (bio)electrochemical system in Chapter 6 resulted in a different bacterial community, due to the establishment of different process conditions. The results in these three chapters clearly demonstrated that bacterial community dynamics are influenced by both substrate composition and reactor configuration, as well as conditions during transient and stable

circumstances, which subsequently leads to a unique bacterial community in each anaerobic digester (Riviere et al., 2009; Pycke et al., 2011; Werner et al., 2011; Li et al., 2013; Lu et al., 2013b).

In short, functionally stable AD contains a static methanogenic community and an ever dynamic bacterial community. The methanogenic community remains unchanged, as long as evolving operational parameters or substrate composition do not influence the optimal conditions for methanogenesis. The bacterial community shows a constant degree of dynamics, which relates to stress tolerance, that is determined by substrate composition, operational conditions and reactor configuration.

2.2. Microbial community composition: the driving factors

Several authors stated in a variety of studies, concerning the microbiology of AD, that a better understanding of the microbial community is necessary to allow in-depth operational control and subsequent increased methane production rates (Briones & Raskin, 2003; Kleerebezem & van Loosdrecht, 2007; Narihiro & Sekiguchi, 2007; Krause et al., 2008; Sabra et al., 2010; Nelson et al., 2011; Koch et al., 2014; Vanwonterghem et al., 2014). However, the dynamic character of the bacterial community, its high richness and the variety of conditions in AD, make it difficult to define this so-called 'average' bacterial community. In Chapter 5, microbial community analysis revealed a relative abundance > 5% of the bacterial phyla Actinobacteria, Bacteroidetes, Chlorobi, Firmicutes, Proteobacteria and WS3 in at least one of the samples, while ten other phyla were also present at a relative abundance > 1%. In correspondence to Chapter 5, the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were also present > 5% in at least one sample described in Chapter 6, together with the Spirochaetes, Tenericutes and WS6 phyla. Bacterial community analysis of full-scale AD plants, as carried out in Chapter 8, led to the identification of no less than 12 phyla present > 5% relative abundance in at least 1 plant. The main phyla were the Bacteroidetes, Firmicutes and Proteobacteria, present > 5% relative abundance in 92%, 92% and 42% of the samples, respectively. Hence, especially the Bacteroidetes and Firmicutes phyla and, to minor extent, the Proteobacteria, can be considered as the main contributors to the AD core microbiome. Representatives of these three phyla are most likely omnipresent in all AD systems, because of their ability to perform several important steps in the hydrolysis, acidogenesis and acetogenesis stages (Ariesyady et al., 2007; Riviere et al., 2009; Nelson et al., 2011; Cardinali-Rezende et al., 2012; Lee et al., 2012; Marshall et al., 2013; Sundberg et al., 2013;

St-Pierre & Wright, 2014). Several attempts were already undertaken to define a core microbiome in AD, yet, to our knowledge, neither driving factors, nor any distinct correlations between certain bacterial populations and operational parameters could be identified, although the substrate type appears to be the main factor determining the bacterial community (Riviere et al., 2009; Nelson et al., 2011; Werner et al., 2011; Li et al., 2013; Lu et al., 2013b; St-Pierre & Wright, 2014; Zhang et al., 2014b). In Chapter 8, however, in-depth analysis of the microbial community in several full-scale plants led to the discovery of three distinct clusters in AD, or 'AD-types'. These three AD-types were characterized by an increased abundance of Bacteroidales, Clostridiales and Lactobacillales, respectively. The discovery of different AD-types relates to the results of other similar ecosystems, yet, in contrast to these ecosystems, in our study clear driving factors for this cluster formation were identified (Arumugam et al., 2011; Mielczarek et al., 2012; Callewaert et al., 2013; Mielczarek et al., 2013; Lesnik & Liu, 2014). Indeed, the combined effect of total ammonia, free ammonia and temperature appeared to be the main driving factor for bacterial community formation.

The methanogenic community has a much lower richness, compared to the bacterial community, making it, together with its static character, relatively easy to determine the main factors driving methanogenic community composition and organization. Indeed, transitions in the methanogenic community only take place when the optimal conditions for the methanogenic community are no longer maintained, hence the factor(s) causing the shift can easily be determined. In Chapter 4, the application of a low OLR of 1 g COD L^{-1} d^{-1}, resulted in a Methanosaetaceae dominated methanogenic community, irrespective of the feeding pattern, which relates to the fact that optimal conditions were maintained in both reactors at all times. In Chapter 3, Methanosaetaceae maintained high copy numbers, between 10^9 and 10^{10} copies g^{-1} sludge, as long as optimal conditions were maintained, irrespective of the selected (co-)substrates. However, an increase in VFA and a decrease in pH resulted in a decreased abundance of Methanosaetaceae. This decrease was not observed in the abundance of Methanobacteriales and Methanomicrobiales, with the exception of those reactors that completely failed. An increase in Methanosarcinaceae abundance was observed at increasing VFA concentrations during molasses co-digestion. In Chapter 5, the selection of a different inoculum for AD of the same substrate resulted in an overall dominance of Methanosaetaceae, with the exception of 2 initial inocula samples and the failing reactor at the end of Phase 1 and 2, all of which were dominated by the hydrogenotrophic Methanobacteriales. However, a 100-

fold increase in Methanosarcinaceae abundance was observed after the application of increasing ammonium pulses in all reactors. It was already stated that active retention of biomass leads to an increase in the relative abundance of archaea in AD, yet, based on the results of Chapter 6 and 7, this increase can be attributed to a strong increase in the absolute abundance of Methanosaetaceae. Indeed, in Chapter 6, a Methanosaetaceae abundance up to 10^{12} copies cm^{-2} of carbon felt was observed, while in the planktonic phase maximum values of only 10^{10} copies g^{-1} sludge were observed. The application of a fixed cell potential and an increase in VFA led to an increased abundance of Methanosarcinaceae. Finally, in Chapter 7, an overall Methanosaetaceae abundance of 10^{10} copies g^{-1} sludge was maintained in all membrane bioreactors, irrespective of the loading rate and the mechanism for fouling prevention.

The overall dominance of Methanosaetaceae, especially under optimal conditions, relates to their high affinity for acetate and low tolerance to common stressors in AD, such as high ammonium, VFA and salt concentrations (Gujer & Zehnder, 1983; Leclerc et al., 2004; Calli et al., 2005a; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b; Nelson et al., 2011; Sundberg et al., 2013; Williams et al., 2013). Screening of full-scale samples in Chapter 8 showed a significant negative correlation between TAN, VFA, FA and conductivity, thus confirming the preference of Methanosaetaceae for 'easy' or optimal conditions with narrow boundaries. In several of our lab-scale experiments, an increase in Methanosarcinaceae was observed at increasing VFA, TAN or salt concentrations, which relates to several other studies, indicating that so-called 'deteriorating' conditions drive a transition from a Methanosaetaceae to a Methanosarcinaceae dominated acetoclastic methanogenesis (Collins et al., 2003; McMahon et al., 2004; Calli et al., 2005b; Conklin et al., 2006; Vavilin et al., 2008b; Blume et al., 2010; Shin et al., 2010; Shin et al., 2011; Merlino et al., 2012; Ho et al., 2013; Merlino et al., 2013; Williams et al., 2013; Lins et al., 2014). However, not only Methanosarcinaceae, but also the hydrogenotrophic orders Methanobacteriales and Methanomicrobiales increased or maintained a constant abundance at deteriorating conditions, results that were also observed in several other lab-scale experiments (Jang et al.; Schnurer et al., 1999; Delbes et al., 2001; Munk et al., 2010; Song et al., 2010; Hao et al., 2013; Lins et al., 2014).

Hence, it appears that the main factors driving bacterial clustering also determine the methanogenic community in AD. At optimal conditions, Methanosaetaceae are the uncontested dominant methanogens in AD, irrespective of the substrate, operational

conditions or reactor configuration. However, increasing ammonium (both TAN and FA), salt and VFA concentrations cause a shift from a Methanosaetaceae to a Methanosarcinaceae, Methanobacteriales and/or Methanomicrobiales dominated methanogenesis.

2.3. Full-scale vs. lab scale: the microbial discrepancy

In the field of AD, new technological applications and substrate combinations for future full-scale application are continuously being investigated. In most cases, prior to full-scale application, lab-scale and pilot-scale experiments are carried out to validate the suitability of the new application or substrate combination. However, due to a higher degree of operational and hydrodynamic fluctuations in full-scale plants, operational data from lab-scale experiments should not be directly projected to full-scale plant designs (Fdz-Polanco et al., 1999; Kaparaju et al., 2009; Bouallagui et al., 2010). The difference in operational and hydrodynamic conditions between lab-scale and full-scale systems might also lead to the evolvement of a different microbial community in lab-scale vs. full-scale plants, whether or not influencing methane production.

In-depth analysis of the bacterial community in Chapter 5 and 6 by means of amplicon sequencing revealed an overall dominance of the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria phyla. These results are in agreement to the results of the full-scale plants that were analysed in Chapter 8, as well as other full-scale systems, showing an overall dominance of the Bacteroidetes, Firmicutes and Proteobacteria phyla (Chouari et al., 2005; Ariesyady et al., 2007; Riviere et al., 2009; Nelson et al., 2011; Cardinali-Rezende et al., 2012; Lee et al., 2012; Regueiro et al., 2012; St-Pierre & Wright, 2014). However, the overall co-dominance of the Actinobacteria in the lab-scale reactors in Chapter 5 and 6 does not relate to the full-scale plant results of Chapter 8. This apparent inconsistent result can be explained by the influencing effect of waste activated sludge in both Chapter 5 and 6. Indeed, in Chapter 5, waste activated sludge is used as co-substrate, and in Chapter 6, the inoculum sludge in all reactors originated from a full-scale sludge digester. Increased (relative) abundances of Actinobacteria are often observed in (waste activated) sludge digesters, due to their ability to degrade a wide variety of organic substrates, even xenobiotic compounds, and their preference for mesophilic AD at low OLR values (Chouari et al., 2005; Rincon et al., 2006; Ariesyady et al., 2007; Krakat et al., 2011; Sundberg et al., 2013). In general, a similar bacterial community can be observed in lab-scale anaerobic digesters compared to full-scale plants.

Methanogenic community analysis of the different lab-scale reactors in Chapter 3-7, using real-time PCR or amplicon sequencing, confirmed an overall dominance of the Methanosaetaceae at stable conditions, with operational parameters within the (narrow) boundaries for optimal methanogenesis. A strong deviation outside these boundaries resulted in a decrease in (relative) abundance of Methanosaetaceae and/or an increase in Methanosarcinaceae, Methanobacteriales and/or Methanomicrobiales. In several cases, such as during co-digestion of molasses and A-sludge (Chapter 3), a strong increase in Methanosarcinaceae abundance was observed, due to the application of increased TAN concentrations (Chapter 5), and under the influence of an applied cell potential (Chapter 6). In other reactors, an increase in Methanobacteriales and/or Methanomicrobiales was observed, for instance during co-digestion of kitchen waste and A-sludge and mono-digestion of kitchen waste (Chapter 3). In all cases, the decrease in Methanosaetaceae and/or increase in Methanosarcinaceae, Methanobacteriales and/or Methanomicrobiales related to an increase in residual VFA. The overall dominance of Methanosaetaceae, especially at 'easy' conditions, relates to the results of the full-scale plant samples in Chapter 8, as well as several other studies, both lab- and full-scale (McHugh et al., 2003; Leclerc et al., 2004; Conklin et al., 2006; Blume et al., 2010; Nelson et al., 2011; Chen et al., 2012; Sundberg et al., 2013; Williams et al., 2013). An increase in Methanobacteriales and Methanomicrobiales at deteriorating conditions, as observed in Chapter 3, also relates to several other studies (Schnurer et al., 1999; Hao et al., 2013; Lins et al., 2014). In Chapter 8, both Methanobacteriales and Methanomicrobiales were observed in all full-scale plant samples, with the exception of one sample in which Methanomicrobiales abundance was below detection limit. However, based on the results of Chapter 8, only Methanobacteriales showed a significant positive correlation with biogas production and OLR, indicating that Methanobacteriales are the crucial hydrogenotrophic methanogens in so-called high-rate AD systems at 'heavy' conditions (Delbes et al., 2001; McMahon et al., 2004; Bialek et al., 2011; Steinberg & Regan, 2011). The increase in Methanosarcinaceae that was observed in several lab-scale studies (Chapter 3,5 and 6), however, does not relate to the full-scale plant results of Chapter 8. Indeed, Methanosarcinaceae were detected in only 55% of the full-scale plant samples, and negatively correlated to TAN, VFA, FA and conductivity, though not as strong as Methanosaetaceae. Thus, this contradicts the results of Chapter 3, 5 and 6 and other lab-scale AD studies, in which Methanosarcinaceae were dominating at 'heavy' conditions, whether or not after taking over from Methanosaetaceae (Shigematsu et al., 2003; Conklin et al., 2006; Karakashev et al., 2006; Vavilin et al., 2008b; Garcia et al., 2011; Hao et al., 2011;

Karlsson et al., 2012; Westerholm et al., 2012b; Ho et al., 2013; Lins et al., 2014). This discrepancy may relate to the operational and hydrodynamic conditions in full-scale plants, compared to lab-scale plants, mainly caused by the much higher degree in substrate variation and, sometimes, the lack of appropriate operational control (Wiese & Haeck, 2006; Kardos et al., 2009). Mixing can be considered one of the most important control strategies in AD, as it maintains the solids in suspension and allows close contact between the substrate and the microbial community, thus, enhancing the methane production process (Karim et al., 2005; Kaparaju et al., 2008). However, vigorous mixing has been shown to have a negative effect on methane production, as it induces foaming, and destroys microbial flocks and filamentous micro-organisms, thus, disturbing syntrophic interactions (Brown & Sale, 2002; Hoffmann et al., 2008; Kaparaju et al., 2008; Kougias et al., 2014). In lab-scale systems, most often intensive mixing is applied to ensure efficient operation, in contrast to full-scale plants, yet, this may severely disturb syntrophic interactions between the hydrogenotrophic methanogens and the SAO (syntrophic acetate oxidizing bacteria). Hence, it can be hypothesized that syntrophic acetate oxidation, one of the main processes for acetate removal at 'heavy' conditions, is hampered, and acetate starts to accumulate (Hattori, 2008; Nettmann et al., 2010). Methanosarcinaceae are the only known methanogens able to perform acetoclastic methanogenesis, together with Methanosaetaceae, yet, due to their higher growth rates at increased acetate concentrations, they can take over from Methanosaetaceae (Gujer & Zehnder, 1983; Masse & Droste, 2000; Conklin et al., 2006; Yu et al., 2006; Qu et al., 2009b; Tomei et al., 2009). This explains the observed increases in abundance in Methanosarcinaceae in lab-scale reactors, in contrast to full-scale plants. Another explanation might be attributed to the fact that due to a lower degree of mixing in full-scale plants, operating at high TS concentrations, acetate concentration might not be homogenous in the digesters. Hence, the actual concentration available for the acetoclastic methanogens is lower than measured in the bulk liquid, which favours Methanosaetaceae.

In general, a high degree of similarity exists in the bacterial community composition and organization between lab-scale and full-scale plants. The methanogenic community is dominated by the acetoclastic Methanosaetaceae in both lab-scale and full-scale plants. However, at 'deteriorating' conditions at lab-scale a transition to a Methanosarcinaceae dominated methanogenesis can be observed, while this shift is not observed in full-scale plants. In fact, only Methanobacteriales are positively correlated to biogas production and

OLR in full-scale plants, making them the crucial methanogens in high-rate full-scale AD systems, instead of Methanosarcinaceae.

3. The microbial community in anaerobic digestion: what are they doing?

The microbial community in AD is highly diverse and continuously changes over time, especially the bacterial community, even at constant or stable operational conditions, as stated earlier, which makes it difficult to attribute specific processes to certain microbial groups. At this point, it is generally accepted that bacteria carry out the first three steps in AD, whereas archaea are responsible for the fourth and final step, methanogenesis. However, the microbial community in AD is still considered a 'black box', despite several attempts to construct a clear overview of the microbial community in AD (Riviere et al., 2009; Werner et al., 2011; Vanwonterghem et al., 2014). Hence, future discoveries in the field of microbial ecology might still cause a revolution in our understanding of the microbial processes in AD. In this research, in-depth analysis of the bacterial community in samples from full-scale plants (Chapter 8) revealed the presence of 3 clusters or AD-types, determined by an increased relative abundance in Bacteroidales, Clostridiales and Lactobacillales, and characterized by specific operational parameter values. These results may serve as a basis for further interpretation of the exact role of specific bacterial groups in the different stages in AD.

3.1. Three dominant phyla in the bacterial community

Three main dominant bacterial phyla, i.e. Bacteroidetes, Firmicutes and Proteobacteria, were identified in AD, based on full-scale plant sample (Chapter 8) and lab-scale reactor (Chapter 5 and 6) analyses. Despite the fact that these three phyla contain representatives able to carry out several reactions in the hydrolysis, acidogenesis and acetogenesis, based on pure culture test and genome analyses, it is still possible to establish a relation between the abundance of these specific phyla, classes and/or orders and specific processes, by using operational data.

Bacteroidetes were identified in each of the different reactors in Chapter 5 and 6, irrespective of the present conditions, however, with the exception of an inoculum sample originating from a full-scale UASB plant, always at a relative abundance < 20%. Bacteroidetes are reported to possess high hydrolytic activity, but also the ability to engage in sugar

fermentation, making them key-players in both the hydrolysis and acidogenesis stages (Riviere et al., 2009; Cardinali-Rezende et al., 2012; Regueiro et al., 2012). Their overall presence in AD, both lab- and full-scale systems, irrespective of the prevalent conditions, with the exception of one reactor sample that showed an accumulation in VFA up to 27.0 g COD L^{-1} at a pH of 6.03 and complete methane production inhibition, indicates their versatile and stress-tolerant character in AD.

The relative abundance of Firmicutes in lab-scale systems was, on average, much higher, compared to Bacteroidetes, with values up to 72.8% of the total bacterial community, yet, a higher degree of variation was also observed. This indicates that Firmicutes are more strongly influenced by changing conditions in AD, than the Bacteroidetes. In Chapter 5, a decrease in the relative abundance of Firmicutes was observed in the reactor showing severe acidification, in contrast to the other reactors operating at optimal conditions. In Chapter 6, a high degree of variation was observed between the different genera belonging to the Firmicutes phylum, which relates to the diverse conditions in the different reactors. This indicates that not only total Firmicutes abundance, but also in-phylum changes take place under influence of changing conditions. Firmicutes are mainly involved in three main processes in AD. Several representatives possess the ability to hydrolyse lipids, proteins and polymeric carbohydrates, while others are involved in syntrophic propionate and butyrate oxidation (Lynd et al., 2002; Riviere et al., 2009; Nelson et al., 2011; Sundberg et al., 2013; Vanwonterghem et al., 2014). The Firmicutes phylum also comprises species able to perform syntrophic acetate oxidation, such as *Tepidanaerobacter acetatoxydans*, *Clostridium ultunense*, *Thermacetogenium phaeum*, and *Syntrophaceticus schinkii* (Schnurer et al., 1996; Hattori et al., 2000; Westerholm et al., 2010; Westerholm et al., 2011b). The dynamic character of the Firmicutes phylum, hence, most likely relates to the increase or decrease in abundance of these SAO under the influence of changing conditions in AD. Indeed, in several studies an increase in SAO (relative) abundance has been observed at conditions deviating from the optimal range for AD (Schnurer et al., 1999; Hao et al., 2011; Karlsson et al., 2012; Westerholm et al., 2012b; Hao et al., 2013; Sun et al., 2014; Werner et al., 2014).

Proteobacteria were observed in lab- and full-scale reactor systems, but in contrast to the Bacteroidetes and Firmicutes, they were, with the exception of 1 sample, never the dominating phylum in full-scale plants. This was observed in Chapter 8, although contradictory results are reported in literature concerning the dominance of Proteobacteria in AD (Chouari et al., 2005; Ariesyady et al., 2007; Riviere et al., 2009; Nelson et al., 2011; Lee

et al., 2012; Sundberg et al., 2013; St-Pierre & Wright, 2014). In our lab-scale experiments, an increase in abundance of Proteobacteria was associated with digester failure, as a decrease in methane production and pH and an increase in residual VFA was observed in relation to increased Proteobacteria abundances (Chapter 5 and 6). Since Proteobacteria in AD are mainly involved in sugar fermentation, leading to the formation of butyrate, propionate and acetate, and further (syntrophic) VFA degradation, their connection to AD process failure is apparent (Ariesyady et al., 2007; Nelson et al., 2011). The main niche of Proteobacteria, hence, appears to be VFA production. Especially at high residual VFA concentrations (up to 27.0 g COD L^{-1}) and pH values as low as 4.75, they are key players in fermentation processes at low pH, also explaining their only lingering presence in AD at optimal conditions (Escalante et al., 2008; Illeghems et al., 2012; Lyu et al., 2013).

3.2. Acetoclastic or hydrogenotrophic methanogenesis as the main pathway?

Up until now, two main pathways for the production of methane have been identified in AD, i.e. acetoclastic and hydrogenotrophic methanogenesis. The prevalence of each pathway depends on several (operational) factors, leading to a dominance of one of these pathways.

The overall dominance of Methanosaetaceae as the main (acetoclastic) methanogens has been observed in different reactor conditions and configurations throughout this research (Chapter 3-7). Their overall supremacy in these lab-scale digesters relates to full-scale results (Chapter 8), indicating that Methanosaetaceae can be considered the main methanogens at optimal conditions, performing acetoclastic methanogenesis, as this is considered the only possible methane production pathway for Methanosaetaceae (Figure 9.2a) (Raskin et al., 1994; Zhu et al., 2012). However, the addition of granular activated carbon, combined with an in-depth study of these anaerobic granules in UASB reactors, revealed the potential of several *Methanosaeta* sp. to produce methane by reducing CO_2 by means of DIET (Morita et al., 2011; Liu et al., 2012; Rotaru et al., 2014). The high electrical conductivity is considered to stimulate DIET, a comparable effect may also have taken place on the electrode surfaces in the reactors in Chapter 6, as carbon felt has a similar high electrical conductivity. The high abundance, up to 10^{12} copies cm^{-2}, of Methanosaetaceae on these electrodes indicates their preference for these highly conductive materials, thus indicating the potential for CO_2 reduction by means of DIET, because of the (potential) availability of free electrons. Selective enrichment of *Methanosaeta* sp. on bamboo charcoal, which is considered to be highly conductive, depending on the carbonization temperature, also indicates their preference for

conductive materials and potential DIET (Jiang et al., 2004; Nomura et al., 2008; Belaid et al., 2013). However, the ability of *Methanosaeta* sp. to produce methane by reducing CO_2 via DIET has thus far only been demonstrated with ethanol as substrate and with a *Geobacter* sp. as syntrophic partner (Morita et al., 2011; Rotaru et al., 2014). Hence, further research will be required to unveil the exact potential of *Methanosaeta* in anaerobic digestion.

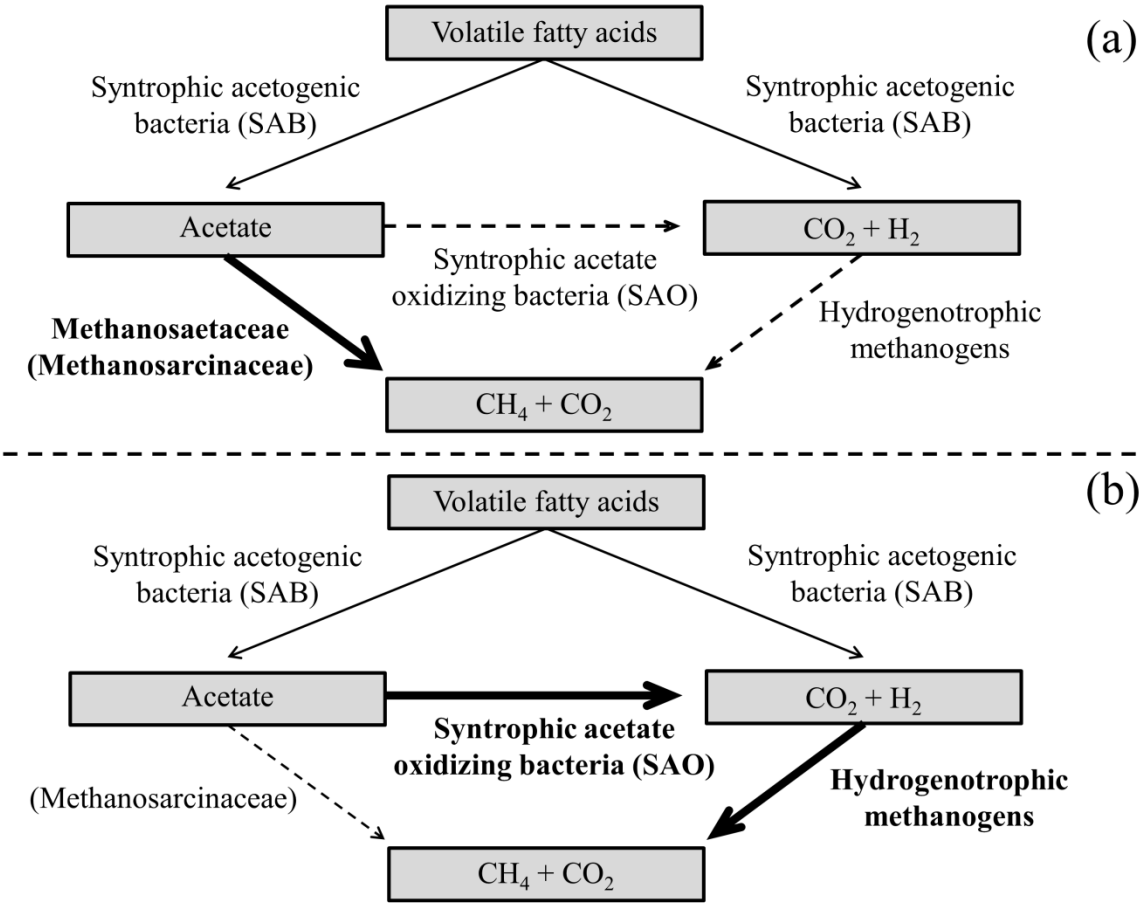

Figure 9.2 *Schematic overview of the dominant methanogenic pathway at (a) so-called 'easy' or optimal conditions and (b) 'heavy' or sub-optimal conditions.*

Methanobacteriales and Methanomicrobiales are able to use only the hydrogenotrophic pathway for methane production, whereas Methanosarcinaceae are mixotrophic, and, therefore, able to use both the acetoclastic and hydrogenotrophic pathway. It has been shown that the contribution of these three methanogenic populations becomes more important at sub-optimal conditions, at the expense of acetoclastic methane production by Methanosaetaceae (Figure 9.2b) (Jang et al.; Schnurer et al., 1999; Delbes et al., 2001; Shigematsu et al., 2003; McMahon et al., 2004; Conklin et al., 2006; Karakashev et al., 2006; Vavilin et al., 2008b; Blume et al., 2010; Munk et al., 2010; Shin et al., 2010; Song et al., 2010; Garcia et al., 2011; Hao et al., 2011; Shin et al., 2011; Karlsson et al., 2012; Merlino et al., 2012; Westerholm et

al., 2012b; Hao et al., 2013; Ho et al., 2013; Merlino et al., 2013; Williams et al., 2013; Lins et al., 2014; Sun et al., 2014). However, because of their mixotrophic character, the exact methane production pathway of the Methanosarcinaceae in AD often remains unknown. Notwithstanding, isotope fractionation has shown that Methanosarcinaceae use the hydrogenotrophic pathway, rather than the acetoclastic pathway, at sub-optimal conditions (Qu et al., 2009a; Lu et al., 2013a). This relates to the increased contribution of syntrophic acetate oxidation, as the main process for acetate removal, at sub-optimal conditions (Schnurer et al., 1999; Angenent et al., 2002; Karakashev et al., 2006; Hattori, 2008; Schnurer & Nordberg, 2008; Nettmann et al., 2010; Hao et al., 2011; Hao et al., 2013; Ho et al., 2013; Sun et al., 2014; Werner et al., 2014). However, in order to confirm the importance of syntrophic acetate production to acetate removal, as well as to determine the main methanogenic pathway, experiments with isotopically labelled substrates will be necessary. Hence, molecular techniques such as stable isotope probing and stable isotope fractionation will be required to reveal the actual acetate removal pathway in AD (Lueders et al., 2004; Penning et al., 2006; Laukenmann et al., 2010; Ho et al., 2013).

In conclusion, Methanosaetaceae perform acetoclastic methanogenesis at optimal conditions, whereas hydrogenotrophic methanogenesis becomes the dominant pathway at sub-optimal conditions, as carried out by Methanobacteriales, Methanomicrobiales and Methanosarcinaceae.

4. Microbial community management in anaerobic digestion: how can we manage them?

4.1. Operational management

In this research, several operational management strategies, including co-digestion (Chapter 2 and 3), feeding pattern variation (Chapter 4), and inoculum selection (Chapter 5), were applied to influence the microbial community, to increase methane production.

It was demonstrated in Chapter 2 and 3 that co-digestion of concentrated A-sludge and kitchen waste resulted in a stable methane production. Co-digestion of A-sludge and molasses led to the accumulation of VFA up to 25.3 g COD L^{-1}, yet, methane production was maintained, in contrast to the mono-digestion of molasses or kitchen waste. This indicates the stabilizing potential of A-sludge in AD, similar to the effects of manure or conventional waste activated sludge, two substrates widely used in AD (Fytili & Zabaniotou, 2008; Fountoulakis

et al., 2010; Fang et al., 2011a; Astals et al., 2012; Westerholm et al., 2012a; Borowski & Weatherley, 2013; Pitk et al., 2013). However, manure (co-)digestion often leads to ammonia inhibition of the AD process, and waste activated sludge has a low biodegradability, in the order of 30-50% (Angelidaki & Ahring, 1993; Hansen et al., 1998; Ekama et al., 2007; Verstraete & Vlaeminck, 2011; Cao & Pawlowski, 2012; Liu et al., 2013; Rajagopal et al., 2013). Due to its low Kjeldahl Nitrogen content (1.0 - 3.0 g N L^{-1}), compared to manure, and high biodegradability, up to 70%, A-sludge can be considered a perfect co-substrate to serve as stabilizing agent in AD.

The main stabilizing effect of A-sludge was attributed to nutrient supplementation, rather than additional inoculation or bioaugmentation of the digester. The high nutrient content of A-sludge, as observed in Chapter 2 and 3, is in relation to manure and waste activated sludge that also lead to an increased nutrient availability during AD. (Park et al., 2006; Fang et al., 2011a; Razaviarani et al., 2013). In Chapter 3, the sterilization of the A-sludge did not influence methane production in any of the treatments, which confirms the fact that A-sludge hand no inoculating effect on AD. However, the intermittent inoculation of AD by means of compost has been shown to increase biogas production (Neumann & Scherer, 2011). Furthermore, in Chapter 5, a clear effect of the initial inoculum on methane production and process stability was observed. The inability of A-sludge to act as an inoculum for AD, most likely relates to the fact that A-sludge operates at aerobic conditions and lower temperatures, compared to AD, whereas compost was operated at a much higher temperature (50-70 °C), and most likely contained micro-aerobic or even anaerobic zones (Neumann & Scherer, 2011). The inability of A-sludge for bioaugmentation of AD, also relates to several studies in which (unsuccessful) attempts were made to apply pure cultures for bioaugmentation in AD (Costa et al., 2012; Westerholm et al., 2012b; Fotidis et al., 2013). This indicates that successful bioaugmentation or inoculation strongly depends on the composition, characteristics and origin of the bioaugmentation source.

The selection of a suitable start inoculum did appear to have a strong effect on methane production and stress tolerance, as observed in Chapter 5. An high initial difference was observed between the different inocula, and despite the fact that after 77 days of operation the inocula evolved to a similar community, with the exception of one reactor, a different response in terms of ammonia tolerance was observed. Considering these results, several aspects should be taken into account when selecting a suitable inoculum to (re)start an anaerobic digester. First, a high methanogenic activity and stable operation prior to collection

from the full-scale plant should be warrantied. Methanogenic activity can be evaluated by means of a specific methanogenic activity (SMA) test or available operational data, and usually does not pose any problems. Stable operation prior to collection cannot be validated so easily. Stable methane production was observed in the full-scale plant from which the Vce sample (Chapter 5) originated, yet failure did take place after 30 days of operation at lab-scale conditions. An in-depth analysis of the operational data, such as pH, TAN, FA and residual VFA, prior to collection, might provide information on the stability of the inoculum. However, this is not conclusive, as AD systems have been known to maintain stable methane production at pH, TAN, FA and VFA values far outside the optimal range, as observed in the results of Chapter 8. Second, the 'history' of the inoculum in terms of operational conditions and substrate should be taken into account, as this will affect the selection of a suitable inoculum. For example, when looking for an inoculum to start a manure or slaughterhouse waste reactor, a sample should be selected from an anaerobic digester operating at high TAN and/or FA concentrations, as the microbial community in these kind of systems is already adapted. A similar approach should be applied for high salt and residual VFA concentrations (de Baere et al., 1984; Hashimoto, 1986; Angelidaki & Ahring, 1993; Feijoo et al., 1995; Calli et al., 2005a; Lefebvre et al., 2007; Schnurer & Nordberg, 2008; Spanheimer & Muller, 2008). Finally, microbial community analysis by means of amplicon sequencing of the candidate inoculum sample can be used to relate the inoculum to the AD-typing concept, as proposed in Chapter 8. A deviation from the three cluster concept in terms of bacterial composition and/or TAN, FA and temperature, which are the main factors determining the clustering, might point to an unbalanced or unstable inoculum. The VCE2 sample in Chapter 8, for instance, deviated from the clustering profile, as it clustered within Cluster 1, despite being thermophilic and containing a high TAN and FA concentration. This sample was used as inoculum in Chapter 5, and resulted in process failure after 30 days, thus confirming the former statement.

The results of Chapter 4 emphasized that the feeding pattern also contributes to the overall operational stability and stress tolerance of the AD process, although no direct effects on methane production were observed. A pulse feeding pattern increases overall stress tolerance in AD, which relates to the fact that a higher degree of variation of certain operational parameters is observed through time, thus leading to the stimulation of more micro-organisms, compared to a highly regular feeding pattern, keeping the microbial community 'motivated'. However, a precise balance should be maintained between a high pulse feeding

pattern, with varying composition, and constant feeding at constant low OLR values. A too high degree of pulse feeding may lead to organic overloading, which may drastically alter the methanogenic community of the digester, thus, causing process failure This relates to the aforementioned results that a stable methanogenic community contributes to process stability (Gujer & Zehnder, 1983; Appels et al., 2008; Chen et al., 2008; Ma et al., 2009; Gomez et al., 2011; Krakat et al., 2011; Chen et al., 2012; Ritari et al., 2012; Regueiro et al., 2014; Town et al., 2014).

In short, operational management in AD can take place through (co-)substrate choice, inoculum selection, and feeding pattern variation. However, the optimal strategy highly depends on the targeted AD system, and requires in-depth analysis of the composition of the average substrate to be digested, operational parameters to be anticipated in the anaerobic digester, and microbial community in the inoculum to be selected.

4.2. Technological management

Two technological strategies were applied to improve methane production and process stability in AD. These strategies were (1) the application of a cell potential by means of carbon felt electrodes (Chapter 6), and (2) active biomass retention by means of AnMBR units, using two different membrane fouling prevention strategies (Chapter 7).

The introduction of a (bio)electrochemical system in AD of molasses resulted in stable methane production at optimal conditions, in contrast to the control treatments that did not contain carbon felt electrodes, as observed in Chapter 6. However, neither a direct effect of the applied cell potential, nor of the resulting current, was observed. Several studies, however, reported a clear direct positive effect of the introduction of a (B)ES in AD, yet these experiments were carried out at higher cell potentials and/or did not include a suitable control (Vijayaraghavan & Sagar, 2010; Tartakovsky et al., 2011; Villano et al., 2011; Sasaki et al., 2013; Tartakovsky et al., 2013). The only effect of the applied cell potential observed in our study, was the increased abundance of Methanosarcinaceae in the reactor suspension, after removal of the electrodes, which was observed at the end of both Phase 2 and 3, indicating a hysteresis effect. Hence, the main positive effect of the electrode in the AD reactors, can be attributed to biomass retention on these electrodes. High abundances of Methanosaetaceae, up to 10^{12} copies cm^{-2}, were detected, which can be explained by their preference to attach to conductive materials (Nomura et al., 2008). In this specific case, this resulted in the retention

of an active community of Methanosaetaceae, despite the changing conditions in the reactor suspension, due to the feeding of the molasses, and therefore most likely explains the maintained stability of the methanogenesis process. In relation to this, in several mature anaerobic biofilm-based reactor systems a *Methanosaeta* sp. dominated methanogenic community was observed, thus confirming the results of Chapter 6 (Encina & Hidalgo, 2005; Fernandez et al., 2008; Ribas et al., 2009).

The application of an anaerobic membrane bioreactor (AnMBR) in Chapter 7 led to stable methane production, as long as diluted molasses were used. Fouling control by means of biogas recirculation appeared to be the best strategy to limit or even avoid membrane fouling, compared to a vibrating membrane. In all three reactors an overall dominance of Methanosaetaceae over Methanosarcinaceae was observed, reaching average values of 6.4 x $10^9 \pm 2.7 \times 10^9$, 1.5 x $10^{10} \pm 9.9 \times 10^9$ and 1.8 x $10^{10} \pm 9.1 \times 10^9$ copies g^{-1} in the HL-AnMBR, NV-AnMBR and V-AnMBR, respectively. Similar results were obtained in an other AnMBR reactor system, showing an overall dominance of *Methanosaeta* sp., most likely due to the high SRT (sludge retention time) values that were maintained in these AnMBR systems, although this also depends on the inoculum that was selected (Zamalloa et al., 2012). However, the constant high *Methanosaeta* sp. abundance in our study did not relate to a constant high methane production, as complete failure was observed in the HL-AnMBR after 46 days. This indicates that active retention of Methanosaetaceae in AD as such is insufficient to maintain high methane production and COD removal rates.

In conclusion, both the introduction of carbon felt electrodes and the application of an AnMBR unit resulted in stable constant methane production. In the case of the carbon felt electrodes, stable methane production was obtained irrespective of the applied cell potential. In the AnMBR system, however, the application of concentrated molasses as feed, resulted in processes failure, which indicates that active retention of Methanosaetaceae outside their optimal growth conditions only leads to stable methane production within a certain range.

5. Perspectives and future challenges

5.1. Methanosaetaceae vs. mixo- and hydrogenotrophic methanogens: a practical application

Anaerobic digestion is evolving from an organic waste treatment system to an energy factory. However, for AD to become a cost-effective technology, high methane production rates need

to be obtained. Hence, based on the obtained knowledge on the exact role of Methanosaetaceae and mixo- and hydrogenotrophic methanogens in AD, we propose a new 2-stage AD concept to maximize energy output and removal efficiency (Figure 9.3).

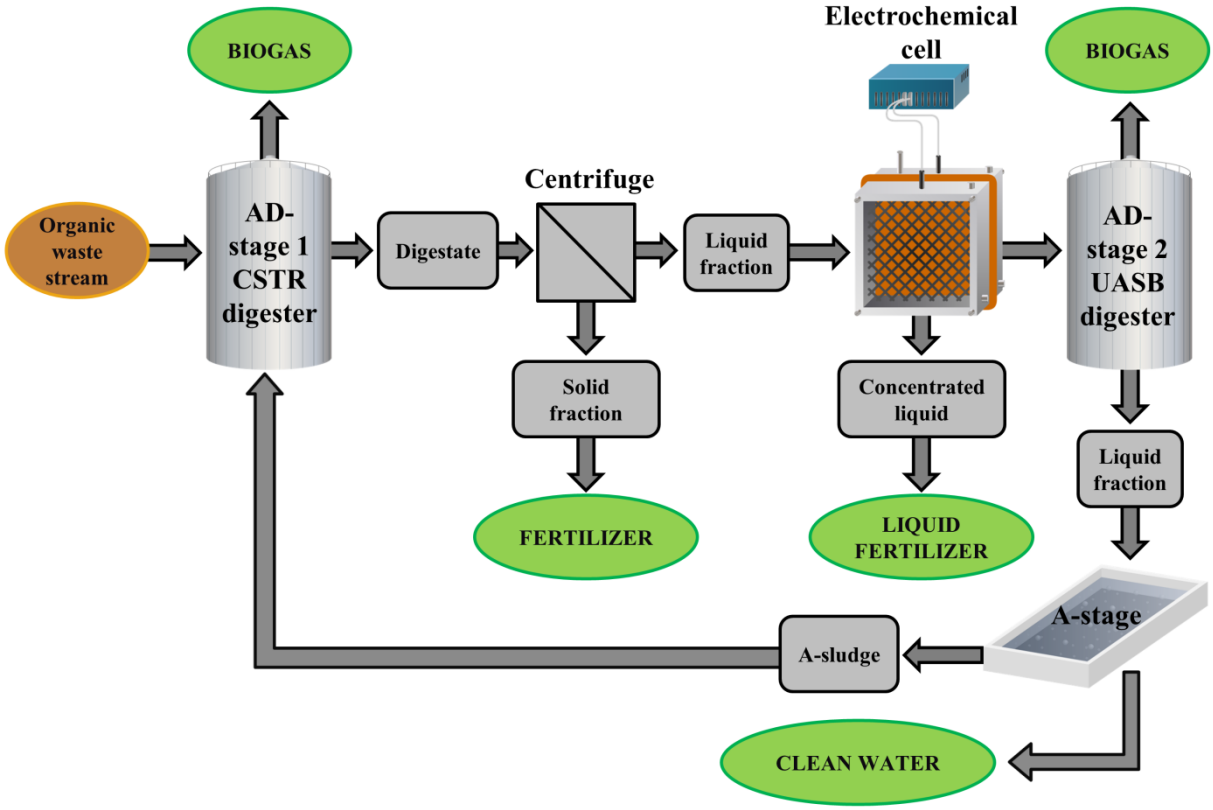

Figure 9.3 *Schematic overview of the 2-stage anaerobic digestion concept for maximal energy recovery and complete nutrient and water recovery.*

The first AD-stage is a high-loaded CSTR digester, with high OLR values, in which common (semi-)solid organic waste streams can be converted to biogas. The organic loading rate will be selected based on laboratory tests, and will vary, depending on the reactor system to be selected between 5-10 kg COD m^{-3} d^{-1} for common semi-solid CSTR digesters and between 10-15 kg COD m^{-3} d^{-1} or even higher for solid waste (DRANCO) digesters (Six & Debaere, 1992). This first AD-stage can, hence, be considered a highly-loaded first stage of the TPAD system in which (hydrogenotrophic) methanogenesis can still take place (Lv et al., 2010; Lv et al., 2013). This system could operate at thermophilic conditions, to increase hydrolysis rate, and at low SRT, to allow high loading rates (Veeken & Hamelers, 1999; Leven et al., 2007). Anaerobic digestion of manure, slaughterhouse waste, the organic fraction of municipal solid waste (OFMSW) and molasses or vinasses may, however, lead to high TAN and salt concentrations (Hansen et al., 1998; Alvarez & Liden, 2008; Satyawali & Balakrishnan, 2008;

El Hadj et al., 2009; Banks et al., 2011; Fang et al., 2011b; Zhang et al., 2012c; Borowski & Weatherley, 2013; Pitk et al., 2013). Hence, the main pathway for methane production in these reactor systems most likely is hydrogenotrophic methanogenesis, due to the fact that Methanosaetaceae are unable to maintain activity at high TAN and salt concentrations, which will lead to high H_2 partial pressure values (Schnurer et al., 1999; Calli et al., 2005a; Calli et al., 2005b; Karakashev et al., 2006; Schnurer & Nordberg, 2008; Vavilin et al., 2008b; Goberna et al., 2010). This then leads to high methane production rates, however, at the cost of increased residual VFA concentrations (Jang et al.; Schnurer et al., 1999; Delbes et al., 2001; Demirel & Scherer, 2008; Munk et al., 2010; Song et al., 2010; Retfalvi et al., 2011; Hao et al., 2013; Lins et al., 2014).

The presence of high levels of residual VFA, up to 15 g COD L^{-1} in the digestate of the first AD-stage, similar to the results of Chapter 6, however, still creates a high methane production potential. To guarantee the cost-effectiveness of the system, these residual VFA could be converted to methane as well, hence, a second AD-stage is implemented. This second AD stage is a UASB reactor in which the liquid fraction of the digestate after the first AD-stage is treated to allow efficient conversion of the residual VFA to biogas at low H_2 partial pressure values. Hence, the 2-stage AD system can be considered as a plug flow system, which allows higher treatment efficiency and process stability, especially for thermophilic systems (Lettinga, 1995). However, since the granular sludge in UASB reactors is dominated by Methanosaetaceae, additional steps are included to reduce the ammonia and salt concentration in the liquid phase (Macleod et al., 1990; Quarmby & Forster, 1995; Hulshoff Pol et al., 2004; Satoh et al., 2007). First, centrifugation of the digestate from the digester of the first AD-stage is carried out to obtain a separation of the solid and liquid fraction. The solid fraction can then be used as fertilizer, however, care should be taken concerning heavy metal accumulation (Walsh et al., 2012; Vaneeckhaute et al., 2013). Second, the liquid fraction after centrifugation requires a reduction in TAN and salt concentration, which can be carried out by means of electrochemical cell, to which power can be provided by burning the biogas (Desloover et al., 2012; Zhao et al., 2013). The concentrated TAN and salt solution can then be used as liquid fertilizer. The liquid fraction of the digestate can then be further treated in the UASB reactor, which can be operated at low HRT (hydraulic rentention time), if necessary, provided that a sufficient removal efficiency of the TAN and salts was obtained in the electrochemical cell. Hence, additional biogas production and treatment of the liquid fraction is acquired in the second AD-stage.

The effluent of the UASB reactor, however, is not yet suitable to be discharged in natural water bodies, as it still contains too high COD and TAN concentrations (Seghezzo et al., 1998; Chong et al., 2012). Hence, the introduction of a post-treatment aerobic polishing step is required. In this case, an A/B system is applied, which allows the removal of residual COD in the UASB effluent in the A-stage, whereas TAN is removed in the B-stage. Due to the high TAN-content and low COD-content, especially after the A-stage, the application of anammox in the B-stage is the appropriate technology (Verstraete & Vlaeminck, 2011; Bernat et al., 2012). After the A/B system, the purified water can be discharged into the environment, whereas the A-sludge, produced in the A-stage, is a suitable substrate for co-digestion in the first AD-stage, as demonstrated in Chapter 2 and 3.

In conclusion, this application leads to a maximal energy recovery by means of 2 AD-stages and the production of highly degradable A-sludge, since a higher process efficiency and stability can be obtained. Furthermore, clean water and organic fertilizer (both solid and liquid) are generated, which coincides with a complete nutrient and water recovery.

5.2. The anaerobic digestion microbiome: what's next?

In Chapter 8, in-depth analysis of 38 samples from 29 full-scale plants resulted in the discovery of (1) the dominance of three main phyla Firmicutes, Bacteroidetes and Proteobacteria, and (2) the clustering of these samples in three clusters, that were named AD-types. These AD-types were determined by TAN concentration, FA concentration and temperature, and characterized by an increased abundance of the Bacteroidales, Clostridiales and Lactobacillales, respectively. Hence, this study provided a clear view on the main factors determining the microbial community in AD, which may serve as a basis for engineering the microbial community in AD, to increase methane production and process stability.

The exact contribution of the potential AD-typing concept towards direct engineering of AD, however, still requires handling of several challenges/problems. First, the main factors determining the clusters and dominant bacterial groups have been determined, yet in most cases the exact role of these groups in the AD process remains unknown, or they possess a very wide metabolic potential, making it impossible to attribute them to a specific pathway or stage (Nelson et al., 2011; Lee et al., 2012; Sundberg et al., 2013; Vanwonterghem et al., 2014). Second, further validation of the clustering results is required, using amplicon sequencing and/or real-time PCR data from other full-scale plants, as well as other clustering

methods and other factors that may influence these results (Koren et al., 2013). Despite the fact that a large dataset of 38 samples from 29 full-scale plants was used, which surpasses the amount of samples or plants that was surveyed in several other similar studies, further validation with other samples from stable operating full-scale plants could confirm, and even extend or refine the AD-type concept (Narihiro et al., 2009; Riviere et al., 2009; Sundberg et al., 2013; St-Pierre & Wright, 2014). Third, the clustering accounted for samples from full-scale digesters with stable biogas production. Comparison of samples from lab-scale reactors and failing full-scale plants would clarify whether (1) the microbial community in lab-scale reactors corresponds to full-scale plants, and (2) the microbial community in failing or failed plants deviates from the AD-type concept. The discrepancy in Methanosarcinaceae abundance between full-scale and lab-scale system already points to a difference in microbial community between full-scale plants and lab-scale reactors, yet, the amplicon sequencing results from the lab-scale reactors in Chapter 5 showed the dominance of the same three phyla, as observed in the full-scale plants. In Chapter 8 cluster 1 contained 8 samples, mainly from mesophilic sludge digesters and UASB reactors, with the exception of the VCE2 sample, which originated from a thermophilic AD plant at high TAN and FA concentrations. The dissimilarity of the operational parameters of this sample, in comparison to the other samples in this cluster, indicates that the VCE plant was operating at sub-optimal conditions at the time of sampling. This result was confirmed by the fact that application of this sample as an inoculum for a lab-scale digester in Chapter 5 resulted in complete process failure after 30 days of operation.

In retrospect, the identification of the three potential AD-types can serve as a basis for unravelling the AD microbiome. Further in-depth research is, however, required to determine the exact role of the core micro-organisms in each cluster to allow microbial community based engineering of AD ecosystems.

5.3. Abundance vs. activity in anaerobic digestion

In the last few years, microbial community analysis in AD, using high-throughput sequencing methods, increased exponentially, resulting in a general overview of the microbial consortium involved in the different stages of the AD process. However, as these methods are, with the exception of fluorescent in-situ hybridization (FISH), DNA-based, exclusively information concerning the abundance of the micro-organisms, and no information concerning their activity, is presented. The exclusive application of DNA-based methods, hence, leaves several

key aspects of the microbial community in AD out of account. First, it has been observed in the few studies performing RNA- protein- and/or metabolite-based community analysis that the methanogens had a much higher activity than expected, based on their absolute or relative abundance (Abram et al., 2011; Zakrzewski et al., 2012; Hanreich et al., 2013; Vanwonterghem et al., 2014). Second, the application of RNA- protein- and/or metabolite-based methods estimates the real contribution of specific groups to certain pathways, thus, enabling a better understanding of the micro-organisms involved in specific processes, which may lead to more thorough process engineering of AD. Third, the response time on RNA, protein or metabolite level to changes in operational parameters is much higher than on DNA level, especially for the slow growing methanogens, which allows the possibility for a more accurate response, e.g. an adjustment of certain operational parameters, to instabilities in the microbial community (Marzorati et al., 2013).

The application of DNA-based methods has led to the discovery of the overall picture of the microbial community in AD, allowing, to a certain extent, knowledge-based process engineering of AD, as it can no longer be considered a 'black box'. However, RNA, protein and metabolite based methods, such as metatranscriptomics, metaproteomics and metabolomics, are essential to estimate the effective metabolic activity of the microbial community in AD, thus allowing more in-depth process control.

6. Conclusions

Anaerobic digestion can be considered a key technology for renewable energy production in the present and future bio-based economy. However, despite its widespread application, the apparent lack of knowledge concerning the microbial community in AD still poses problems, as it causes the implementation of ill-informed operational decisions, which may lead to subsequent process failure. In this research, several strategies to improve biogas production and process stability, both on an operational and technological level, were successfully implemented.

Co-digestion of A-sludge with kitchen waste and molasses, two substrates that usually cause process failure during mono-digestion, resulted in stable methane production. The application of a pulse feeding pattern, together with the selection of a suitable inoculum proved to be highly important to maintain process stability, and increase stress tolerance. The introduction of a bioelectrochemical system in AD led to an increased methane production and process

stability during molasses digestion, yet, the main stabilizing effect was attributed to biomass retention. The implementation of an anaerobic membrane bioreactor for the treatment of molasses wastewater resulted in stable methane production, as long as diluted molasses was used and biogas recirculation was implemented as the main mechanism for fouling prevention.

In-depth analysis of the microbial community of several full-scale AD plants revealed the overall dominance of three main phyla and resulted in a potential identification of three bacterial clusters, that could be considered as AD-types. However, further research will be required to validated the actual existence of these three clusters in AD. The main drivers determining the microbial community in AD were identified as total ammonia concentration, free ammonia concentration and digester temperature. A comparison of the lab-scale reactor results with full-scale plant microbial community analysis results confirmed the overall importance of Methanosaetaceae as the main (acetoclastic) methanogens in AD. However, the assumed important role of Methanosarcinaceae, as observed at lab-scale conditions, could not be confirmed by full-scale installation results. Hence, instead of Methanosarcinaceae, the Methanobacteriales are to be considered as the main drivers of the so-called high-rate AD.

In short, this research demonstrated the potential of several strategies to improve methane production and process stability. At all times, Methanosaetaceae proved to be the crucial methanogens to ensure process stability. Although high-rate AD requires the presence of a stable Methanobacteriales community, sustaining a stable active community of Methanosaetaceae is of vital importance to maintain an efficient AD process.

ABSTRACT

The production of renewable energy from organic waste streams is one of the most important aspects in the concept of sustainable development. Anaerobic digestion can be considered one of the main techniques to treat organic waste streams, allowing both waste stabilization and renewable energy production in the form of biogas. Its widespread application on full-scale relates to the fact that anaerobic digestion has, apart from biogas production and organic waste stabilization, several other advantages over alternative biological processes, e.g. a low cell yield, a high organic loading rate, limited nutrient demands, and low costs for operation and maintenance of the reactor system. The methanogenic archaea are responsible for the final and critical step of anaerobic digestion, as they produce valuable methane. One of the major drawbacks of anaerobic digestion is, however, the sensitivity of the methanogenic community to different environmental factors or stressors.

At this point, our knowledge of the microbial community taking care of the different stages in anaerobic digestion is still limited and, therefore, anaerobic digestion can still be considered a 'black box'. Indeed, our knowledge of the bacterial community is restricted to the attribution of the first three steps in anaerobic digestion, i.e. hydrolysis, acidogenesis and acetogenesis. Although several key populations have already been identified, the exact contribution of the different bacterial phyla remains, however, to be elucidated. Methanogenesis, the last step, is carried out by archaea. The methanogenic community can be divided into two different groups, related to their main methanogenic pathway, i.e. hydrogenotrophic and acetoclastic methanogens. Thus far, only two genera, *Methanosaeta* and *Methanosarcina*, are reported to be able to carry out acetoclastic methanogenesis. Due to a distinct difference in physiology, morphology and metabolic potential, these two genera are expected to occupy different niches in anaerobic digestion. However, up until now, little is known about the specific contribution of both genera to methanogenesis in anaerobic digestion.

The main objective of this research was to unravel the 'black box' of anaerobic digestion to allow better and more solid process engineering. Several strategies were applied to improve biogas production and process stability, by (in)directly influencing the microbial community. A main focus was placed on the methanogenic community, as methanogenesis can be considered the weak link in the chain, because of the sensitivity of the methanogenic community to different environmental factors. However, to reach stable methane production,

a close interaction between the bacterial and methanogenic community is required, hence, the bacterial community was also examined in terms of composition and organization.

In Chapter 2, A-sludge originating from the A-stage of the 'Adsorptions-Belebungsverfahren', was co-digested with kitchen waste to increase biogas production. This Fe-rich A-sludge appeared to be a suitable co-substrate for kitchen waste, as methane production rate values of 1.15 ± 0.22 and 1.12 ± 0.28 L L^{-1} d^{-1} were obtained during mesophilic and thermophilic co-digestion, respectively, of a feed-mixture consisting of 15% kitchen waste and 85% A-sludge. Mono-digestion of kitchen waste resulted in process failure. The thermophilic process led to higher residual volatile fatty acid concentrations, up to 2070 mg COD L^{-1}, hence, the mesophilic process can be considered the most 'stable'.

The optimal combination of A-sludge and kitchen waste served as a basis for the co-digestion of A-sludge with kitchen waste or molasses at mesophilic conditions in Chapter 3. In this chapter the objective was to evaluate the exact stabilizing mechanism of A-sludge as co-substrate in anaerobic digestion. Co-digestion of kitchen waste and molasses with A-sludge resulted in stable methane production, as values up to 1.53 L L^{-1} d^{-1} for kitchen waste and 1.01 L L^{-1} d^{-1} for molasses were obtained. The stabilizing effect of A-sludge in anaerobic digestion could not be attributed to bioaugmentation, despite its indigenous methanogenic activity, and therefore was dominated by nutrient addition. Methanosaetaceae maintained high copy numbers, between 10^9 and 10^{10} copies g^{-1} sludge, as long as optimal conditions were maintained, irrespective of the selected (co-)substrates. However, an increase in volatile fatty acids and a decrease in pH resulted in a decreased abundance of Methanosaetaceae.

In Chapter 4, a different feeding pattern was applied to obtain a higher degree of functional stability by (in)directly changing the evenness, dynamics and richness of the bacterial community. A short-term stress test revealed that pulse feeding leads to a higher tolerance of the digester to an organic shock load of 8 g COD L^{-1} and total ammonia levels up to 8000 mg N L^{-1}. The bacterial community showed a high degree of dynamics over time, yet the methanogenic community remained constant. These results suggest that the regular application of a limited pulse of organic material and/or a variation in the substrate composition might promote higher functional stability in anaerobic digestion.

In Chapter 2-4, the anaerobic sludge originating from the same sludge digester was used as inoculum. The contribution of the inoculum to stable methane production and stress tolerance was investigated in Chapter 5. A different response in terms of start-up efficiency and ammonium tolerance was observed between the different inocula. *Methanosaeta* was the

dominant acetoclastic methanogen, yet *Methanosarcina* increased in abundance at elevated ammonium concentrations. A shift from a Firmicutes to a Proteobacteria dominated bacterial community was observed in failing digesters. Methane production was strongly positively correlated with Methanosaetaceae, but with several bacterial populations as well. Overall, these results indicated the importance of inoculum selection to ensure stable operation and stress tolerance in anaerobic digestion.

In several studies, the positive effect of a bioelectrochemical system on biogas production in anaerobic digestion is described, however, the main mechanism behind this remained unsolicited, and primary controls were not executed. In Chapter 6, the stabilizing ability of a bioelectrochemical system for molasses digestion was evaluated in a 154 days experiment. A high abundance of *Methanosaeta* was detected on the electrodes, however, irrespective of the applied cell potential. This study demonstrated that, in addition to other studies reporting only an increase in methane production, a bioelectrochemical system can also remediate anaerobic digestion systems that exhibited process failure. However, the lack of difference between current driven and open circuit systems indicates that the key impact is through biomass retention, especially Methanosaetaceae, rather than electrochemical interaction with the electrodes.

Anaerobic membrane bioreactors with different fouling prevention strategies, i.e. biogas recirculation or membrane vibration, were applied to increase the retention of slow growing methanogens in Chapter 7. Biogas recirculation was the best mechanism to avoid membrane fouling, while the trans membrane pressures in the vibrating membrane bioreactor increased over time, due to cake layer formation. Stable methane production, up to 2.05 L L^{-1} d^{-1} and a concomitant COD removal of 94.4%, were obtained, only when diluted molasses were used, since concentrated molasses resulted in process failure. Real-time PCR results revealed a clear dominance of Methanosaetaceae over Methanosarcinaceae as the main acetoclastic methanogens in both anaerobic membrane bioreactor systems.

In Chapter 8, an extensive evaluation of 38 samples from 29 full-scale anaerobic digestion plants was carried out to relate operational parameters to microbial community composition and organization. The bacterial community was dominated by representatives of the Firmicutes, Bacteroidetes and Proteobacteria, covering 86.1 ± 10.7% of the total bacterial community. Acetoclastic methanogenesis was dominated by Methanosaetaceae, yet, only Methanobacteriales significantly positively correlated to biogas production. Three potential clusters, that could be considered as ‘AD-types’, were identified. These so-called ‘AD-types’

were determined by total ammonia concentration, free ammonia concentration and temperature, and characterized by an increased abundance of the Bacteroidales, Clostridiales and Lactobacillales, respectively. The identification of these three potential AD-types may serve as a basis for directly engineering the microbial community in anaerobic digestion. However, further research will be required to validated the actual existence of these three clusters in AD.

This research demonstrated the potential of several operational and technological strategies to improve biogas production and process stability in anaerobic digestion. Stable anaerobic digestion hosts a static methanogenic community, as long as evolving operational parameters or substrate composition do not influence the optimal conditions for methanogenesis, and an ever dynamic bacterial community. Methanosaetaceae are the uncontested dominant methanogens in anaerobic digestion, irrespective of the substrate, operational conditions or reactor configuration. However, increasing ammonium, salt and volatile fatty acid concentrations cause a shift from acetoclastic methanogenesis by Methanosaetaceae to hydrogenotrophic methanogenesis. Comparison of the lab-scale reactor results with full-scale plant microbial community analysis results showed a high similarity on bacterial level. However, at 'deteriorating' conditions at lab-scale a transition to a Methanosarcinaceae dominated methanogenesis was observed, while this shift could not be observed in full-scale plants. Hence, instead of Methanosarcinaceae, the Methanobacteriales are to be considered as the main drivers of so-called high-rate anaerobic digestion. The identification of the three AD-types can serve as a basis for unravelling the anaerobic digestion microbiome. Further in-depth research, however, will be required to determine the exact role of the core micro-organisms in each cluster to allow microbial community based engineering of anaerobic digestion ecosystems. The application of RNA, protein and metabolite based methods will be essential to estimate the effective metabolic activity of the microbial community in anaerobic digestion, thus, allowing more in-depth process control and further unravelling of the anaerobic digestion 'black box'.

SAMENVATTING

De productie van hernieuwbare energie, gebruik makend van organische afvalstromen, is één van de meest cruciale aspecten binnen het concept van duurzame ontwikkeling. Anaerobe vergisting wordt beschouwd als een van de meest belangrijke technologieën voor de behandeling van organische afvalstromen, waarbij zowel stabilisatie van het organisch afval, als de productie van hernieuwbare energie, onder de vorm van biogas, worden beoogd. De wijdverbreide toepassing van deze technologie op volle schaal kan verklaard worden door het feit dat anaerobe vergisting, naast biogas productie en stabilisatie van het organisch afval, verschillende andere voordelen bezit in vergelijking met andere biologische processen, zoals een lage cel opbrengst, hoge organische belasting, beperkte nutriënten vereisten en lage operationele en onderhoudskosten van de reactor. De methanogene archaea zijn verantwoordelijk voor de finale en meest cruciale stap van het vergistingsproces, aangezien ze instaan voor de productie van het energierijke methaan. Eén van de nadelen van anaerobe vergisting is echter de sensitiviteit van de methanogene gemeenschap ten opzichte van verschillende omgevingsfactoren en stressoren.

Op dit moment is onze kennis van de microbiële gemeenschap die instaat voor de verschillende fasen van het anaeroob vergistingsproces nog vrij beperkt. Daardoor wordt anaerobe vergisting nog steeds beschouwd als een ‘black box’ proces. De huidige kennis betreffende de bacteriële gemeenschap in anaerobe vergisting is beperkt tot de toekenning van de eerste drie fasen, namelijk hydrolyse, acidogenese en acetogenese. Hoewel reeds verschillende sleutelgemeenschappen werden geïdentificeerd, is de exacte bijdrage van de verschillende bacteriële fyla echter nog niet opgehelderd. De laatste fase in anaerobe vergisting, namelijk methanogenese, wordt uitgevoerd door archaea. De methanogene gemeenschap kan ingedeeld worden in twee groepen, naargelang de methanogene pathway, namelijk hydrogenotrofe en acetoclastische methanogenen. Tot op heden werden slechts twee genera, namelijk *Methanosaeta* en *Methanosarcina*, geïdentificeerd die in staat zijn methaan te produceren via de acetoclastische pathway. Vanwege hun groot verschil in fysiologie, morfologie en metabolisch potentieel, wordt verondersteld dat deze twee genera verschillende niches innemen in anaerobe vergisting. Tot op heden is echter weinig gekend betreffende de contributie van beide genera ten aanzien van de methanogenese in anaerobe vergisting.

Dit onderzoek had als belangrijkste objectief het in kaart brengen van de ‘black box’ van anaerobe vergisting om het vergistingsproces meer gefundeerd te kunnen sturen.

Verschillende strategieën werden toegepast om de biogas productie en proces stabiliteit te verbeteren, door (in)directe sturing van de microbiële gemeenschap. Hierbij werd in de eerste plaats gefocust op de methanogene gemeenschap, aangezien deze het meest kwetsbaar is voor veranderingen van de omgevingsfactoren. Om echter een stabiele methaanproductie te verkrijgen, is een nauwe interactie vereist tussen de bacteriële en methanogene gemeenschap. Daarom werden ook de samenstelling en organisatie van de bacteriële gemeenschap geëvalueerd.

In Hoofdstuk 2 werd A-slib, afkomstig van de A-trap van de 'Adsorptions-Belebungsverfahren', co-vergist met keukenafval om een verhoogde biogas productie te verkrijgen. Dit Fe-rijk A-slib bleek een geschikt substraat te zijn voor co-vergisting met keukenafval, hetgeen resulteerde in methaan productie snelheden van 1,15 ± 0.22 en 1,12 ± 0,28 L L^{-1} d^{-1} tijdens mesofiele en thermofiele co-vergisting, respectievelijk, van een mengsel bestaande uit 15% keukenafval en 85% A-slib. Mono-vergisting van keukenafval resulteerde in proces inhibitie. Het thermofiele vergistingsproces leidde tot hogere residuele vluchtige vetzuur concentraties tot 2070 mg COD L^{-1}, waardoor het mesofiele proces als het meest 'stabiel' werd beschouwd.

De optimale combinatie van A-slib en keukenafval werd in Hoofdstuk 3 gebruikt als basis voor de co-vergisting van A-slib met keukenafval of melasse bij mesofiele condities. Het doel van dit hoofdstuk was om na te gaan wat het exacte stabiliserend effect was van A-slib tijdens co-vergisting in anaerobe vergisting. Co-vergisting van A-slib met keukenafval en melasse resulteerde in een stabiele methaanproductie, waarbij waarden van 1,53 L L^{-1} d^{-1} voor keukenafval en 1,01 L L^{-1} d^{-1} voor melasse werden verkregen. Het stabiliserend effect van A-slib kon niet toegewezen worden aan bioaugmentatie, ondanks de endogene methanogene activiteit in het A-slib, en werd daarom toegeschreven aan nutriënt additie. De Methanosaetaceae behielden een hoge abundantie, met waarden tussen 10^9 en 10^{10} kopieën g^{-1} slib, onafhankelijk van het gekozen substraat, zolang optimale omstandigheden behouden werden. Een toename in vluchtige vetzuren resulteerde echter in een afname in de Methanosaetaceae abundantie.

In Hoofdstuk 4 werd een verschillend voedingspatroon toegepast om een hogere functionele stabiliteit te bereiken, door (in)direct de gelijkheid, dynamica en rijkheid van de bacteriële gemeenschap aan te passen. Op basis van een korte-termijn stress test kon afgeleid worden dat een gepulseerd voedingspatroon een hogere tolerantie van de vergister voor een 'shock' belasting van 8 g COD L^{-1} en een totale ammonium concentratie van 8000 mg N L^{-1} kon

teweegbrengen. De bacteriële gemeenschap vertoonde hierbij een verhoogde dynamica in functie van de tijd, terwijl de methanogene gemeenschap gelijk bleef. Deze resultaten suggereren dat de regelmatige applicatie van een gelimiteerde puls van organisch materiaal of een verandering in substraat samenstelling een hogere functionele stabiliteit kan teweegbrengen in anaerobe vergisting.

In Hoofdstuk 2-4 werd anaeroob slib afkomstig van dezelfde slibvergister gebruikt als inoculum. De bijdrage van het inoculum tot stabiele methaan productie en stress tolerantie werd onderzocht in Hoofdstuk 5. Een verschillende respons met betrekking tot opstart efficiëntie en ammonium tolerantie kon geobserveerd worden tussen de verschillende inocula. *Methanosaeta* was de dominante acetoclastische methanogeen, doch *Methanosarcina* nam toe in abundantie bij hogere ammonium concentraties. Een verschuiving in dominantie van een Firmicutes naar een Proteobacteria gedomineerde bacteriële gemeenschap werd waargenomen in falende vergisters. Methaan productie was sterk positief gecorreleerd met de Methanosaetaceae abundantie, eveneens met verschillende bacteriële populaties. Deze resultaten tonen het belang aan van de selectie van een geschikt inoculum om een stabiele operatie en stress tolerantie te verzekeren in anaerobe vergisting.

In verschillende studies werd het positief effect van een bioelektrochemisch system op de biogas productie in anaerobe vergisting beschreven. Het exacte mechanisme hierachter werd echter niet onderzocht en negatieve controles werden vaak niet in beschouwing genomen. In Hoofdstuk 6 werd het stabiliserend effect van een bioelektrochemisch systeem tijdens de anaerobe vergisting van melasse onderzocht in een experiment van 154 dagen. Een hoge Methanosaetaceae abundantie werd geobserveerd op de elektroden, onafhankelijk van de toegepaste cel potentiaal. Deze studie toonde aan dat, in tegenstelling tot andere studies die enkel een toename in methaan productie vaststelden, een bioelektrochemisch systeem ook toegepast kan worden om anaerobe vergisting te remediëren. De afwezigheid van een verschil tussen systemen met een opgelegde cel potentiaal en de open kring systemen bevestigt dat het belangrijkste effect de retentie van biomassa is, vooral van Methanosaetaceae, eerder dan elektrochemische interactie met de elektroden.

Anaerobe membraan bioreactoren met verschillende fouling preventie strategieën, namelijk biogas recirculatie of membraan vibratie, werden toegepast in Hoofdstuk 7 om een verhoogde retentie te verkrijgen van de traag groeiende methanogenen. Biogas recirculatie bleek het meest geschikt om membraan fouling te vermijden, terwijl de trans membraan druk toenam in de membraan bioreactor met vibrerend membraan, ten gevolge van koekvorming. Een stabiele

methaanproductie van 2,05 L L^{-1} d^{-1} en een COD verwijderingsefficiëntie van 94,4 procent werd bereikt tijdens de vergisting van verdunde melasse, terwijl geconcentreerde melasse leidde tot proces inhibitie. Real-time PCR resultaten toonden een duidelijke dominantie aan van de Methanosaetaceae, in vergelijking met de Methanosarcinaceae, als de voornaamste acetoclastische methanogenen in beide anaerobe membraan bioreactor systemen.

In Hoofdstuk 8 werd een uitgebreide evaluatie van 38 stalen van 29 volle schaal anaerobe vergistingsinstallaties uitgevoerd om operationele parameters te relateren aan de samenstelling en organisatie van de microbiële gemeenschap. De bacteriële gemeenschap werd gedomineerd door de Firmicutes, Bacteroidetes en Proteobacteria, die in totaal 86,1 ± 10,7% van de bacteriële gemeenschap vertegenwoordigden. Acetoclastische methanogenese werd gedomineerd door Methanosaetaceae, doch enkel Methanobacteriales vertoonden een significante positieve correlatie met biogas productie. Drie potentiële clusters, die als 'AD-types' beschouwd werden, konden geïdentificeerd worden. Deze zogenaamde 'AD-types' werden bepaald door de totale ammonium concentratie, vrije ammoniak concentratie en de temperatuur, en gekarakteriseerd door een verhoogde abundantie van, respectievelijk, de Bacteroidales, Clostridiales en Lactobacillales. De identificatie van deze drie potentiële AD-types kan aangewend worden als basis om de microbiële gemeenschap in anaerobe vergisting meer gefundeerd te sturen. Verder onderzoek is weliswaar vereist om de validiteit van deze drie clusters in anaerobe vergisting te bevestigen.

Dit onderzoek toonde het potentieel aan van verschillende operationele en technologische strategieën om biogas productie en proces stabiliteit in anaerobe vergisting te verbeteren. Stabiele anaerobe vergisting bevat een statische methanogene gemeenschap, zolang de veranderende operationele parameters of substraat samenstelling de optimale condities voor methanogenese niet verstoren, evenals een (sterk) dynamische bacteriële gemeenschap. Methanosaetaceae kunnen beschouwd worden als de onomstreden dominante methanogenen in anaerobe vergisting, onafhankelijk van het substraat, de operationele condities of de reactor configuratie. Een toename in de concentratie aan ammonium, zout en vluchtige vetzuren veroorzaakt echter een verschuiving van acetoclastische methanogenese door de Methanosaetaceae naar hydrogenotrofe methanogenese. Een vergelijking tussen de reactoren op laboratorium schaal en de volle schaal microbiële gemeenschap analyse resultaten wees op een hoge similariteit op bacterieel niveau. Onder suboptimale omstandigheden werd in de reactoren op laboratorium schaal een transitie naar een Methanosarcinaceae gedomineerde acetoclastische gemeenschap geobserveerd, wat echter niet kon vastgesteld worden in volle

schaal installaties. Dit in acht nemend, dienen de Methanobacteriales, eerder dan de Methanosarcinaceae beschouwd te worden als de echte drivers van de zogenaamde high-rate anaerobe vergisting. De identificatie van drie AD-types kan als basis dienen om het microbioom van de anaerobe vergisting verder in kaart te brengen. Bijkomend onderzoek is echter vereist om de exacte rol van de kern micro-organismen in elke cluster te bepalen om de sturing van anaerobe vergisting op basis van microbiële parameters mogelijk te maken. De toepassing van RNA, proteïne en metaboliet gebaseerde methodes zal noodzakelijk zijn om de effectieve metabolische activiteit van de microbiële gemeenschap in de anaerobe gemeenschap in te schatten, en aldus meer gefundeerde proces controle en verdere ontrafeling van de anaerobe vergisting 'black box' te realiseren.

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Zhang, W.W., Culley, D.E., Nie, L., Brockman, F.J. 2006. DNA microarray analysis of anaerobic *Methanosarcina barkeri* reveals responses to heat shock and air exposure**.** Journal of Industrial Microbiology & Biotechnology, 33 (9), 784-790.

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Zhao, R., Satpradit, O., Rijnaarts, H.H.M., Biesheuvel, P.M., van der Wal, A. 2013. Optimization of salt adsorption rate in membrane capacitive deionization**.** Water Research, 47 (5), 1941-1952.

Zheng, D., Angenent, L.T., Raskin, L. 2006. Monitoring granule formation in anaerobic upflow bioreactors using oligonucleotide hybridization probes**.** Biotechnology and Bioengineering, 94 (3), 458-472.

Zhu, J.X., Zheng, H.J., Ai, G.M., Zhang, G.S., Liu, D., Liu, X.L., Dong, X.Z. 2012. The Genome Characteristics and Predicted Function of Methyl-Group Oxidation Pathway in the Obligate Aceticlastic Methanogens, *Methanosaeta* spp**.** Plos One, 7 (5).

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CURRICULUM VITAE

Personal Information

Name:	Jo De Vrieze
Born:	June 1st 1987, Eeklo, Belgium
Nationality:	Belgian
Marital status:	Legal cohabitation with Jana Wijnsouw
Contact information:	Reytstraat 169, 9700 Oudenaarde, Belgium Jo.DeVrieze@ugent.be devriezejo@gmail.com

Academic background and education

2010- present **PhD Candidate in Applied Biological Sciences**
Ghent University, Belgium
Funding: Ghent University (Assistant – 2010-2014)
Promoters: Prof. Dr. ir. Nico Boon and Prof. em. Dr. ir. Willy Verstraete
Thesis: "*Methanosaeta* vs. *Methanosarcina* in anaerobic digestion: the quest for enhanced biogas production."

2005-2010 **MSc./BSc. Bioscience Engineering: Environmental Technology**
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
MSc. Thesis: "Evolutionary ecotoxicology under field conditions: the potential and realized micro-evolutionary response as a consequence of cadmium exposure in a natural *Daphnia magna* population."
Laboratory of Environmental Toxicology and Aquatic Ecology, Department of Applied Ecology and Environmental Biology, Ghent University, Ghent, Belgium
Promoters: Prof. Dr. ir. Karel De Schamphelaere and Prof. Dr. Colin Janssen

1999-2005 **High School**, College O.-L.-V.-ten-Doorn, Eeklo, Belgium: Science – Mathematics (8hr).

Expertise

- Microbial ecology and technology of anaerobic digestion.
- Molecular techniques and analysis of environmental microbial ecosystems.
- Innovative treatment of wastewater and organic waste in general.
- Bioelectrochemical systems in anaerobic digestion ecosystems.

Professional activities during PhD research

Research supervision

Tutor of master thesis students

- Donaat Mortelmans (2013-2014), 'Study of the causes of inefficient use of biomass in full scale anaerobic digestion'
- Wim Kegels (2013-2014), 'The stabilizing effect of sulphate reducing bacteria in anaerobic digestion'
- Arne Braems (2012-2013), 'Short-circuiting of microbial aggregates in anaerobic digestion'
- Kristof Plovie (2012-2013), 'Valorisation of concentrated A-sludge by means of anaerobic digestion'
- Charlotte Imschoot (2012-2013), 'Anaerobic membrane bioreactor configurations for the anaerobic digestion of molasses'
- Maria João Cardoso Jacinto (2012), 'Enhanced organics recovery from wastewater via a membrane assisted A-stage'
- Sylvia Gildemyn (2011-2012), 'Valorisation of bio-refinery waste streams by means of electro-assisted anaerobic digestion'
- Jens Van den Brande (2011-2012), 'Anaerobic membrane bioreactors for the digestion of waste streams in the bio-refinery'
- Veerle Verrue (2011), 'Novel biokatalysis to upgrade side streams in the bio-refinery'
- Susan Muyindike (2010-2011), 'Cradle-to-cradle wastewater treatment in Uganda

Teaching at Faculty of Bioscience Engineering, Ghent University

- Theoretical PC-exercises coordinator of the course 'Microbial Re-use Technology' – 2012-2014;
- Theoretical PC-exercises coordinator of the course 'Microbial Ecological Processes' – 2012-2014;
- Theoretical PC-exercises coordinator of the course 'Biotechnological Processes' – 2012-2014;
- Theoretical PC-exercises coordinator of the exercise River Modelling in the course 'Environmental Microbiology' – 2012-2014;
- Co-lecturer PC-exercises 'Microbial Re-use Technology' – 2011-2012;
- Co-lecturer PC-exercises 'Microbial Ecological Processes' – 2010-2012;
- Co-lecturer PC-exercises 'Biotechnological Processes' – 2010-2012;
- Ad-interim theory lecturer of the course 'Microbial Ecological Processes' – 2011-2012.

Supervision of traineeship students

- Silke Verbrugge (2013), 'Anaerobic biodegradation of PU-foam'
- Lars De Lathouwer (2012), 'A-sludge as stabilizing agent in anaerobic digestion of kitchen waste'

Publications

Articles included in ISI Web of Science (A1, published)

2014

1. **De Vrieze, J.**, Gildemyn, S., Vilchez-Vargas, R., Jáuregui, R., Pieper, D.H., Verstraete, W., Boon, N. Inoculum selection is crucial to ensure operational stability in anaerobic digestion. *Accepted in Applied Microbiology and Biotechnology*. (IF 3.8)
2. **De Vrieze, J.**, Hennebel, T., Van den Brande, J., Bilad, R.M. Bruton, T.A., Vankelecom, I.F.J., Verstraete, W., Boon N. 2014. Anaerobic digestion of molasses by means of a vibrating and non-vibrating submerged anaerobic membrane bioreactor *Biomass and Bioenergy*, **68**, 95-105. (IF 3.0)
3. **De Vrieze J.**, Gildemyn, S., Arends, J.B.A., Vanwonterghem, I., Verbeken, K., Boon N., Verstraete, W., Tyson, G.W., Hennebel, T., Rabaey, K. 2014. Biomass retention

on electrodes rather than electrical current enhances stability in anaerobic digestion. *Water Research*, **54**, 211-221. (IF: 4.7)

4. Arends, J.A., Speeckaert, J., Blondeel, E., **De Vrieze, J.**, Boeckx, P., Verstraete, W., Rabaey, K. and Boon, N. 2013. Greenhouse gas emissions from rice microcosms amended with a plant microbial fuel cell. *Applied Microbiology and Biotechnology* 98(7), 3205-3217. (IF: 3.7) I performed the molecular analysis, data processing and evaluation and interpretation of the methanogenic community.
5. Atashgahi, S., Maphosa, F., **De Vrieze, J.**, Haest, P., Boon, N., Smidt, H., Springael, D. and Dejonghe, W. (2014) Evaluation of solid polymeric organic materials for use in bioreactive sediment capping to stimulate the degradation of chlorinated aliphatic hydrocarbons. *Applied Microbiology and Biotechnology* **98**(5), 2255-2266. (IF: 3.7; 1 citation) I performed the molecular analysis, data processing and evaluation and interpretation of the methanogenic community.

2013

6. Van Haandel, A., **De Vrieze, J.**, Verstraete, W., Dos Santos, V.S. 2013. Methanosaeta dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. *Journal of Chemical Technology & Biotechnology*, In Press, 10.1002/jctb.4255. (IF: 2.5) I performed the molecular analysis, data processing and evaluation and interpretation of the methanogenic community. I also wrote a large part of the manuscript.
7. Kjerstadius, H., Jansen, J.L., **De Vrieze, J.**, Haghighatafshar, S., Davidsson, A. 2013 Hygienization of sludge through anaerobic digestion at 35, 55 and 60 °C. *Water Science & Technology* **68**(10), 2234-2239. (IF: 1.1) I performed the molecular analysis, data processing and evaluation and interpretation of the methanogenic community.I also wrote the section on microbial community analysis.
8. **De Vrieze, J.**, De Lathouwer, L., Verstraete, W., Boon, N. 2013. High-rate iron-rich activated sludge as stabilizing agent for the anaerobic digestion of kitchen waste. *Water Research*, **47**(11), 3732-3741. (IF: 4.7, 1 citation)
9. **De Vrieze, J.**, Verstraete, W., Boon, N. 2013. Repeated pulse feeding induces functional stability in anaerobic digestion. *Microbial Biotechnology*, **6**(4), 414-424. (IF: 3.2; 1 citation)

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10. **De Vrieze, J.**, Hennebel, T., Boon, N., Verstraete, W. 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. *Bioresource Technology*, **112**(0), 1-9. (IF: 5.0; 37 citations)
11. Zamalloa, C., **De Vrieze, J.**, Boon, N., Verstraete, W. 2012. Anaerobic digestibility of marine microalgae Phaeodactylum tricornutum in a lab-scale anaerobic membrane bioreactor. *Applied Microbiology and Biotechnology*, **93**(2), 859-869. (IF: 3.4; 11 citations) I assisted in the experiment itself and I performed the molecular analysis, data processing and evaluation and interpretation of the microbial community. I also assisted in writing the manuscript.

Complete papers in conference proceedings (C1)

2013

1. **De Vrieze, J.**, Gildemyn, S., Arends, J.B.A., Boon, N., Verstraete, W., Hennebel, T. and Rabaey, K. A Bioelectrochemical System as Stabilizing and Remediating Agent in Anaerobic Digestion. 13th World Congress on Anaerobic Digestion. Santiago de Compostela, Belgium. 25-28 June 2013.
2. Arends, J.B.A., Van Denhouwe, S., **De Vrieze, J.**, Boon, N., Verstraete, W., Rabaey, K. 2013. Integration of wetland wastewater treatment with disinfection via bioelectrochemical H2O2 production. 18th National Symposium on Applied Biological Sciences. Ghent, Belgium. 8th February 2013. *Communications in Agricultural and Applied Biological Sciences*, **78**(1), 173-7.

2012

3. **De Vrieze, J.**, Gildemyn, S., Verstraete, W., Boon, N. Feeding Pattern Variation and Inoculum Selection Induces Functional Stability In Anaerobic Digestion. ISWA World Solid Waste Congress. Firenze, Italy. 17-19 September 2012.
4. Zamalloa, C., **De Vrieze, J.**, Boon, N., Verstraete, W. 2012. Anaerobic digestibility of marine microalgae Phaeodactylum tricornutum in a lab-scale anaerobic membrane bioreactor. Proceedings of the 1st International Conference on Biogas Microbiology. Leipzig, Germany. 14-16 September 2012. Centre of environmental research (UFZ).

Dissertations (C2)

De Vrieze J. (2010). Evolutionary ecotoxicology under field conditions: the potential and realized micro-evolutionary response as a consequence of cadmium exposure in a natural *Daphnia magna* population. Ghent University, Master thesis.

Reviewing and editing

Ad hoc reviewer of *Current Opinion in Biotechnology*, *Microbial Biotechnology*, *Journal of Chemical Technology and Biotechnology*, *Journal of Environmental Engineering*.

Presentations international conferences (the presenting author is underlined)

Oral presentations

1. Arends, J.B.A., **De Vrieze, J.**, Gildemyn, S., Vanwonterghem, I., Verbeken K., Boon, N., Verstraete, W., Tyson, G.W., Hennebel, T., Rabaey, K. A Bioelectrochemical system in anaerobic digestion: Biomass retention prevails over electrochemical parameters.. 2nd European meeting of the International Society for Microbial Electrochemistry and Technology. Alcala, Spain. 3-5 September 2014.
2. **De Vrieze, J.**, Saunders, A.M., He, Y., Fang, J., Verstraete, W., Boon, N. Ammonia and temperature determine three distinct AD-types in the anaerobic digestion microbiome. 2nd International Conference on Biogas Microbiology ICBM. Uppsala, Sweden, 10-12 June 2014.
3. **De Vrieze, J.**, Hennebel, T., Van den Brande, J., Bilad, R.M., Vankelecom, I.F.J., Verstraete, W., Boon, N. Anaerobic digestion of molasses by means of a vibrating and non-vibrating submerged anaerobic membrane bioreactor. 3rd IWA Benelux Young Water Professionals Regional Conference. Belval, Luxembourg. 02-04 October 2013.
4. **De Vrieze, J.**, Gildemyn, S., Arends, J.B.A., Vanwonterghem, I., Boon, N., Verstraete, W., Tyson, G.W., Hennebel, T., Rabaey, K. A bioelectrochemical system in anaerobic digestion: stabilization and remediation. 4th International Microbial Fuel Cell Conference. Cairns, Australia, 01-04 September 2013.
5. **De Vrieze J.**, Gildemyn, S., Arends, J.B.A., Boon, N., Verstraete, W., Hennebel, T., Rabaey, K. A Bioelectrochemical System as Stabilizing and Remediating Agent in Anaerobic Digestion. 13th World Congress on Anaerobic Digestion. Santiago de Compostela, Spain. 25-28 June 2013.

6. Verstraete, W., **De Vrieze, J.** Anaerobic Digestion: About Beauty and Consolation. 13th World Congress on Anaerobic Digestion. Santiago de Compostela, Spain. 25-28 June 2013. **Invited plenary presentation.**
7. Arends, J.B.A., Van Denhouwe, S., **De Vrieze, J.**, Boon, N., Verstraete, W., Rabaey, K. Integration of wetland wastewater treatment with disinfection via bioelectrochemical H_2O_2 production. 18th National Symposium on Applied Biological Sciences. Ghent, Belgium. 8th February 2013.
8. Raport, L., **De, Vrieze J.**, Willems, B., Volcke, E.I.P., Meers, E., Boon, N. Biomethane potential of agro-industrial substrates depends on the inoculum type. 18th National Symposium on Applied Biological Sciences. Ghent, Belgium. 8th February 2013.
9. **De Vrieze, J.**, Gildemyn, S., Arends, J.B.A., Boon, N., Verstraete, W., Hennebel T., Rabaey, K. A Bioelectrochemical System as Stabilizing and Remediating Agent in Anaerobic Digestion. First EU-ISMET European International Society for Microbial Electrochemistry and Technology Meeting. Ghent, Belgium. 26-28 September 2012.
10. **De Vrieze, J.**, Gildemyn, S., Verstraete, W. & Boon, N. Feeding Pattern Variation and Inoculum Selection Induces Functional Stability in Anaerobic Digestion. ISWA World Solid Waste Congress. Firenze, Italy. 17-19 September 2012.
11. Eggermont, B., Bundervoet, B., **De Vrieze, J.**, Hennebel, T., Verstraete, W. Energy from agro-industrial wastes: the quest for more recovery by anaerobic digestion. I. International Conference on Biogas Microbiology. Leipzig, Germany. 13-16 September 2011. **Invited presentation**.
12. Eggermont, B., Bundervoet, B., **De Vrieze, J.**, Hennebel, T., Verstraete, W. Energy from agro-industrial wastes: the quest for more biogas. II International Symposium on Agricultural and Agroindustrial Waste Management (SIGERA). Foz do Iguaçu, Brazil. 14-17 March 2011. **Invited presentation**.
13. **De Vrieze, J.**, Messiaen, M., Janssen, C.R., De Schamphelaere, K.A.C. Putting evolutionary principles into ecotoxicology: at which concentration of cadmium can we expect natural selection in a natural *Daphnia magna* population? The Daphnia Genomics Consortium Meeting 2010. Leuven, Belgium. 26-30 March 2010. Oral flash presentation.

Poster presentations

1. Gildemyn, S., **De Vrieze, J.**, Arends, J.B.A., Verstraete, W., Hennebel, T., Rabaey, K. Enhancement of the anaerobic digestion process by introducing a bioelectrochemical system. Francqui Symposium. Ghent, Belgium. 22nd November 2013.
2. Raport, L., **De Vrieze, J.**, Willems, B., Volcke, E.I.P., Meers, E., Boon, N. Inoculum origin determines biomethane potential of agroindustrial substrates and by-products. 13th World Congress on Anaerobic Digestion. Santiago de Compostela, Spain. 25-28 June 2013.
3. **De Vrieze, J.**, Hennebel, T., Van den Brande, J., Bilad, R.M., Vankelecom, I.F.J., Verstraete, W., Boon N. Anaerobic digestion of molasses by means of a vibrating and non-vibrating submerged anaerobic membrane bioreactor. 18th National Symposium on Applied Biological Sciences. Ghent, Belgium. 8th February 2013.
4. Gildemyn, S., **De Vrieze, J.**, Arends, J.B.A., Verstraete, W., Hennebel, T., Rabaey, K. Enhancement of the anaerobic digestion process by introducing a bioelectrochemical system. 18th National Symposium on Applied Biological Sciences. Ghent, Belgium. 8th February 2013.
5. Arends, J.B.A., Van Denhouwe, S., **De Vrieze, J.**, Boon, N., Verstraete, W., Rabaey K. Integrated wetland and bio-electrochemical H_2O_2 production for wastewater treatment and disinfection. 2nd International PlantPower Symposium. Wageningen, The Netherlands. 22 November 2012.
6. Gildemyn, S., **De Vrieze, J.**, Arends, J.B.A., Hennebel, T., Rabaey K. Reactor types for electro-assisted AD. 2nd International PlantPower Symposium. Wageningen, The Netherlands. 22 November 2012.
7. Gildemyn, S., **De Vrieze, J.**, Arends, J.B.A., Hennebel, T., Rabaey K. Reactor types for electro-assisted AD. First EU-ISMET European International Society for Microbial Electrochemistry and Technology Meeting. Ghent, 26-28 September 2012.
8. Arends, J.B.A., Van Denhouwe, S., **De Vrieze, J.**, Boon, N., Verstraete, W., Rabaey K. Integrated wetland and bio-electrochemical H_2O_2 production for wastewater treatment and disinfection. First EU-ISMET European International Society for Microbial Electrochemistry and Technology Meeting. Ghent, 26-28 September 2012.
9. Raport, L., **De, Vrieze J.**, Willems, B., Volcke, E.I.P., Meers, E., Boon, N. Toekomst voor microbiële opvolging van anaerobe vergistingsinstallaties. 2de Vlaams VergistingsFORUM. Ghent, 19th September 2012.

10. **De Vrieze, J.**, Boon, N., Verstraete, W. Feeding pattern variation induces functional stability in anaerobic digestion. 17th National Symposium on Applied Biological Sciences. Leuven, 10th February 2013.
11. **De Vrieze J.**, Messiaen, M., Janssen, C.R., De Schamphelaere K.A.C. Putting evolutionary principles into ecotoxicology: at which concentration of cadmium can we expect natural selection in a natural *Daphnia magna* population? The Daphnia Genomics Consortium Meeting 2010. Leuven, 26-30 March 2010.

Conference Organization

1. Member of the **organizing committee** of the Francqui Symposium: Recent advances in microbial and enzymatic electrocatalysis. Ghent, 22 November 2013.
2. Member of the **organizing committee** of the First EU-ISMET European International Society for Microbial Electrochemistry and Technology Meeting. Ghent, 26-28 September 2012.
3. Member of the **organizing committee** of the 1 day electrochemistry workshop linked to the First EU-ISMET European International Society for Microbial Electrochemistry and Technology Meeting. Ghent 26 September 2012.
4. Member of the **organizing and scientific committee** of the First International Symposium on Microbial Resource Management in Biotechnology: Concepts and Application. Ghent, Belgium, 30 June – 1 July 2011.

International study leaves and workshops

1. June 14-28, 2014. PhD Course Arctic Microbiology: education and training in field work and analysis, Akureyri (Iceland).
2. March 2014 – May 2014. Research stay at the University of Santiago de Compostela (USC), Santiago de Compostela (Spain).
3. October 23-26, 2012. PhD Course Microbial ecology in wastewater treatment, Aalborg University, Aalborg (Denmark).
4. September 3-7, 2012. Research stay at the Aalborg University, Aalborg (Denmark).

DANKWOORD

Het aanvatten van een doctoraat aan de faculteit Bio-ingenieurswetenschappen en, in het bijzonder, aan het Laboratorium voor Microbiële Ecologie en Technologie (LabMET) kan beschouwd worden als een unieke, doch soms exhaustieve en enigmatische ervaring. Desalniettemin waren de afgelopen vier jaar van mijn doctoraat (en ook de vijf voorgaande jaren als student aan het 'Boerekot' een unieke ervaring en ik kan met opgeheven hoofd en met een zuiver gemoed concluderen dat ik de juiste beslissing genomen heb. Een doctoraat schrijf/draag je uiteraard niet alleen. Een groot aantal mensen hebben mij dan ook de afgelopen jaren met raad en/of daad, al dan niet onbewust, bijgestaan. Bij dezen had ik dan ook graag deze gelegenheid aangegrepen deze mensen hiervoor te bedanken.

Eerst en vooral gaat mijn dank uit naar de juryleden van mijn doctoraat, daar het eindresultaat van deze vier jaar zeker ook hun verdienste is. Bij dezen dus bedankt aan Prof. Dr. ir. Lars Angenent, Prof. Dr. ir. Veerle Fievez, Dr. Pascal Pipyn, Prof. Dr. ir. Alfons Stams en Prof. Dr. ir. Arne Verliefde. Ik had graag Lars Angenent in het bijzonder willen bedanken voor de interessante discussies met betrekking tot de validiteit van het 'AD Microbiome' concept.

Mijn beide promotoren, namelijk Prof. Dr. ir. Nico Boon en Prof. Dr. ir. Willy Verstraete verdienen uiteraard ook mijn opperste en oprechte dank, aangezien dit werk zonder hun constante input en wetenschappelijke motivatie nooit het daglicht zou gezien hebben. Prof. Boon, ik herinner mij nog de dag dat ik uw bureau binnenkwam om te solliciteren voor de assistentenpositie, waarbij ik zelf uitging van het idee 'dat ik het toch maar eens kon proberen'. Uw eerste woorden waren dan ook: "Jo, zeg maar Nico", wat ik vanaf dan ook ten harte heb genomen en wat mij direct het idee gaf dat ik uitermate welkom was op LabMET. Uw wetenschappelijke bijdrage aan dit werk kan niet genoeg benadrukt worden en ook voor een enthousiast gesprek buiten het wetenschappelijke om stond u steeds paraat, wat ik dan ook uitermate geapprecieerd heb. Door uw doorzettingsvermogen en overtuigingskracht heb ik ook steeds met vol enthousiasme kunnen doorwerken aan dit doctoraat, ook als het even wat minder ging. Nico, in alle eerlijkheid, bedankt hiervoor en ik hoop dat we in de toekomst nog verder een sterke samenwerking kunnen onderhouden. Prof. Verstraete (het duurde iets langer voor ik u met 'Willy' durfde aan te spreken), het was mij een erg grote eer u als promotor van mijn doctoraat te hebben en ik ben er dan ook erg trots op uw laatste doctoraatstudent op LabMET te mogen zijn. Hier verzinkt mijn betoog in alle clichés die over u bestaan, maar uw eeuwig durend enthousiasme en het spuien van ontelbaren ideeën waren

mij soms een kwelling van de geest, maar in het merendeel van onze meetings (soms wel erg vroeg op zaterdagmorgen) heb ik er waarlijk van genoten met u samen te werken.

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In the final stage of my PhD, I had the opportunity to work at the BioGroup of the Universidade de Santiago de Compostela with Dr. Marta Carballa and Prof. Dr. Juan Lema. Marta, it was a pleasure to work with you during those three months, and I think we had some very interesting discussions, including those during lunchtime concerning Real Madrid and FC Barcelona. Juan, I would also like to thank you for the opportunity to work at the USC and for the lunch invitation in that amazing restaurant near the Atlantic coast (I forgot the exact name). Muchas gracias a todos.

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