Influence of Substrate Type, Solids Retention Time, and Temperature on Digester Process Performance and Microbial Community Dynamics

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ABSTRACT

This study explored the effects of modifying important parameters in anaerobic digestion (AD) such as substrate characteristics, solids retention time (SRT), and temperature on microbial community dynamics and process performance of anaerobic digesters treating a mixture of primary settled sludge and secondary sludge produced during activated sludge treatment. To elucidate the effects of substrate characteristics, more specifically, proteins and carbohydrates, four laboratory-scale reactors were fed thickened sewage sludge collected from one of two water reclamation plants (WRP1 or WRP2) for 20 months. Protein concentrations were significantly higher than those of carbohydrates in both substrates, and on average were four times higher in WRP2 than in WRP1 substrates. It was hypothesized that proteins would be favored when their concentrations were higher than carbohydrates; hence, they could play a role in enhancing AD.

When the substrates were switched from WRP1 to WRP2, biogas production increased tenfold. Proteins played a bigger role in this increase because average protein removal increased by 20% while there was no significant change in average carbohydrate removal. Results of 21-day batch tests where either bovine serum albumin (BSA) or sucrose to WRP2 substrate was added to substrate from WRP2 confirmed that proteins were preferentially used over carbohydrates. Additionally, when basal medium plus a combination of BSA and sucrose at two ratios (30:1 and 5:1) was fed to reactors, sucrose was not utilized, whereas BSA was degraded. The results confirmed that proteins were the preferred substrates. The order W5 (Spirochaetes), which contains putative protein users, was enriched when reactors were fed with WRP2 substrates. In conclusion, proteins may play an important role in enhancing digestion since they were preferentially utilized over carbohydrates as long as relative concentrations were higher.
I further analyzed the effects of changing the SRT from 30 days to 15 days on the performance of the reactors and hypothesized that step-wise SRT reduction would not affect hydrolysis and hydrolytic bacteria. Additionally, methanogenesis would be enhanced at the shorter SRT since the SRT was the same as the HRT in reactors. SRT reduction would also increase the abundance of archaea. To test these hypotheses, I used four laboratory-scale reactors grouped into two clusters. Each cluster was operated at different SRTs. Results supported the hypothesis that step-wise SRT reduction did not affect hydrolysis and hydrolytic bacteria. Although biogas production increased, overall archaeal abundance did not change significantly. In contrast, the abundance of *Methanoseta* decreased noticeably. This decrease, coupled with an increase in biogas production, suggested that methanogens were more active at the shorter SRT as revealed by specific methanogenic activity tests. Considering that the reduction in SRT doubled the amount of sludge that could be treated using existing reactor capacity, and increased biogas production by up to 65% without significant changes to the community, step-wise SRT reduction could be considered as optimization method for full-scale digesters that are operating at longer SRT.

Lastly, I probed the effects of pulsed temperature perturbations on AD since temperature variation is a common disturbance in anaerobic digestion. I hypothesized that short-term temperature changes would affect both the methanogenic community dynamics and methanogenesis itself. To answer this hypothesis, I operated two laboratory-scale anaerobic reactors, labeled R1 and R2, at 35 ± 1°C for 140 days with an SRT of 30 days. On a specified day, the temperature in the specific reactor was reduced to 25 °C for 24 h before returning to 35 °C. R1 received its first set of perturbations on Days 49 and 51. To confirm that changes observed in R1 were caused by temperature perturbations, a similar perturbation was applied to both R1 and R2 on Day 97.
Hydrolysis was not impacted by temperature cycling. Yet, despite similar biogas production and composition, a sharp increase in acetate concentration was observed in R1 after perturbations on Days 49 and 51, and in R2 after the perturbation on Day 97, suggesting that acetoclastic methanogenesis was affected. After the first set of perturbations in R1 there was a sharp decrease in *Methanoseta* abundance as assessed by metagenomic analysis. Concomitantly, the abundance of hydrogenotrophic methanogens such as *Methanobacterium* and *Methanospirillum* increased. Similar trends were also observed in R2 after its first perturbation on Day 97. Lastly, the effects of the additional Day 97 perturbation in R1 were less severe suggesting increased community resistance to such perturbations. Despite its brevity, pulsed temperature perturbations may significantly alter the methanogenic community, even in the absence of any apparent effect on biogas production and composition. The increase in the abundance of hydrogenotrophic methanogens following the perturbations could be beneficial since they were more resilient than their acetoclastic counterparts. Moreover, accumulated acetic acid could also be harvested from the digested sludge. Finally, higher archaeal diversity following repeated pulse perturbations could improve community robustness and long-term process stability.

Overall, this study showed that modifying simple parameters can improve AD without the high costs and energy requirements typically associated with conventional optimization methods such as pretreatment and usage of additives. Minimal manipulation of these parameters also led to desirable changes in the methanogenic community.
CHAPTER 1

Introduction

1.1 Anaerobic digestion as sludge stabilization technique

Sewage sludge is an unavoidable by-product from various chemical, physical, and biological treatment steps in wastewater treatment. It comprises of primary sludge (PS) that is generated in the primary settling tank and secondary or waste activated sludge (WAS) from the secondary settling tank (Tezel et al. 2011). The cost of sewage sludge disposal can contribute up to 50-60% of the whole treatment plant operational cost due to the sheer amount of sludge that is typically generated (Appels et al. 2008, Weemaes and Verstraete 1998). Additionally, up to 90% of the sewage sludge is comprised of putrescible organics that can generate offensive odor and cause health-related hazards (Appels et al. 2008, Weemaes and Verstraete 1998). Therefore, strict treatments to stabilize those organics is needed before the sludge can be safely disposed of.

One commonly used sludge stabilization method is anaerobic digestion (AD). AD can reduce the sludge volume by converting the sludge organics into energy-rich biogas containing mainly methane and carbon dioxide (Batstone and Jensen 2011, Tezel et al. 2011). It is also more advantageous than its aerobic counterpart because of lower operational costs.

1.2 Steps in anaerobic digestion

Anaerobic digestion is a complex process involving four different but tightly interconnected steps in the absence of oxygen as electron acceptor (Batstone and Jensen 2011, Gerardi 2003a). These steps are hydrolysis, fermentation/acidogenesis, acetogenesis, and methanogenesis.
1.2.1 Hydrolysis

Hydrolysis involves the solubilization of organic particulates such as proteins, carbohydrates, and fats into their monomers such as amino acids, sugars, and long chain fatty acids (Angelidaki and Sanders 2004, Gujer and Zehnder 1983). Most sludge organics are present as polymers in which smaller molecules are held together by chemical bonds that cause them to be insoluble (Gerardi 2003b). Thus, hydrolysis becomes crucial because microorganisms in the digester cannot utilize those organics unless they are converted into substrates that can be transported through the microbial cell envelope (Kim et al. 2003). This process is mediated by enzymes such as proteases, cellobiases, lipases, and amylases produced by both facultative and obligate anaerobes (Batstone and Jensen 2011, Gujer and Zehnder 1983, Tezel et al. 2011).

Hydrolysis has been identified as one of the potentially rate-limiting steps in AD, especially when the sludge solids concentration is high. Hydrolysis also becomes rate limiting in the presence of highly complex molecules in the sludge such as carbohydrates with a high lignin content or proteins with fibrous tertiary structure (Batstone and Jensen 2011).

The problem with hydrolysis is further aggravated by the different biodegradability potentials of primary and secondary sludge. Primary sludge contains mostly proteins and fats that are generally easier to degrade than secondary sludge (Tezel et al. 2011). Secondary sludge contains mostly microbial cells whose membranes are formed by glycan strands. These strands are crosslinked with peptide chains that are difficult to biodegrade (Weemaes and Verstraete 1998).

Besides hydrolysis, methanogenesis is another potential rate-limiting step in the digestion process. This scenario is especially pertinent when the sludge retention time is
very short or when the materials digested are easily degradable (Appels et al. 2008, Batstone and Jensen 2011).

1.2.2 Fermentation

The solubilized organic materials from the hydrolysis step will be utilized in the ensuing fermentation step. Fermentation is defined as a process where the solubilized organics are converted into their reduced or oxidized forms without the presence of obligate electron acceptors (Batstone and Jensen 2011). Fermentative bacteria obtain their energy from this oxidation-reduction process.

In AD, amino acids, sugars, and fatty acids produced from the hydrolysis phase are converted into short-chain organic acids such as butyric acid, propionic acid, oxaloacetic acid, or purivic acid during fermentation (Gujer and Zehnder 1983). In addition, hydrogen, carbon dioxide, and alcohols are also produced in this phase (Appels et al. 2008, Batstone and Jensen 2011, Tezel et al. 2011).

The fermentation of amino acids to organic acids is done either through the transamination pathway or the Stickland pathway. In the transamination reaction, purivic acid and oxaloacetic acid are produced through the reactions between the amino acids and α-ketoglutaric acid with the help of enzymes such as glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. Both of the aforementioned enzymes have been found in anaerobic digesters (Tezel et al. 2011).

Another pathway for the conversion of amino acids to organic acids is the Strickland pathway. This pathway is also known as the coupled fermentation pathway because the reaction involves a pair of amino acids. One of the amino acids acts as a reducing agent while another acts as an oxidizing agent (Figure 3) (Schink 1997, Tezel et al. 2011). The reducing agent/ the electron donor will be oxidized to CO₂, NH₃, and a carboxylic acid that contains one less carbon on its carbon chain than the original form.
Meanwhile, the oxidizing agent/the electron acceptor will also be reduced to NH$_3$ and carboxylic acid. However, the carboxylic acid here contains the same amount of carbon on its chain as its original form (Batstone and Jensen 2011).

Besides amino acids, sugar is another major group that is typically fermented in AD. Their fermentation proceeds through the Embden-Meyerhof-Parnas (EMP) or the acetyl-CoA pathways to produce end products such as propionic acid, butyric acid, acetic acid, ethanol, butanol, and hydrogen (Figure 4) (Batstone and Jensen 2011, Gujer and Zehnder 1983, Tezel et al. 2011).

1.2.3 Acetogenesis

In this phase, acetate-forming bacteria will utilize the hydrogen ions to convert the intermediates from the previous fermentation phase to acetic acid, carbon dioxide, and hydrogen. Some of these conversions are actually thermodynamically unfavourable (McInerney et al. 1979). One example is the conversion of propionate to acetate, carbon dioxide, and hydrogen. This conversion has a positive Gibbs energy which makes it an endergonic reaction (Schink 1997). In order for this process to progress, the hydrogen partial pressure needs to be low. To achieve this, acetogenesis is typically coupled with a hydrogen utilizing process such as methanogenesis (Batstone and Jensen 2011, Gujer and Zehnder 1983, Tezel et al. 2011). Hence, it is important for the acetogens to maintain a symbiotic relationship with the hydrogenotrophic methanogens. This symbiotic relationship is usually described as syntrophy.

Syntrophy is a phenomenon in which two different types of organisms depend on each other to perform activities that neither could have achieved without the presence of the other (Schink 1997). In anaerobic digestion, the hydrogen utilizing methanogens rely on the acetogens for their substrates while the latter depend on the methanogens to maintain the low hydrogen partial pressure required for a successful acetogenesis. The
ailure to achieve syntrophy between the two can be catastrophic and it has been reported as one of the major causes of digestion instability (Amani et al. 2010).

1.2.4 Methanogenesis

Following the acetogenesis step, the acetate, hydrogen and carbon dioxide produced will be converted into the final digestion product which is methane and carbon dioxide gas. This process can be further classified into several different types based on the substrates utilized. Of all the types, the most common one is the acetoclastic methanogenesis. Acetoclastic methanogenesis refers to production of methane and carbon dioxide by the cleaving of acetate (Batstone and Jensen 2011). Typically, this type of methanogenesis contributes up to 70% of the methane production in a mature digester, making acetate the most prominent precursor of methane (Gujer and Zehnder 1983).

Another type of methanogenesis that typically occurs in sewage sludge anaerobic digestion is hydrogenotrophic methanogenesis (Batstone and Jensen 2011, Gerardi 2003b). In this process, hydrogen and carbon dioxide are used as electron donor and acceptor, respectively, to produce methane (Appels et al. 2008). Since hydrogen in the digester is limited, hydrogenotrophic methanogenesis only contributes up to 30% of the overall methane production in a digester (Amani et al. 2010).

Besides through methanogenesis, methane can also be produced from a process called syntrophic acetate oxidation. In this process, acetate is first oxidized to hydrogen. The hydrogen is then converted into methane (Hattori 2008). This mechanism usually occurs when there is a high level of inhibitors such as ammonia at the thermophilic temperature range (50-65°C) (Fotidis et al. 2013, Karakashev et al. 2006). Moreover, a low acetate concentration in thermophilic digesters also increases the likelihood of methane production through syntrophic acetate oxidation (Karakashev et al. 2006).
1.3 Governing parameters in anaerobic digestion

A balance must be maintained among the four different steps to ensure a successful digestion. This balance is very delicate and can be easily upset by disturbances. Therefore, there are several parameters that must be controlled to ensure that the balance can be maintained. Some of the parameters include temperature, substrate characteristics, and solids retention time (Tezel et al. 2011, Vanwonterghem et al. 2015b)

1.3.1 Temperature

Temperature can affect the physiochemical properties of digester substrates (Appels et al. 2008). It can also influence microbial community dynamics since it governs the metabolic and growth rates of organisms in anaerobic digesters (Appels et al. 2008). Therefore, it is crucial that the digestion temperature be kept within an optimal range. Changes to the operating temperature must also be applied slowly, not exceeding 1°C/day (Tchobanoglous et al. 2003)

Of the four AD stages, acidogenesis and hydrolysis are the steps that are affected more severely by temperature changes. A 5°C decrease in the operating temperature has been shown to cause a 50% and 10% decrease in the acidogenesis and hydrolysis kinetics, respectively (Donoso-Bravo et al. 2009). Higher temperature also increases methanogenic activities which then lead to an increase in biogas production (Zábranská et al. 2000).

Despite the increase in gas production, a decrease in gas quality has been reported at higher digestion temperatures. This decrease is caused by an increase in the water and carbon dioxide contents in the gas. Moreover, there is usually an elevated level of soluble organics such as VFA in the digesters that are operated at higher temperatures. One explanation for this is because there is an increase in maintenance requirement and substrate-saturation level at higher temperature (Batstone and Jensen 2011). Another possible explanation for this increase is the enhanced fermentation at higher temperature. Since the production rate is higher than the
consumption rate, accumulation of soluble products is inevitable (Labatut et al. 2014, Vanwonterghem et al. 2015b).

Based on its operating temperature, anaerobic digesters can be classified into three different categories: psychrophilic where the operating temperature is kept in the range of 10-30°C, mesophilic where the operating temperature ranges from 30-40°C, and thermophilic where operating temperature is kept within 40-70°C. Of the three, psychrophilic digestion usually occurs naturally while both mesophilic and thermophilic are usually engineered. (Batstone and Jensen 2011).

A mesophilic digester (MAD) is the most common type of digester utilized in wastewater treatment plants although the use of thermophilic digestion (TAD) has started to gain popularity. This is due to the improved biogas production, higher hydrolysis rate, and better pathogen removal observed in TAD (Labatut et al. 2014, Zábranská et al. 2000). However, despite the seemingly improved performance, thermophilic digestion is more prone to process instability, possibly due to the less diverse microbial community, higher reaction rate, and higher susceptibility to inhibitors (Chae et al. 2008, Chen et al. 2008, Levén et al. 2007)

1.3.2 Substrate characteristics

Besides temperature, digester substrate is another important parameter that drives the digestion process (De Francisci et al. 2015, Labatut et al. 2014, Tezel et al. 2011, Vanwonterghem et al. 2015b). This is because different types of substrates can lead to different microbial community structures (De Francisci et al. 2015, Satpathy et al. 2016). The constituents of complex substrates can also be the precursors to different inhibitors (Chen et al. 2008, De Francisci et al. 2015). Hence, digestion is often regarded as a substrate-driven process.
Extensive studies have been carried out to understand the effects of different types of substrates such as animal manure, sewage sludge, and food waste on AD (Mata-Alvarez et al. 2014). Through this deeper understanding, many have acknowledged the shortcomings of single substrate digestion due to, among others, inadequate organic loading and a suboptimal C:N ratio (Mata-Alvarez et al. 2014). To overcome these problems, the concept of mixing different substrates with complementary properties (i.e., co-digestion) to enhance digestion has been widely explored (Dias et al. 2014, Mata-Alvarez et al. 2014, Zhang et al. 2013). Results of these studies show that co-digestion can indeed produce more biogas than mono-digestion.

1.3.3 Solids Retention Time (SRT)

Solids retention time, which refers to the time the solids stay inside a digester, is another important parameter that needs to be taken into account in designing and operating anaerobic digesters (Tchobanoglous et al. 2003). This is because SRT can be a major selective force that dictates the types of organisms that eventually dominate in a digester (Elefsiniotis and Oldham 1994a). SRT also affects the extent of hydrolysis and the amount of biogas produced (Tezel et al. 2011). Therefore, a digester SRT must be selected carefully. Although most digesters are currently operated at a relatively longer SRTs of 20-30 days to avoid failures, operating an anaerobic digester at a shorter SRT has been shown to be feasible (Appels et al. 2008, Nges and Liu 2010).

A shorter SRT allows for the digesters to be operated at higher organic loading rates. This increases the overall efficiency of the digester and enables more sludge to be stabilized in a given period of time. However, the trade-off for shorter SRT digester operation is that this condition might be detrimental for the slower growing organisms. Hence, it is important that the SRT applied is adequate to cater for the slowest growing organisms, the methanogens (Elefsiniotis and Oldham 1994a, Vanwonterghem et al. 2015b).

1.4 Microorganisms involved in anaerobic digestion
It is crucial that the aforementioned operating parameters be maintained within the optimum range for the microorganisms within an anaerobic digester (Amani et al. 2010, Tezel et al. 2011). This is not an easy task considering that there are many different groups of organisms in the digesters and all of them have different growth rates, metabolic activities, and physiochemical requirements (Amani et al. 2010). The different groups of organisms can be classified into two domains; the Bacteria that drive the first three digestion stages and the Archaea that spearhead the last digestion stage (Gerardi 2003b).

1.4.1 Bacteria

Bacteria in anaerobic digesters are archetypally classified into three groups: the core groups of bacteria that are typically found in a majority of anaerobic digesters, the common groups of bacteria that are found in many digesters, and the specific groups of bacteria that are found only in specific digesters (Rivière et al. 2009).

The core groups are generally represented by five different phyla. Those phyla are Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, and Synergistetes or Deferribacteres (Carballa et al. 2015, Narihiro and Sekiguchi 2007, Rivière et al. 2009, Tezel et al. 2011).

1.4.1.1 Proteobacteria

Several genera have been identified within the Proteobacteria phylum. These genera include Desulfovibrio, Syntrophomonas, and Thauera (Tezel et al. 2011). Desulfovibrio is capable of performing incomplete oxidation of organic acids into acetate (Hao et al. 2014, Tezel et al. 2011). Several species of this genus are also involved in syntrophic relationships with other organisms to perform different functions in an anaerobic digester. An example of this syntrophy is the cooperation of Desulfovibrio with Syntrophobacter wolinii to degrade propionate (Boone and Bryant 1980). Another example is the ability of Desulfovibrio genus to degrade lactate to
methane and carbon dioxide by collaborating with *Methanosarcina barkeri* (McInerney and Bryant 1981)

However, despite its positive contribution, *Desulfovibrio* is also one of the most common sulfate reducing bacteria (SRB) in anaerobic digesters (Novaes 1986). SRB can pose as competitors for methanogens or other anaerobes in anaerobic digesters. This is because many SRB utilise similar substrates as the methanogens or other anaerobes that are responsible for different digestion steps (Chen et al. 2008). Activities of SRB can also produce an elevated amount of sulphide which is toxic to the methanogens (Chen et al. 2008).

### 1.4.1.2 Firmicutes

Firmicutes is another phylum that is commonly clusters within the core bacterial groups of an anaerobic digester. Examples of the genera within this phylum are *Ruminococcus, Clostridium, Acetobacterium, Lactobacillus*, and *Syntrophomonas* (Tezel et al. 2011). Most of the said genera are involved in the hydrolysis process. For example, species from the *Ruminococcus* genus can hydrolyse cellulose which is one of the most abundant plant cell wall polysaccharides (Shi and Weimer 1996). In addition to hydrolysis, *Lactobacillus, Clostridium, and Acetobacterium* are also involved in the fermentation step (Cabezas et al. 2015, Gerardi 2003b, Novaes 1986, Tezel et al. 2011). Some species within the *Acetobacterium* and *Clostridium* genera (i.e., *Acetobacterium woodii* and *Clostridium aceticum*) are also involved in the conversion of hydrogen and carbon dioxide into acetate, a process commonly known as homoacetogenesis (Batstone and Jensen 2011, Novaes 1986).

Members of the *Syntrophomonas* genus such as *Syntrophomonas wolfei* and *Syntrophomonas zehnderi* are mainly involved in the acetogenesis process in which they oxidize different fatty acids into acetate and hydrogen (Novaes 1986, Schmidt et
al. 2016). The hydrogen produced from the aforementioned oxidation is then utilized syntrophically by the hydrogenotrophic methanogens (Rivièr et al. 2009).

1.4.1.3 Chloroflexi

Chloroflexi has been found to be the major phylum in many digesters (Narihiro and Sekiguchi 2007, Rivièr et al. 2009, Tezel et al. 2011). However, the actual metabolic functions of this phylum are still unclear due to the lack of cultivable representatives (Rivièr et al. 2009, Tezel et al. 2011). Recently, four filamentous strains have been successfully isolated from Chloroflexi subphylum 1 (Sekiguchi et al. 2003). These four strains are classified into the genera Anaerolinea, Leptolinea, and Levilinea which are found mainly in the granules of UASB digesters (Yamada et al. 2006). Since they are able to proliferate using carbohydrate and yeast as substrates, their role in the hydrolysis of carbohydrates and amino acids has been proposed (Narihiro and Sekiguchi 2007).

1.4.1.4 Bacteroidetes

Another phylum in the core bacterial group, Bacteroidetes, is a known group of proteolytic bacteria since they can ferment the amino acids produced from protein hydrolysis into acetate (Kindaichi et al. 2004, Rivièr et al. 2009). Genera of this phylum include Bacteroides, Cytophaga and Flavobacterium which are also involved in the fermentation process (Tezel et al. 2011). An example of a species from this phylum is Flavobacterium johnsoniae which is able to digest polysaccharides (McBride et al. 2009). Some unclassified members of this phylum are also known to be able to oxidize butyrate (Schmidt et al. 2016).

1.4.1.5 Synergestes

Unlike the four previously mentioned phyla, Synergestes is one phylum in the core bacterial group that is often overlooked. This is because its abundance in anaerobic
digesters is typically low (Godon et al. 2005). All the same, *Synergestes* has been found in many digesters; hence, its inclusion as a member of the anaerobic digester core group. With regard to its function, members of this phylum are known to ferment amino acids to acetate (Godon et al. 2005, Tezel et al. 2011)

1.4.2 Archaea

In addition to Bacteria, members of the Archaea are important players in anaerobic digestion. Archaea are mainly involved in the methanogenesis step and most of the methanogens currently identified belong to this domain. These methanogens can be clustered into two major groups based on their substrates: the acetoclastic and the hydrogenotrophic methanogens.

Acetoclastic methanogens are the methanogens that cleave acetate into methane and carbon dioxide (Batstone and Jensen 2011). Examples of the genera belonging to this group are *Methanosaeta, Methanothrix* and *Methanosarcina* (Batstone and Jensen 2011). Members of the *Methanosaeta* genus (e.g., *Methanosaeta consilii, Methanosaeta soehngenii, and Methanococcus mazeii*) are obligate acetoclastic methanogens, meaning that they can only utilize acetate as substrate (Batstone and Jensen 2011, Jetten et al. 1992, Novaes 1986). On the contrary, *Methanosarcina* species are metabolically more versatile since they can utilize not only acetate, but also hydrogen, carbon dioxide, and methanol (Batstone and Jensen 2011, Schmidt et al. 2016). Hence, their growth rates are typically higher than those of *Methanosaeta* (Jetten et al. 1992).

*Methanosaeta* species typically dominate over *Methanosarcina* in the low acetate condition because they have lower acetate requirement threshold. The *Methanosaeta* acetate requirement typically ranges from 7 to 70 µM as compared to *Methanosarcina* whose acetate requirement threshold is typically > 100 µM (Jetten et al. 1992). Therefore, *Methanosarcina* usually thrive in conditions where acetate is abundant.
Besides acetoclastic methanogens, another type of methanogens present in anaerobic digester are the hydrogenotrophic methanogens. These hydrogen-utilizing methanogens play an important role in the digestion process, both as methane producers as well as syntrophic partners to the other organisms in the digester. Hydrogenotrophic methanogens produced methane by using hydrogen to convert carbon dioxide into methane; hence, keeping the hydrogen at the low level required by the acetogenic bacteria (Gerardi 2003b).

*Methanomicrobiales* is an example of the order belonging to this group (Tezel et al. 2011)

### 1.5 Microbial ecology concepts in bioreactors

The Bacteria and Archaea in the digesters form a microbial community. The microbial community structure in one digester might be different from the structure in another digester although their operating parameters are similar (Rivière et al. 2009). This has led to the question as to what factors actually drive the formation of a certain digester community structure.

Community assembly is a main objective of ecological studies (Pavoine and Bonsall 2011). Overall, the mechanisms of the formation of a certain assembly are still poorly understood (Logue and Lindström 2010). Some studies reported that deterministic processes such as environmental filtering are the main factor that shape a certain community structure (Falk et al. 2009, Fierer and Jackson 2006, Vanwonterghem et al. 2014). Yet others proposed that neutral or stochastic processes dominate a community assembly process (Gilbert et al. 2012, Gravel et al. 2006, Ofițeru et al. 2010, Stegen et al. 2013). In practice, it may be hard to properly differentiate the contribution of each type of process in shaping a community (Powell et al. 2015) although a theoretical framework has been provided for bioreactor studies (Santillan et al., submitted).
Despite the ongoing debate between proponents of the neutral-based and the niche-based theories, processes that affect the formation of a community can generally be differentiated into four types: selection, drift, dispersal, and speciation (Vellend 2010).

Selection is a deterministic process that shapes a microbial community profile (Nemergut et al. 2013). The outcome of selection is dictated by different abiotic factors such as substrate availability (Logue and Lindström 2010). These abiotic factors are often called environmental filters and they can help or prevent a certain species from proliferating in a particular location (Kraft et al. 2015). Notwithstanding the importance of those abiotic factors in influencing the outcome of the selection process, it is also important to consider the roles of biotic factors. Biotic factors like competition and predation can affect the outcome of a selection process (HilleRisLambers et al. 2012, Kraft et al. 2015, Stegen et al. 2013).

Unlike selection, the other three processes (i.e drift, dispersal, and speciation) are more stochastic (Chase and Myers 2011). Drift is defined as the changes in species abundance due to chance and it is usually dominant in a community where there is weak selection (Chase and Myers 2011, Nemergut et al. 2013, Stegen et al. 2013, Vellend 2010). This type of process has been shown to affect the less abundant species more severely. This is because only minimal negative changes are needed to push species at low abundance toward local extinction (Nemergut et al. 2013).

Speciation or diversification is the creation of new species at a certain location (Nemergut et al. 2013, Vellend 2010). Therefore, it is an important aspect that needs to be considered when studying the formation or evolution of a community (Chase 2003, Ricklefs 1987). Unfortunately, there is still a lack of knowledge about the temporal and spatial dynamics in microbial evolution because of the complexities involved (Nemergut et al. 2013). Hence, most studies only use inferences based on the distribution of genetic diversity across different temporal and spatial scales (Nemergut et al. 2013). Another important aspect
that needs to be taken into consideration when studying the evolution of a certain community is the concept of dormancy. It is possible that dormant species might be reawakened/recovered at certain conditions and disrupt the evolutionary pattern of a community (Buerger et al. 2012, Nemergut et al. 2013). Horizontal gene transfer (HGT) is another force that needs to be reckoned with when studying the evolution of a certain community. This is because HGT has the ability to change the rate of evolution (Nemergut et al. 2013). For example, one organism gains resistance to some toxins through evolution while another organism acquires this same resistance through HGT. This recombination process accelerates the process for the other organism to develop resistance to the same toxins (Nemergut et al. 2013, Papke and Gogarten 2012).

Dispersal is the movement of organisms across different spaces and is usually affected by other processes such as selection and drift (Vellend 2010). There are several models that attempt to better explain this process. One of them is the Mainland-Island model where it is assumed that the organisms disperse in a one-way direction, from an infinite-sized source community (i.e., the Mainland) to the smaller, discrete communities (i.e., the Island). Another model is the Island model. This model assumes that the dispersal occurs between islands or networks of smaller communities or metacommunities without the presence of a mainland (Leibold et al. 2004, Vellend 2010).

Overall, the process of microbial assembly is affected by the combinations of these four different types of processes. Different theories and models focusing on different combinations of the four groups have been suggested (Hubbell 2005, Ricklefs 1987, Tilman 1982). Moreover, the dominant processes that can affect a microbial community differ from one system to another. This difference has stimulated the application of ecological concepts in different biological systems to better understand the microbial communities that drive the systems. This is essential because the microbial community is the crux of any biological
system (Stark et al. 2014). Therefore, by understanding the processes that affect the microbial community formation, recovery, and resilience, a more robust and efficient biological system can be created (Cavender-Bares et al. 2009, Vanwonterghem et al. 2014, Vuono et al. 2015). This understanding also allows for predictions of community fluctuations under different conditions or perturbations (Valentín-Vargas et al. 2012). This ability can be beneficial in preventing process failure which can be extremely costly to recover (Stark et al. 2014).

Bioreactors play an integral part in this endeavour because they give researchers the ability to control and manipulate the bioreactors to fit into their specific research questions. This ability is an advantage that is not available when dealing with full-scale systems (Rivière et al. 2009, Vuono et al. 2015). Ecological concepts have therefore been used to explain the microbial communities in several bioreactors such as anaerobic digesters, membrane bioreactors, and sequencing batch reactors (Falk et al. 2009, Vanwonterghem et al. 2014, Vuono et al. 2015).

1.6 Use of metagenomics to describe microbial communities

Despite the importance of ecology in the studies of different biological systems, earlier studies have suffered from the biases that might be introduced by the culture-based molecular analysis techniques such as Sanger-based capillary sequencing. This because the nature of the aforementioned techniques can lead to the oversimplification of a community diversity (de Castro et al. 2013, Garza and Dutilh 2015). The emergence of culture-independent techniques that are able to sequence samples isolated directly from different sources has helped overcome this (Garza and Dutilh 2015). The increased depth and coverage coupled with lower cost has made the so-called next-generation sequencing (NGS) a powerful tool to gain a better understanding of a microbial community assembly (Cabezas et al. 2015, Soon et al. 2013, Stark et al. 2014).
NGS enables the simultaneous sequencing of thousands to millions of molecules (Soon et al. 2013). This is a significant improvement over conventional capillary sequencing based on the Sanger method which is labour-intensive (Garza and Dutilh 2015, Soon et al. 2013). Hence, the application of NGS to sequence environmental metagenomes has become widespread.

The application of NGS coupled with bioinformatics to elucidate the metagenomes in a given environment has immensely improved the field of metagenomics (de Castro et al. 2013, Garza and Dutilh 2015, Kröber et al. 2009, Thomas et al. 2012). Metagenomics itself is often defined as the study of the genetic materials within an environment in a shotgun or untargeted way (Garza and Dutilh 2015, Thomas et al. 2012). Metagenomics can provide more thorough genetic information on microbial community composition, functional characteristics and novel enzymes than other techniques that are based on a single gene such as 16S rRNA (Ju et al. 2016, Li et al. 2015, Thomas et al. 2012). Examples of platforms for NGS that are commonly applied in metagenomics are:

1.6.1 454/Roche

This system uses emulsion polymerase chain reaction (ePCR) to amplify random DNA fragments which will be attached to microscopic agarose beads containing millions of oligomers on their surface (Mardis 2008). These beads will then be deposited in the wells of a picotitre plate and four deoxynucleoside triphosphates (dNTPs) are sequentially added (Thomas et al. 2012). If complementary to the template strands, the dNTPs are incorporated by a DNA polymerase (Thomas et al. 2012). Light is emitted through the pyrophosphate that is produced from this polymeration. This light is then detected by a charge-coupled device (CCD) camera and subsequently converted to the actual sequence of the template (Mardis 2008, Thomas et al. 2012)

1.6.2 Illumina Sequencing
This sequencing platform adopts the sequencing by synthesis (SBS) technology (Mardis 2008). Random DNA fragments are first immobilized on a surface of a glass flow cell (Mardis 2008, Thomas et al. 2012). These fragments are then hybridized to the complementary oligonucleotides in the flow cell surface through heating and cooling steps. They are then amplified as clusters on the flow cell surfaces (Mardis 2008). Finally, these amplified fragments are sequenced with reversible terminators in the SBS process (Bentley et al. 2008).

Both of the aforementioned techniques have their own advantages and disadvantages. Some of the more prominent ones are listed in Table 1 (Mardis 2008). Despite these differences, NGS has revolutionized many fields of study including metagenomics and ecology.

2. **Motivation**

2.1 **Challenges in anaerobic digestion**

The application of NGS has deepened the understanding of complex systems such as anaerobic digestion that have been previously acknowledged as “black-box” due to the lack of suitable methods to routinely describe all taxa involved in the process (Rivière et al. 2009). However, despite this achievement, many digesters are still beleaguered by suboptimum performance such as poor operational stability, long start-up times, long solids retention times, and high sensitivity to changes in operational conditions and the presence of inhibitors (Amani et al. 2010, Appels et al. 2008, Schink 2002). One of the main reasons for this is the lack of complete understanding of the dynamics of the complex microbial community that drives the digestion process (De Francisci et al. 2015). Although several methods have been proposed to improve digester performance through pre-treatment, co-digestion, and use of additives, these methods could be energy and cost intensive (Mottet et al. 2009).
2.2 Research objectives and questions

The possibility of improving digestion by modifying parameters such as substrate composition, solids retention time, and temperature needs to be explored. However, the effects of changes in these parameters on the microbial community are not fully understood (De Francisci et al. 2015, Labatut et al. 2014). It is, therefore, important to close this knowledge gap as much as possible because understanding of a digester microbial community is essential in creating a stable and efficient digestion (Stark et al. 2014).

To achieve this goal, several main research questions needed to be answered:

1. Will a change in substrate characteristics significantly impact anaerobic reactor process performance and the microbial community?
2. Of proteins and carbohydrates that are present in the substrate, which play a bigger role in enhancing anaerobic digestion?
3. What are the effects of step-wise SRT reduction on the anaerobic digestion of sewage sludge?
4. Can SRT reduction significantly impact the hydrolytic and methanogenic communities if the lowest SRT applied is still within the methanogenic range?
5. Will short temperature cycling be significant enough to alter the process performance and microbial community in anaerobic reactors?
6. Can microbial community develop resistance toward similar perturbation?

Questions 1 and 2 are addressed in Chapter 2 where it is hypothesized that proteins would be better utilized than carbohydrates when protein concentrations were higher. I also hypothesized that proteins would be favored over carbohydrates when the concentrations of the latter were lower. Hence, proteins could play a big role in enhancing anaerobic digestion of sewage sludge that contained significantly higher proteins than carbohydrates. Chapter 3
addresses questions 3 and 4. In this chapter, I hypothesized that reducing the SRT from 30 to 15 days would not affect hydrolysis and hydrolytic bacteria. However, methanogenesis would be enhanced due to the increase in substrate concentrations since SRT in our system was equal to HRT. Additionally, SRT reduction could alter the methanogenic community. The last two questions are explored in Chapter 4. It was hypothesized that despite its brevity, pulsed perturbation would affect methanogenesis and methanogenic community, which could also develop resistance toward similar disturbances.
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CHAPTER 3

Role of proteins in anaerobic digestion of sewage sludge

ABSTRACT

Anaerobic digestion of sewage sludge is a substrate-driven process with proteins and carbohydrates comprising more than 50% of the feed. Lower protein than carbohydrate removal has been reported due to the latter’s inhibition of protein hydrolysis. We hypothesized that higher levels of proteins than carbohydrates in the digester feed would favor protein degradation. To test this hypothesis, four laboratory-scale reactors were fed with thickened sewage sludge collected from two water reclamation plants (WRP1 and WRP2) for 20 months. Protein levels in WRP2 were on average 4.3 times higher than in WRP1, while carbohydrates were on average 3.8 times higher in WRP2. Following the change in feed from WRP1 to WRP2 there was a 10 times increase in biogas production in the reactors. While average protein removal increased by 20%, there was no significant change in average carbohydrate removal. Results of batch tests (21 days) adding either bovine serum albumin (BSA) or sucrose to WRP2 substrate showed that proteins were preferentially used over carbohydrates. In additional tests employing basal medium and a combination of BSA and sucrose at two ratios (30:1 and 5:1), sucrose was not utilized whereas BSA was degraded, further supporting the observation that proteins were the preferred substrates.

Changes in microbial communities also suggested that proteins were the preferred organics in our study. The abundance of the order W5 (Spirochaetes), which contains putative protein users, significantly increased with higher protein feed. Among the hydrogenotrophic methanogens, Methanospirillum and Methanobacterium replaced Methanolinea, likely due to an increase in hydrogen partial pressure resulting from the higher organic content in WRP2 substrate. We conclude that proteins are preferentially utilized over carbohydrates in anaerobic
digestion as long as their relative concentrations are higher. Hence, proteins may play an important role in enhancing digestion.

Keywords: wastewater treatment, metagenomics, carbohydrates, *Spirochaetes*. 
1. Introduction

Anaerobic digestion (AD) is a common sewage sludge stabilization technique and achieves reduction of sludge solids content by converting organics into energy-rich biogas (Tezel et al. 2011). Since up to 50% of wastewater treatment plant operational costs are usually designated for solids disposal, AD is an attractive option to lower operational costs (Ariunbaatar et al. 2014).

Many full-scale anaerobic digesters still experience suboptimal performance. This might be caused by the complex nature of the digestion process itself, which involves chemical and biochemical conversions. AD can be divided into four distinct but tightly interconnected steps that are driven by distinct microbial groups possessing different metabolic requirements (Amani et al. 2010, Appels et al. 2008). The first digestion step is hydrolysis where complex particulate organics such as proteins, carbohydrates, and lipids are solubilized into their monomers including amino acids, sugars, and fatty acids. These monomers are then converted into short-chain fatty acids, alcohols, hydrogen, and carbon dioxide in the acidogenesis or fermentation step (Tezel et al. 2011). The intermediates from the fermentation step are further converted into acetic acid, hydrogen, and carbon dioxide in the acetogenesis step. The success of acetogenesis depends largely on the orchestrated activity between the acetogens and hydrogenotrophic methanogens, where the latter will utilize the hydrogen produced by the acetogens to maintain a low hydrogen partial pressure required for the success of acetogenesis (Appels et al. 2008, Tezel et al. 2011). The fourth and last step is methanogenesis in which acetic acid as well as hydrogen and carbon dioxide are converted into methane and carbon dioxide (Batstone and Jensen 2011). A balance must be struck among the four steps to ensure a successful digestion. There are several parameters that are important in maintaining this balance such as solids retention time, temperature, and substrate characteristics (Nielfa et al. 2015, Tezel et al. 2011).
Substrate characteristics that can affect the performance of an anaerobic digester include the ratio of primary to secondary sludge (Batstone and Jensen 2011, Tezel et al. 2011) as well as the type and composition of the substrate being digested (Cesaro and Belgiorno 2014, Mata-Alvarez et al. 2014, Nielfa et al. 2015). Proteins and carbohydrates make up more than 50% of the sludge organics, and their roles in anaerobic digestion have been explored (Elefsiniotis and Oldham 1994b, Neyens and Baeyens 2003). Most of the previous studies reported higher removal of carbohydrates than of proteins (Elefsiniotis and Oldham 1994b, Shao et al. 2013, Yang et al. 2015). This is because the presence of other organics such as carbohydrates might suppress the degradation of proteins by inhibiting the production of enzymes required for protein hydrolysis (Breure et al. 1986, Tommaso et al. 2003). These studies utilized simpler proteins and carbohydrates such as gelatin, bovine serum albumin (BSA), glucose, or yeast (Breure et al. 1986, Elbeshbishy and Nakhla 2012, Tommaso et al. 2003). The kinetics affecting degradation of these simpler organics may be different from those of complex proteins and carbohydrates that are present in sewage sludge, and different proportions of proteins and carbohydrates in the digester substrate have been shown to affect digestion efficiency (Vidal et al. 2000). Additionally, many of the studies on the effects of substrate characteristics were done under thermophilic conditions, although digesters are often operated at mesophilic temperatures (De Francisci et al. 2015, Tezel et al. 2011, Wagner et al. 2013).

The objectives of the present study were to explore the effects of different substrate characteristics on (i) process performance and (ii) microbial community dynamics of sludge digesters operated at mesophilic temperatures, with an emphasis on the role of proteins and carbohydrates. We hypothesized that proteins in the substrate sludge could be utilized more efficiently than carbohydrates if their concentrations were higher because carbohydrates would no longer act as inhibitor of protein utilization. We further stipulated that proteins would be the
preferred substrates over carbohydrates, leading to lower carbohydrate utilization in anaerobic digestion.

Sewage sludge from two different full-scale wastewater treatment plants was added to four laboratory-scale anaerobic digesters operated in two Phases. Metagenomic analysis was employed to analyze the effect of changing substrates on microbial communities. Batch tests to analyze biogas production using different substrates were then conducted by adjusting the solids concentration in one substrate type to ensure that the intrinsic characteristics were similar in both substrates and to monitor whether there was any difference in biogas production. Protein and carbohydrate degradation profiles were also analyzed in these batch tests. Lastly, degradation tests using simpler proteins and carbohydrates at different protein-to-carbohydrate ratios were conducted to further test preferential utilization of proteins over carbohydrates.

2 Materials and Methods

2.1 Laboratory-scale anaerobic digesters

Four 5-L double-jacket anaerobic reactors were used in this study. They were labelled R1 to R4 and served as replicates. Each reactor had a working volume of 4.2 L and was stirred at 180±1 rpm with a top mounted mechanical stirrer. The reactor covers were fitted with a rubber O-ring to ensure airtight sealing. After inoculation, the reactors were flushed with nitrogen gas to purge the residual oxygen in the headspace.

The operating temperature for all reactors was kept at 35±1°C by circulating heated water in the reactor jacket. The biogas produced was quantified using a U-shaped gas collection apparatus. The apparatus was filled with thermal oil as the barrier solution. For each 50 mL of biogas collected, a click was triggered and the biogas collected was released. The number of clicks was recorded in the computer connected to the reactors.

The reactor study was divided into two Phases. During each Phase, the reactors were fed in a semi-continuous feeding mode on a daily basis where 140 ml of sludge was discharged
from the reactors and an equal amount of WRP substrate was fed into the reactors. The solids retention time was 30±1 d and it was equal to the hydraulic retention time.

2.2 Anaerobic sludge and digester substrate

Seed sludge was obtained from an egg shaped anaerobic digester at the WRP1 in Singapore. The solids retention time (SRT) for this digester was 30±2 days and the working temperature was 30±1°C. During the first phase of lab-scale reactor operation, the substrate used was the thickened mixed primary and secondary sludge from WRP1. During the second phase, the substrate was collected from a different facility, WRP2. Phase 1 lasted six months and Phase 2 lasted 14 months.

After collection substrates were immediately sieved to remove larger sized particulates like gravel or hair that could hinder the feeding and discharging process. They were kept at 4°C prior to usage.

2.3 Activity tests

All activity tests were performed in triplicate using serum bottles that were inoculated with fresh anaerobic sludge obtained from WRP2. The working volume for each serum bottle was 80 mL, comprised of 77.3 mL of seed sludge and 2.7 mL of feed sludge. To gain a better understanding of the protein and carbohydrate degradation potential without introducing a bias in the form of different substrate solids concentrations, the WRP2 feed was diluted to match the solids concentration of WRP1 substrate. Bovine serum albumin (BSA) or sucrose was added at 1014 mg /L or 162 mg /L, respectively, to some of the bottles fed with the adjusted WRP2 substrate to double concentrations of the naturally occurring proteins and carbohydrates in the substrate sludge (Figure 1).

All serum bottles were closed with butyl rubber stoppers and capped with aluminum crimped seals before they were flushed with a mixture of nitrogen and carbon dioxide gas to
remove traces of oxygen in the headspace. The bottles were then stored in a shaking incubator kept at 35°C and 180 rpm.

2.4 Sample collection

Samples were collected from each of the reactors through a sampling port located at mid-height of the reactors. Sampling from the reactors was done twice weekly and samples were collected from the serum bottles on operational days 1, 2, 3, 8, 12, 14, and 21 (Fig. S3). All samples were immediately filtered through a 0.45-μm pore size filter, except for the samples designated for solids and total chemical oxygen demand analysis.

2.5 Physicochemical analysis

Solids measurements were done in accordance with Standard Methods (APHA 2012). Both total and soluble chemical oxygen demand (COD) were measured using HACH COD test kits with a detection range of 20-1500 mg/L. Volatile acids (VA) were measured using HACH VFA TNTplus 872 (HACH, Loveland, CO, USA) adopting the esterification method (Montgomery et al. 1962). The concentration of four main volatile acids (acetate, propionate, butyrate and valerate) was quantified in accordance with Standard Methods (APHA, 2012), using gas chromatography (GC 2010 plus, Shimadzu, Japan) equipped with TCD and FID detectors and a DB-FFAP capillary column from Agilent Technologies. Total alkalinity was determined using the HACH TNT 870 kit adopting the colorimetric method, while total Ammonia was quantified with HACH TNT 832 kit adopting the salicylate method (Loveland, CO, USA). The gas composition was measured using gas chromatography (GC 2010 plus, Shimadzu, Japan) equipped with TCD and ECD detectors. Total carbohydrates were measured using the Dubois method (Dubois, 1951) while total proteins were measured using the DC Protein Assay kit from Bio-Rad Laboratories (Hercules, CA, USA). The assay is based on the protein quantification by Lowry et al. (1951).

2.6 DNA Extraction and Sequencing
DNA was extracted from laboratory-scale digesters sludge using FastDNA\textsuperscript{©} Spin Kit for Soil and the FastPrep\textsuperscript{©} Instrument (MP Biomedicals, Santa Ana, CA, USA). The manufacturer’s protocol was modified by increasing the numbers of lysing steps of cells using the FastPrep\textsuperscript{©} instrument to four, each lasting 40 s. The extracted DNA was further purified using DNA Clean and Concentrator™-10 purification kit (Zymo Research Corp., Irvine, CA, USA). The quality and concentration of the extracted and purified DNA was evaluated using NanoDrop\textsuperscript{©} 2000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA), and Qbit \textsuperscript{©} 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. An agarose gel stained with ethidium bromide, using a 1-kb ladder as reference, was used to ensure that the extracted DNA was not too fragmented. The DNA sequencing was done using the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Prior to sequencing, the quality of DNA samples was checked on Bioanalyzer 2100 using a DNA 12000 Chip (Agilent, Santa Clara, CA, USA). Sample quantitation was carried out using Invitogen’s Picogreen assay. Library preparation was performed according to Illumina’s TruSeq Nano DNA Sample Preparation protocol. Following manufacturer’s recommendation, DNA samples were sheared on a Covaris S220 or E220 to approximately 450 bp and uniquely tagged with Illumina barcode to allow pooling of libraries for sequencing. The finished libraries were quantified using Invitogen’s Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent, Santa Clara, CA, USA). Library concentrations were normalized to 4 nM and validated using qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems, CA, USA) using KAPA Illumina Library Quantification Kit (Kapa Biosystems, Roche). The libraries were pooled at equimolar concentrations and sequenced on Illumina HiSeq 2500 sequencer. Rapid mode at a read-length of 250 bp paired-end was used. Raw reads generated from the sequencing were quality checked and adaptors were removed using cutadapt version 1.9.1 with default parameters (except --overlap 10 -m 30). After a quality check, the reads were processed.
using Ribotagger-v.8.1.r106 with default parameters targeting the V4 hyper variable region (Xie et al. 2016).

2.7 **Theoretical calculation of volatile matter reduction**

Theoretical achievable volatile matter reduction in reactors (Vd) was calculated using the following equation (Tchobanoglous et al. 2003):

\[
Vd = 13.7 \times \ln (\text{SRT}) + 18.9
\]

(Eq. 1)

where \( Vd \) (%) is the theoretical volatile matter reduction that can be achieved at a specific SRT (d).

2.8 **Statistical Analysis**

A two-sample t test assuming unequal variance was used in this study. The t test for unequal variance has been suggested to be equally or more robust against types I and II errors than the t test for equal variance (Ruxton 2006).

Multivariate analysis of microbial community was done using PRIMER v6 with PERMANOVA+ software (Clarke and Gorley 2006). The processed read counts were first standardized based on the total counts per sample. Square-root transformation was applied to the standardized data to minimize effects of dominant organisms before Bray-Curtis distance matrix was constructed. Both PERMANOVA (permutational multivariate analysis of variance) and ANOSIM (analysis of similarity) were used to check the null hypotheses that communities in Phase 1 and Phase 2 were similar (Anderson and Walsh 2013).

PERMANOVA and ANOSIM were suitable alternatives for multivariate analysis of variance (MANOVA) since MANOVA required assumption of multivariate normality to be met (Anderson 2001, Clarke 1993). The number of permutations was set to be 9999 and substrate was used as fixed factor. PERMDISP analysis was done to determine homogeneity of variance among samples (Anderson 2006).
3. RESULTS

3.1 Laboratory-scale anaerobic reactor operation

The concentrations of organics in WRP2 substrate were significantly higher than in WRP1 (Table 1). This led to higher reactor effluent concentrations when the substrate was switched from WRP1 in Phase 1 to WRP2 in Phase 2. The volatile solids concentration in the four replicate reactor effluents ranged from 7,000 to 10,000 mg/L when reactors were fed with WRP1 substrate, and from 17,000 to 20,000 mg/L in the second phase when they were received WRP2 substrate (Figure 1).

Although we operated four replicate reactors throughout the study, some unplanned temperature perturbations occurred in reactors R3 and R4 on days 246 and 292 (Figure S1), which affected reactor performance. In the two undisturbed reactors, the pooled average volatile matter reduction (VMR) increased slightly from 39±16% in Phase 1 to 46±13% in Phase 2 (P=0.012, unpaired t-test) (Table 2). Average tCOD removal was not statistically different between phases (49 ± 23 % in Phase 1 and 52 ± 11% in Phase 2, P=0.42, unpaired t-test).

Due to technical issues when reactors were connected directly to the U-shaped meter, the biogas collection method was changed to gas bags in the latter stage of Phase 2. The gas in the bags was then pushed manually through the U-shaped meter for quantification purposes. The mean biogas production in Phase 1 was 550 ± 465 mL of biogas/day. The average increased to 5,920 ± 2,076 mL biogas/day in Phase 2. This increase was independent of the collection method used.

Enhanced biogas production could have been due to either protein or carbohydrate digestion. The mean protein removal in R1 increased by 20% from 36 ± 17% in Phase 1 to 56 ± 18% in Phase 2 (Table 2), which was statistically significant with a p-value of 0.0001. Unlike
proteins, average carbohydrate removal did not change significantly when the substrate was switched to WRP2 in Phase 2 (49 ± 17 % in Phase 1 and 56 ±22 % in Phase 2; P=0.11, unpaired t-test) (Table 2). The lack of increase in carbohydrate removal suggests that proteins were responsible for the increase in biogas production in Phase 2.

3.2 Protein and carbohydrate degradation test

We considered that the observed performance differences could have been due to the fact that the two substrates originated from different wastewater treatment plants. One of the substrates also had significantly higher volatile solids concentrations. To exclude these alternate explanations, we conducted activity tests for 21 days using WRP1 and WRP2 substrates. The substrate from WRP2 was diluted with water so that its organics concentration was similar to that in WRP1 substrate (t-test between the WRP1 substrate and diluted WRP2 substrate dilution yielded p-value of 0.88) (Table 3). Biogas production under these two conditions was highly similar, indicating that location did not cause the difference observed in digestion efficiency (Figure 2).

We performed additional degradation tests using basal medium with simple proteins and carbohydrates such as BSA and sucrose to minimize effects of different types of complex carbohydrates and proteins in the substrate sludge. Since different organics might have different degradation potentials at different conditions, BSA and sucrose were selected to ensure that the hydrolysis step could be bypassed. This was done to ensure that the true degradation potentials of proteins and carbohydrates could be elucidated. In this test, either BSA or sucrose was added to selected bottles fed with the adjusted WRP2 substrate to double the original protein and carbohydrate concentrations to 2,919 mg BSA /L and 378 mg glucose/L, respectively. The bottles augmented with BSA produced the highest cumulative
amount of biogas, while the bottles fed with additional sucrose had a negligible biogas increase when compared to the non-augmented WRP2 bottles (Figure 2).

We also investigated the degradation kinetics of proteins and carbohydrates in the activity test to determine which of the two was preferentially used in the digestion process. The concentrations of particulate proteins and carbohydrates increased sharply on day 1 (Figure 3a and b). Particulate proteins increased from 963 mg BSA/L to 2,098 mg BSA/L in the bottles fed with WRP1 substrate and from 943 to 2,209 mg BSA/L in the bottles fed with WRP2 substrate, while particulate carbohydrates increased from 110 mg sucrose/L and 109 mg sucrose/L to 250 mg sucrose/L and 314 mg sucrose/L for WRP1 and WRP2, respectively. This increase was possibly due to hydrogen partial pressure. Particulate protein concentrations decreased by 24% for WRP1 and 36% for WRP2 the next day while particulate carbohydrates in WRP1 and 2 bottles only decreased by 5% and 17% from day 2 onward (Figure 3A and B). Particulate protein concentrations in WRP1 bottles kept on decreasing until day 12 of the degradation test, while particulate proteins in WRP2 bottles decreased until day 8, before the concentrations became more or less constant. The larger overall decrease in proteins suggested that proteins were utilized better than carbohydrates. This observation was further supported by a larger decrease in soluble proteins than carbohydrates (Figures 3 C and D).

Particulate protein profiles in bottles augmented with BSA were similar to non-BSA augmented bottles (Figure 3A). This was expected since additional BSA affected soluble proteins and not particulate proteins. This addition further reduced the hydrolysis of carbohydrates as shown by the increase in particulate carbohydrates (Figure 3B). In addition, soluble carbohydrate concentrations in the bottles fed with additional BSA did not decrease, suggesting that carbohydrates were not used (Figure 3D).
The additional carbohydrates in the form of sucrose did not have any palpable impact on the utilization of carbohydrates and proteins in the activity test. Particulates carbohydrates and proteins in these bottles did not differ noticeably from the non sucrose augmented WRP2 (Figures 3A and B). After the additional carbohydrates were utilized at the beginning of the test, soluble carbohydrate concentration increased until a final concentration of 246 mg sucrose/L on day 21 of the test, implying that the additional sucrose did not enhance carbohydrate utilization (Figure 3D).

Lastly, to further confirm potential inhibitory effects of proteins on carbohydrate utilization, an additional degradation test was conducted using two different protein-to-carbohydrate ratios. Basal medium, BSA and sucrose were added to selected serum bottles containing anaerobic sludge. For a P:C ratio of 5:1, 54% decrease of BSA was detected on day 3 while 84% decrease of BSA was observed at a 30:1 ratio (Figure 4A). In contrast, the sucrose concentration on day 3 for a 5:1 and 30:1 ratio only decreased by 14% and 22%, respectively (Figure 4B).

3.3 Microbial community dynamics

Microbial community analysis was performed for reactors R1 and R2 only since reactors R3 and R4 had been exposed to unplanned temperature perturbations. Samples were collected from R1 and R2 during steady state periods for both Phases (i.e., more than three SRTs had passed since the substrate change occurred). R1 and R2 community profiles were highly similar. The average bacteria and archaea abundance in R1 was 74% and 7%, respectively, while in R2, the abundance was 74% for bacteria and 6% for archaea. The rest of the OTUs were unclassified (Table 4). Both reactors were dominated by the bacterial phyla Actinobacteria, Proteobacteria, Chloroflexi, Bacteroidetes and the archael genus Methanosaeta (Figure 5A and B). Once R1 and R2 were fed with WRP2 substrates, bacteria
abundance in R1 and R2 increased by 12% and 13%, respectively (Table 4). In contrast, mean archaea abundance decreased to 4% in both R1 and R2 (Table 4). The dominant phyla were similar to those in Phase 1. The only exception was the emergence of Spirochaetes as one of the most dominant phyla in Phase 2 (Figure 5).

Clustered separation of sample points using NMDS ordination further showed that the communities in the two phases were different (Figure 6). PERMANOVA p-values were 0.0001 and 0.0005 with Pseudo-F values of 13.65 and 15.59 for R1 and R2, respectively, confirming that communities were significantly different when fed with different substrates. Large ANOSIM R values further supported this observation (0.81 for R1 and 0.89 for R2). Moreover, p-values obtained from PERMDISP analysis were 0.81 for R1 and 0.88 for R2, indicating homogenous dispersion between the two groups. This showed that differences between communities were caused by different substrates and not due to internal variances among samples.

The abundance of the phylum Spirochaetes in Phase 2 was noticeably higher than in Phase 1 (Figure 5). There was enrichment over time of the OTUs classified as members of W5, an order within the Spirochaetes class in this phylum. The average abundance of W5 increased by 12% and 17% in R1 and R2, respectively, after both reactors had received WRP2 substrates for an extended period of time. The changes in the abundance of the other phyla were less evident (less than 5% change in abundance).

Archaea only made up a small portion of the community; hence, we analyzed archaea separately since they play a substantial role in anaerobic digestion. We selected OTUs assigned as archaea and re-standardized them based on total counts of archaea only. Both PERMANOVA and ANOSIM analyses showed that archaea differed significantly in the two
phases (p-value: 0.0056 and Pseudo-F: 7.57; ANOSIM R: 0.21). PERMDISP analysis yielded a p-value of 0.58, indicating homogenous dispersion among samples in both Phases.

The differences in archaea were mainly caused by a shift in hydrogenotrophic methanogens. The abundance of *Methanolinea* genus in R1 and R2 decreased sharply after the reactors had received WRP2 substrates (Figure 7). When the reactors received WRP1 substrates, *Methanolinea* made up 22% of the archaea community in R1 and 31% of the archaea community in R2, respectively, making it the second most abundant methanogen after *Methanoseta*. However, this genus was not detected in Phase 2. Instead, different types of hydrogenotrophic methanogens occupied its niche, namely, *Methanospirillum* and *Methanobacterium*. In Phase 2, *Methanospirillum* accounted for 11% of the average archaea community in R1, an increase of 10% from Phase 1. This genus represented 17% of the archaea community in reactor 2, an increase of 15% from Phase 1. The average abundance of *Methanobacterium* in R1 increased to 8% of the archaea communities from 2% in Phase 1 while in R2, this genus rose from 1% to 21% of the archaea communities in Phase 2.

Microbial community analysis in R3 and R4 was limited to the samples collected at the beginning of Phase 2 prior to unplanned temperature perturbations that occurred in R3 and R4. Microbial community dynamics at the beginning of Phase 2 in R3 and R4 were similar to those in R1 and R2 at the end of Phase 2, indicating that changes had been detected shortly after the substrate was changed. The mean bacterial abundance in R3 and R4 was 73% for both in Phase 1 and increased to 79% and 77% in Phase 2. The average abundance of archaea in reactors 3 and 4 decreased by 1% from 6% to 5% in both reactors (Table S1).

Similar to the profiles observed in reactors R1 and R2 at a much later stage of Phase 2, NMDS profiles of R3 and R4 also showed a clear separation between the two substrate groups (Figure S2). PERMANOVA analysis of the two reactors also yielded p-values lower than 0.05.
(0.047 and 0.0001 and pseudo-F values of 3.06 and 6.06 for reactors 3 and 4) while ANOSIM results yielded R values of 0.49 and 0.50 for both reactors. PERMDISP p-value was 0.77 (pseudo-F of 0.15) for reactor 3 and 0.88 (pseudo-F<0.1) for reactor 4. These analyses indicated that changes in microbial communities were already detected shortly after the substrate had changed.

Moreover, the average abundance of the phylum Spirochaetes started to increase in R3 and R4 at the beginning of Phase 2 (2.83% and 0.5% in R3 and R4, respectively). Similarly, phylum Methanomicobia started to decrease by 1.21% and 1.23% in R3 and R4 respectively. This further showed that constant feeding of the WRP2 feed had induced the emergence of protein users over time despite a temperature perturbation, supporting the hypothesis that protein was used preferentially in Phase 2.

4. DISCUSSION

A change in substrate from WRP1 to WRP2 led to improved biogas production. We hypothesized that the relative abundance of proteins and carbohydrates in the substrate played a role since they make up a large portion of substrate organic matter (Elefsiniotis and Oldham 1994b, Yang et al. 2015). Of the two, proteins seemed to be more important as supported by the more significant increase in protein removal when compared to carbohydrate removal in Phase 2, although protein and carbohydrate concentrations increased comparably from Phase 1 to Phase 2.

Study results differ from previous reports that carbohydrates had a higher degradation efficiency compared to proteins (Elefsiniotis and Oldham 1994b, Yang et al. 2015). Hydrolytic coefficients for carbohydrates were reported to range from 0.025 to 0.54 d⁻¹ while protein coefficients were between 0.015 and 0.075 d⁻¹ (Christ et al. 2000, Gujer and Zehnder 1983, Pavlostathis and GiraldoGomez 1991). Hence carbohydrate degradation in anaerobic digestion should be faster that protein degradation (Pavlostathis and GiraldoGomez 1991). A possible
explanation for the divergent results in this study is the overall higher protein concentration in substrates. It has been reported that the presence of carbohydrates may suppress the production of enzymes required for protein hydrolysis (Breure et al. 1986, Elefsiniotis and Oldham 1994b, Tommaso et al. 2003). Hence the low carbohydrate concentrations in the substrates could have boosted the degradation of proteins in this study.

The notion that proteins played a more important role in our study was further supported by the biogas production. The theoretical biogas production from carbohydrate-rich substrate at standard conditions (0°C and 1 bar absolute pressure) is 0.415 L/g of VS while the theoretical methane yield from protein is 0.496 L/g of VS (Angelidaki and Sanders 2004). Since the theoretical gas production did not differ much and protein removal increased more than did carbohydrate removal, the increase in biogas production in Phase 2 could be contributed to proteins. The ratio of methane and carbon dioxide in our biogas was in the range of 60-70% CH₄ and 30% CO₂. This corresponds to the theoretical ratio of CH₄ to CO₂ in the biogas produced by protein-rich feed, which was 70-71% to 29-30%, compared to the 50:50 theoretical composition produced by carbohydrate-rich feed (Weiland 2010).

### 4.1 Effect of protein and carbohydrate addition to substrates

Degradation tests showed that the substrate constituents were the main driver of the differences in digestion efficiencies observed. Higher biogas production observed in bottles augmented with BSA also supported the hypothesis. Significantly, there was negligible increase in biogas production in the bottles augmented with sucrose when the carbohydrate levels were double those of unamended bottles. Proteins were solubilized better than carbohydrates, although previous studies had reported faster and higher carbohydrate solubilization (Breure et al. 1986, Miron et al. 2000, Yang et al. 2015). One possible reason for this discrepancy is that the carbohydrate concentrations in the substrates used in our study were much lower than those of proteins.
Despite the overall decreasing trends, there were fluctuations in particulate protein concentrations. An increase on day 1 was observed in all the samples collected. It has been reported that extracellular polymeric proteins and carbohydrates are produced during the first days of digestion, possibly due to the high hydrogen partial pressure observed during this period of time (Jia et al. 1996). The possible presence of high hydrogen partial pressure on the first day of the activity test is supported by the presence of 35 to 116 mg/L propionate in samples on day 1. High hydrogen partial pressure can inhibit propionate conversion to acetate (Appels et al. 2008, Batstone and Jensen 2011). Propionate was not detected on subsequent days, indicating that the hydrogen partial pressure might have decreased. This decrease coincided with the decrease in the particulate protein concentration observed on subsequent days.

Another possible reason for the intermittent increase in particulate proteins is accumulation on the surface of bacterial cells (Liu and Fang 2002). Proteins are major components of extracellular polymeric substances (EPS) and bacteria produce enzymes required to perform digestion of macromolecules within their microenvironment (Laspidou and Rittmann 2002, Park et al. 2008, Zorel et al. 2015). Therefore, it is possible that the recurring increase in particulate protein levels, especially from day 3 onward, was caused by the secretion of hydrolytic enzymes on the surface of the bacterial cells to aid in the degradation of particulate organics. The secretion of enzymes is typically recurrent until the organics are completely broken down into smaller fragments (Confer and Logan 1998).

Soluble proteins started to increase from day 12 onward, possibly due to depletion of nutrients and available carbon sources since the activity test was a batch test where no additional substrates were added. Thus, to prolong their survival, microorganisms may produce soluble microbial products such as proteins to scavenge for necessary nutrients to prolong their survival (Aquino and Stuckey 2003, Barker and Stuckey 1999, Boero et al. 1991, Laspidou and
Rittmann 2002). The lack of a noticeable increase in biogas production at this stage seemed to support this rationale. Moreover, cell lysis might also explain the increase in soluble proteins since occurrence of cell lysis at longer SRTs has been observed before (Elefsiniotis and Oldham 1994b). This is supported by the decrease in the volatile solids concentration near the end of the activity test period.

Carbohydrates were not consumed as well as proteins in the activity test, suggesting that high levels of proteins could slow carbohydrate utilization. A previous study reported reduced carbohydrate utilization when protein concentrations were equal or higher than those of carbohydrates (Elbeshbishy and Nakhla 2012). Another possible reason was the rigorous mixing of the serum test bottles. Elfsiniotis and Oldham (1994) reported that the degradation of carbohydrates was more effective in an upflow anaerobic sludge blanket (UASB) than in a continuously mixed system due to the slow rate of diffusion occurring in the former. This coupled with the short test period might have resulted in incomplete hydrolysis of the particulate carbohydrates.

Near the end of the activity test, particulate carbohydrates started to be solubilized. It is possible that protein concentrations were too low at that stage to affect utilization of carbohydrates. A higher carbohydrate hydrolytic coefficient was observed when protein concentrations were lower (Elbeshbishy and Nakhla 2012). Cell lysis may have caused this decrease and resulted in organic materials in the sludge being released into the bulk solution (Barker and Stuckey 1999). This observation was corroborated by the increase in soluble carbohydrate concentrations, because carbohydrates released from biomass are considered one of the sources of soluble microbial products (SMPs) (Aquino and Stuckey 2003, Barker and Stuckey 1999, Boero et al. 1991, Laspidou and Rittmann 2002). Moreover, the notion of cell lysis was supported by the low biogas production observed near the end of the digestion period.
Furthermore, in bottles fed substrates with protein-to-carbohydrate ratios of 5:1 or 30:1, proteins inhibited the uptake of carbohydrates as evident from the lack of removal of easily degradable carbohydrates that had been added. The sucrose in the bottles was not utilized until the BSA concentrations were noticeably lower from day 2 onward.

The aforementioned trends in the carbohydrate and protein concentrations coupled with the rapid increase in biogas production at the beginning of the test suggested that proteins may be used to drive biogas production. However, proteins are also known to be the precursor of ammonia, a possible inhibitor in the digestion process (Astals et al. 2014, Kayhanian 1999). Hence addition of proteins must be considered with caution. The ammonia generated during the activity test did not seem to have any negative effect on biogas production (Table 2). The low volatile fatty acids observed in reactors also implied that methanogenesis was proceeding well in all samples tested.

### 4.2 Microbial community dynamics when fed with sewage sludge with significantly different proteins and carbohydrates

#### 4.2.1 Bacteria

In both Phases, the dominant bacterial phyla were hydrolytic, fermentative, and syntrophic organisms that have been reported to dominate in many anaerobic digesters (Ariesyady et al. 2007, Guo et al. 2015, Rivière et al. 2009, Rui et al. 2015, Tezel et al. 2011). Microbial community dynamics in Phase 1 and Phase 2 were affected by the change in substrate which is in agreement with previous findings (De Francisci et al. 2015, Lu et al. 2013, Pavlostathis and Giraldo-Gomez 1991, Sundberg et al. 2013).

The enrichment over time of *Spirochaetes* in reactors 1 and 2 was striking. Although many members of this phylum can be pathogenic, causing diseases such as syphilis and Lyme
disease (Cabello et al. 2001), some of the non-pathogenic members are glucose-consumers in anaerobic digestion, especially members of the class *Spirochaete* (Ariesyady et al. 2007, Delbès et al. 2000, Gou et al. 2016). It is, therefore, of interest that the relative abundance of this phylum should increase when the carbohydrate utilization remained the same in Phase 2. The reason was that the members of this phylum, whose abundance increased conspicuously in Phase 2, were affiliated to order W5. Although this order has been classified here as part of the class *Spirochaete*, there is ongoing debate about its appropriate classification, as it is also part of the candidate phylum *Cloacimonetes*. Some have proposed that this candidate phylum should be recognized as a stand-alone group, while others prefer to include it in the *Spirochaetes* phylum as a member of the class *Spirochaete* (Rinke et al. 2013, Rui et al. 2015, Stolze et al. 2016).

Similar to *Spirochaetes*, *Cloacimonetes* is a novel group of bacteria whose ecological roles are still widely unknown due to lack of reference organisms (Pelletier et al. 2008a, Stolze et al. 2016). Only one representative species of this group has been completely sequenced. This reference bacterium, *Candidatus cloacamonas acidominovorans*, was reported to be involved in the metabolism of amino acids (Pelletier et al. 2008a). Hence, the enrichment of *Cloacimonetes* members over time, coupled with an increase in protein utilization when reactors 1 and 2 were fed with WRP2, supports the hypothesis that proteins rather than carbohydrates were utilized in Phase 2. Although this bacterium has also been suggested to use carbohydrates, the lack of a noticeable increase in carbohydrate utilization in Phase 2 contradicts this notion. Overall, microbial community analysis strengthens the hypothesis that high protein levels hampered the utilization of carbohydrates since organisms that could use both proteins and carbohydrates did not seem to use the additional carbohydrates available in WRP2 substrates.
4.2.2 Archaea

With regard to archaea, the abundance of *Methanosaeta* was expected since this methanogen has been reported to dominate in stably operating anaerobic digesters (McHugh et al. 2003b, Nelson et al. 2011, Rivière et al. 2009). Their abundance in our reactors might be due to the low concentrations of volatile acids, a condition that selected for *Methanosaeta* over *Methanosarcina*, another common acetoclastic methanogens, due to the former’s lower acetate threshold (Demirel and Scherer 2008, Jetten et al. 1992). The low volatile acids levels may explain the absence of *Methanosarcina* in both phases.

Of the hydrogenotrophic methanogens, the disappearance of *Methanolinea* in Phase 2 was likely caused by the hydrogen partial pressure. This genus is a member of the order *Methanomicrobiales* whose high affinity toward hydrogen means that it can thrive under conditions with low partial pressure of hydrogen; hence, its abundance in the reactors decreased as the hydrogen partial pressure increased (Sakai et al. 2009). Hydrogen was the intermediate following the anaerobic conversion of the organics in the substrates, (Batstone and Jensen 2011, Pavlostathis and Giraldo-Gomez 1991, Tezel et al. 2011). Therefore, although the concentrations of the hydrogen partial pressure in the reactor headspace remained too low to be detectable, an increase in hydrogen partial pressure was possible due to the increase in the organics in Phase 2.

The possible increase in hydrogen partial pressure was further confirmed by the emergence of *Methanospirillum* and *Methanobacterium*. Although *Methanospirillum* is a member of the *Methanomicrobiales* order, it displays a low affinity toward hydrogen partial pressure; hence, its abundance increases when the hydrogen partial pressure increases, a trend that is also observed in the genus *Methanobacterium* (Sakai et al. 2009). *Methanobacterium*
is also a fast growing methanogens; hence, it is likely that they outgrew *Methanolinea*, a slower growing methanogens, in Phase 2 (Sakai et al. 2007).

The lack of a control reactor in this study might make it difficult to separate the substrate effects from temporal effects. However, the community dynamics in reactors R3 and R4 revealed that similar changes were already happening at the early stage of Phase 2. Moreover, the changes observed in R3 and R4 were similar to those observed in R1 and R2 (i.e., the enrichment of the phylum Spirochaetes and the decrease of Methanomicrobia), although the magnitude of changes was less than the changes observed in R1 and R2. The eventual enrichment or demise of certain organisms in R1 and R2 showed that constant feeding using WRP2 substrates had intensified whatever changes were observed at the beginning of the Phase, suggesting that changes observed at the latter stage of Phase 2 were mainly caused by the substrates.

These trends further highlight the importance of not only the type of substrate but also the intrinsic characteristics of the substrates used. This is so because changes in both process performance and microbial community were observed in Phase 2 despite the fact that the reactors were fed with sewage sludge in both Phases.

It would also be of interest to elucidate the nature of proteins and carbohydrates in the different substrates to distinguish the types of proteins and carbohydrates that could be most beneficial for anaerobic digestion.

5. **CONCLUSIONS**

- Protein degradation was favored over carbohydrate degradation in the anaerobic digestion of substrates that contained more proteins than carbohydrates, suggesting the potential of proteins to enhance the digestion process and biogas production.
- Some organisms that could use both proteins and carbohydrates were acclimated to using only proteins. This was achieved by continuously subjecting them to substrates containing more proteins than carbohydrates.

- Adopting a targeted optimization method based on the intrinsic characteristics of digester substrate can be beneficial. For example, when the digester substrate contains more proteins than carbohydrates, instead of augmenting with carbohydrates, which would not be utilized efficiently, it would be better to augment with proteins to increase biogas production.

ACKNOWLEDGEMENTS

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Table 1. Comparison of substrates from WRP1 and WRP2. Values represented the mean with standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Substrate source</th>
<th>P-value$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WRP1$^2$</td>
<td>WRP2$^3$</td>
</tr>
<tr>
<td>Total Solids (mg/L)</td>
<td>20,626 (5,630)</td>
<td>66,320 (16,918)</td>
</tr>
<tr>
<td>Volatile Solids (mg/L)</td>
<td>17,260 (4,794)</td>
<td>49,925 (11,314)</td>
</tr>
<tr>
<td>Total COD (mg/L)</td>
<td>33,895 (11,319)</td>
<td>92,840 (23,595)</td>
</tr>
<tr>
<td>Soluble COD (mg/L)</td>
<td>1,406 (380)</td>
<td>7,381 (5,934)</td>
</tr>
<tr>
<td>Total Protein (mg/L)</td>
<td>931 (680)</td>
<td>4,011 (5,170)</td>
</tr>
<tr>
<td>Total Carbohydrate (mg/L)</td>
<td>169 (143)</td>
<td>645 (211)</td>
</tr>
</tbody>
</table>

$^1$ Mean with standard deviation in parentheses

$^2$ n= 23 samples

$^3$ n= 20 samples

$^4$ P value was calculated from two samples t-test assuming unequal variance. T-test for unequal variance had been suggested to be equally or more robust against types I and II errors in hypothesis testing notwithstanding the underlying variances (Ruxton 2006). The null hypothesis that WRP1 and WRP2 substrates were similar was rejected when the P value was < 0.05.

Table 2: Process performance in replicate laboratory-scale anaerobic reactors. Values represent the pooled mean with standard deviation in parentheses.
<table>
<thead>
<tr>
<th>Parameter (unit of measurement)</th>
<th>Substrate source</th>
<th>P-value$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WRP$^{1,3}$</td>
<td>WRP$^{2,3}$</td>
</tr>
<tr>
<td>Volatile matter reduction (%)</td>
<td>39 (16)</td>
<td>46 (13)</td>
</tr>
<tr>
<td>Total COD removal (%)</td>
<td>49 (23)</td>
<td>52 (11)</td>
</tr>
<tr>
<td>Soluble COD removal (%)</td>
<td>82 (6)</td>
<td>89 (6)</td>
</tr>
<tr>
<td>Volatile acids (mg/L of acetic acid)</td>
<td>41 (20)</td>
<td>122 (38)</td>
</tr>
<tr>
<td>Biogas production (mL)</td>
<td>550 (465)</td>
<td>5,920 (2,076)</td>
</tr>
<tr>
<td>Ammonia (mg/L)</td>
<td>563 (78)</td>
<td>1,467 (227)</td>
</tr>
<tr>
<td>Average protein removal (%)</td>
<td>36 (17)</td>
<td>56 (18)</td>
</tr>
<tr>
<td>Average carbohydrate removal (%)</td>
<td>49 (17)</td>
<td>56 (22)</td>
</tr>
</tbody>
</table>

$^1$ n = 46

$^2$ n = 40

$^3$ R1 and R2 were replicates and they received WRP1 substrates for 6 months and WRP2 substrates for 14 months. The samples used were those collected during the last 4 to 5 months of each phase.

$^4$ P value was calculated from two samples t-test assuming unequal variance. T-test for unequal variance had been suggested to be equally or more robust against types I and II errors in hypothesis testing notwithstanding the underlying variances (Ruxton 2006). The null hypothesis that WRP1 and WRP2 substrates were similar was rejected when the P value was < 0.05.

Table 3: Physiochemical properties of substrates used in protein and carbohydrate batch degradation tests.

The substrate from WRP2 was diluted so that its total solids concentration was similar to that from WRP1.
<table>
<thead>
<tr>
<th>Constituent or ratio (unit)</th>
<th>Substrate source</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (mg/L)</td>
<td>WRP1</td>
<td>25,900</td>
<td>26,000</td>
</tr>
<tr>
<td>VS (mg/L)</td>
<td>WRP1</td>
<td>21,300</td>
<td>18,700</td>
</tr>
<tr>
<td>VS/Ts</td>
<td>WRP1</td>
<td>0.82</td>
<td>0.72</td>
</tr>
<tr>
<td>Protein (mg/L)</td>
<td>WRP1</td>
<td>1,608</td>
<td>1,014</td>
</tr>
<tr>
<td>Carbohydrate (mg/L)</td>
<td>WRP1</td>
<td>193</td>
<td>162</td>
</tr>
<tr>
<td>P to C</td>
<td>WRP1</td>
<td>8.33</td>
<td>6.26</td>
</tr>
<tr>
<td>TCOD (mg/L)</td>
<td>WRP1</td>
<td>37,880</td>
<td>31,920</td>
</tr>
<tr>
<td>SCOD (mg/L)</td>
<td>WRP1</td>
<td>1360</td>
<td>1440</td>
</tr>
<tr>
<td>VFA (mg/L)</td>
<td>WRP1</td>
<td>1240</td>
<td>1280</td>
</tr>
<tr>
<td>Alkalinity(mg/L CaCO₃)</td>
<td>WRP1</td>
<td>508</td>
<td>510</td>
</tr>
<tr>
<td>Ammonia (mg/L)</td>
<td>WRP1</td>
<td>165</td>
<td>118</td>
</tr>
<tr>
<td>Nitrate (mg/L)</td>
<td>WRP1</td>
<td>0.56</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 4: Average abundance of bacteria, archaea, eukaryote and unclassified in replicate reactors R1 and R2. The values represented mean with standard deviation in parentheses. The reactors received WRP1 substrate in Phase 1 and WRP2 substrate in Phase 2.
<table>
<thead>
<tr>
<th>Group (unit of measurement)</th>
<th>Phase 1&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Phase 2&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>R2</td>
</tr>
<tr>
<td>Bacteria (%)</td>
<td>74 (3)</td>
<td>74 (1)</td>
</tr>
<tr>
<td>Archaea (%)</td>
<td>7 (2)</td>
<td>7 (1)</td>
</tr>
<tr>
<td>Eukaryote and Unclassified (%)</td>
<td>18 (3)</td>
<td>19 (2)</td>
</tr>
</tbody>
</table>

<sup>1</sup> n: 8 samples per reactor

<sup>2</sup> n: 8 samples per reactor
**Figure 1:** Mean effluent volatile solids concentrations in replicate reactors R1 and R2. The reactors were fed WRP1 substrate in Phase 1 and WRP2 substrate in Phase 2.
Figure 2: Biogas production from activity test as function of time. Time points represent the sampling days ((1, 2, 3, 8, 12, 15, and 21) when samples were collected from the serum bottles. , WRP1, bottles fed with substrate collected from WRP1; , WRP2, fed with substrate collected from WRP2 which solids concentration had been adjusted to match that in WRP1 substrate; , 2P, adjusted WRP2 with additional bovine serum albumin to double the substrate protein concentration; , 2C, adjusted WRP2 substrate with additional sucrose to double the substrate carbohydrate concentration. Error bars represented the standard error of the means among triplicates at each time point. Some of the error bars are contained within symbols.
Figure 3: Protein and carbohydrate concentrations during activity tests. Time points represent the sampling days ((1, 2, 3, 8, 12, 15, and 21) when samples were collected from the serum bottles. Particulate (A) and soluble (C) protein concentrations in triplicate bottles as a function of time. Particulate (B) and soluble (D) carbohydrate concentrations in triplicate bottles as a function of time. WRP1, bottles fed with substrate collected from WRP1; WRP2, fed with substrate collected from WRP2 which solids concentration had been adjusted to match that in WRP1 substrate; 2P, adjusted WRP2 with additional bovine serum albumin to double the substrate protein concentration; 2C, adjusted WRP2 substrate with additional sucrose to double the substrate carbohydrate concentration. Error bars represented the standard deviation among triplicates at each time point. Some of the error bars are contained within symbols.
Figure 4: Protein and carbohydrate degradation in bottles fed substrates with different P to C ratios. Time points represent the sampling days (1, 2, 3, 8, 12, 15, and 21) when samples were collected from the serum bottles. (A) Protein concentrations in triplicate bottles as a function of time. (B) Carbohydrate concentrations in triplicate bottles as a function of time. Red +, bottles fed with substrate containing a P to C ratio of 5:1. Blue ●, bottles fed with substrate containing a P to C ratio of 30:1. Error bars represent the standard error of the mean among triplicates at each time point. Some of the error bars might be contained within the symbols.
Figure 5: Average phylum abundance in reactors 1 and 2. Each data point represents the average abundance with error bars denoted standard deviation. All phyla belong to the Bacteria except Methanobacteria, Methanomicrobia, Thermoplasmata, which belonged to Archaea, Eukaryota, and Unclassified. Figure A refers to samples collected from reactor 1, while (B) refers to samples collected from reactor 2. ●, samples collected in Phase 1 (WRP1 substrate); ○, samples collected in Phase 2 (WRP2 substrate).
Figure 6: nMDS showing clustering of samples in each Phase. (A) nMDS profile from R1. (B) nMDS profile from R2. The graphs were generated using Primer 6 software. The data used to generate this plot were transformed using square-root transformation to minimize the bias that might be introduced by the most dominant OTUs. ●, refers to samples collected when the reactor was fed with WRP1 (Phase 1); ○, refers to samples collected when the reactor was fed with WRP2 (Phase 2). Numbers denote the day of reactor operation.
Figure 7: Average archaea abundance in reactors 1 and 2. Each data point represents the average abundance with error bars denoting the standard deviation.

Figure (A) was for reactor 1 while (B) was for reactor 2. ●, samples collected in Phase 1 (WRP1 substrate); ○, samples collected in Phase 2 (WRP2 substrate).
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CHAPTER 3

Influence of solids retention time on process performance and microbial community dynamics in mesophilic anaerobic digestion of sludge

ABSTRACT

A short solids retention time (SRT) increases the amount of sludge that can be treated per unit volume of a digester thus increasing digester efficiency. However, it can also lead to incomplete hydrolysis (if the SRT is equal to the hydraulic retention time) and wash out of slow-growing organisms such as methanogens. We hypothesized that step-wise SRT reduction from 30 to 15 days would not affect hydrolysis since neither abundance nor community structure of hydrolytic bacteria would be affected. Additionally, methanogenesis would be enhanced because a reduction in SRT would increase archael abundance.

To test these hypotheses, we operated four laboratory-scale reactors for 14 months. Reactors 1 and 2 served as replicates and were grouped into Cluster 1, while replicate reactors 3 and 4 were in Cluster 2. The SRT of these two clusters was reduced in a step-wise fashion. Notwithstanding of the SRT applied, the SRT of Cluster 1 was always longer than the SRT of Cluster 2 (that is, the SRT was staggered). In Cluster 1, the SRT was 30, 25, and 15 days in Phases 1, 2 and 3, respectively; in Cluster 2 it was 25 and 15 days in Phases 1 and 2, respectively.

A comparison across different phases for each cluster showed that bacterial abundance, composition, and diversity (as measured by Hill’s N2 index) did not differ throughout the study, suggesting that a step-wise reduction of SRT was not an important factor for community assembly. Similarly, temporal drift and substrate fluctuations had no effect. When the two clusters were compared for each phase neither hydrolytic activity nor the hydrolytic bacterial community differed significantly. Step-wise reduction of SRT did enhance biogas production,
although the archaeal community in general was largely unaffected. The abundance of *Methanosaeta* decreased noticeably at the shorter SRTs, suggesting that it was more susceptible to changes in SRT. Overall, methanogens became more active at shorter SRTs as confirmed by specific methanogenic activity tests.

Considering that step-wise SRT reduction from 30 to 15 days doubled the amount of sludge that could be treated using existing reactor capacity, and increased biogas production by up to 65% without significant changes to the community, step-wise SRT reduction can be considered an optimization method for full-scale digesters that are operating at longer SRT.
1. INTRODUCTION

Sewage sludge is an inevitable byproduct in wastewater treatment and it contains putrescible organics that can cause offensive odor and health-related problems if left untreated (Appels et al. 2008, Weemaes and Verstraete 1998). One of the most common ways to treat sludge is sludge stabilization through anaerobic digestion (AD) prior to disposal. AD converts organics in sludge into energy rich biogas without the need for oxygen as electron acceptor (Batstone and Jensen 2011, Gerardi 2003). Hence, the operational cost is lower than for aerobic treatment of sludge.

AD is an intricate process involving four different yet tightly interconnected steps, namely, hydrolysis, acidogenesis/fermentation, acetogenesis, and methanogenesis (Appels et al. 2008, Tezel et al. 2011). In hydrolysis, complex organics in sludge are solubilized into their simpler monomers (Angelidaki and Sanders 2004, Gujer and Zehnder 1983). These monomers are converted into volatile acids such as butyric, propionic, and oxaloacetic acids as well as hydrogen, carbon dioxide, and alcohols in fermentation step (Appels et al. 2008, Batstone and Jensen 2011, Gujer and Zehnder 1983). Intermediates from the fermentation step are then converted into acetic acids, hydrogen and carbon dioxide before being converted into methane and carbon dioxide in the last two steps (Appels et al. 2008, Tezel et al. 2011). Due to the complexity of the process, many full-scale digesters are still burdened by operational instability, sensitivity to changes in environmental conditions, and long retention times (Amani et al. 2010).

An important parameter to ensure the success of AD is the solids retention time (SRT), which refers to the length of time sludge resides in a digester (Appels et al. 2008, Clara et al. 2005, Tchobanoglous et al. 2003). Based on the range of SRT applied, anaerobic digesters can be classified into two groups: methanogenic digesters (SRT> 8 days) and acidogenic digesters where the methanogens are usually suppressed (SRT≤ 8 days) (Miron et al. 2000). Full-scale
anaerobic digesters are typically operated at methanogenic conditions to avoid failure due to washout of slow-growing key players (Appels et al. 2008, Li et al. 2017). SRT can also affect volatile matter reduction (VMR) in anaerobic digestion if SRT is equal to HRT. Longer SRT typically equates to higher reduction (Clara et al. 2005, de la Rubia et al. 2006). However, longer SRTs also lead to a higher digester volume, which can increase the operational cost (Clara et al. 2005). On the other hand, a shorter SRT allowed for more sludge to be treated using existing digester capacity which translated into faster sludge turnover (Ziganshin et al. 2016). Moreover, if the SRT was within the methanogenic state, SRT reduction could lead to higher volumetric biogas production (Kiyohara et al. 2000, Lee et al. 2011). Hence, stable operation at short SRTs is desirable because it boosts the efficiency of a digester.

Many full-scale anaerobic digesters are operated at longer SRTs (up to 40 days) in part because of limited knowledge of microbial communities in digesters (Bolzonella et al. 2005, Parkin and Owen 1986, Rivière et al. 2009). For process performance it is crucial to understand the effects on the microbial community when the SRT is altered (Stark et al. 2014, Wagner and Loy 2002).

It is known that anaerobic digesters can be operated stably at shorter SRTs of 10-20 days (Elefsiniotis and Oldham 1994, Kim et al. 2014, Lee et al. 2011, Nges and Liu 2010). Some authors used either primary or secondary sludge as sole substrate or substrates such as swine manure, olive-mill solids residue, fodder beet silage or synthetic wastewater (Bolzonella et al. 2005, Bouzas et al. 2007, Clara et al. 2005, Elefsiniotis and Oldham 1994, Krakat et al. 2010, Li et al. 2017, Manser et al. 2015), while one study used two-phase anaerobic digestion (Kim et al. 2014). Other studies investigated the effects of different SRTs on thermophilic digesters (de la Rubia et al. 2006, Nges and Liu 2010). Previous works have also investigated the effects of SRT on microbial community dynamics but emphasized the effects observed in
acidogenic conditions where methane production was suppressed (Bouzas et al. 2007, Lee et al. 2011, Miron et al. 2000, Vanwonterghem et al. 2015).

To date no replicated step-down study has been conducted on the effect of SRT on microbial communities in methanogenic digesters of mixed sewage sludge at mesophilic temperatures. It is important to expound the fate of microbial communities as a function of SRT within the methanogenic range (that is, when SRT > 8 days) to ensure that SRT reduction does not alter the community. The stability of a biological system depends on functional redundancies and the interactions among different functional groups in the system (Briones and Raskin 2003). Hence, community dynamics can affect both stability and functionality of a system by changing how members of the community interact with each other or altering the functional redundancies of certain members of the community (Allison and Martiny 2008).

The objectives of this study were (i) to investigate the effects of step-wise SRT reduction from 30 days to 15 days on anaerobic digestion of mixed primary and secondary sewage sludge at mesophilic conditions and (ii) to study the concurrent dynamics in the microbial community using metagenomics, with a replicated study design to avoid potentially biased interpretation of community assembly in single reactors. We hypothesized that the decrease in SRT would not affect hydrolysis. However, since the SRT in our reactors was equal to the hydraulic retention time (HRT), shortening the SRT meant increasing the available substrate. Therefore, methanogenesis should be enhanced. We also hypothesized that the reduction in SRT would not affect bacteria since the range used in this study was adequate for bacterial doubling times (lowest SRT applied in this study > 10 d), but the archaea community.

2. MATERIALS and METHODS

2.1 Laboratory-scale anaerobic digesters
Four 5-L double-jacket anaerobic reactors were used in this study and they served as replicates. Each reactor had a working volume of 4.2 L and was stirred at 180 ± 1 rpm with a top mounted mechanical stirrer. The reactor covers were fitted with a rubber O-ring to ensure airtight sealing. After inoculation, the reactors were flushed with nitrogen gas to purge the residual oxygen in the headspace.

The operating temperature for all reactors was kept at 35 ± 1°C by circulating heated water in the reactor jacket. The biogas produced was collected in air-tight gas bags connected to the reactors. The amount of gas collected in the bag was quantified by pushing the content through a U-shaped gas collection apparatus. The apparatus was filled with thermal oil as the barrier solution. For each 50 mL of biogas collected, a click was triggered and the biogas collected was released. The number of clicks was recorded in the computer connected to the reactors.

2.2 Reactor operation and SRT reduction scheme

All reactors had been operated stably at an SRT of 30 d for more than six months prior to commencing the study. The work was divided into three phases during two years (Figure S1). Reactors 1 and 2 were grouped into Cluster 1 and reactors 3 and 4 into Cluster 2. The study design involved a step-down with two reactors remaining at the previous SRT and two reactors stepping down to the lower SRT. In all phases, the SRT in Cluster 1 was always longer than the SRT in Cluster 2. In Phase 1, Cluster 1 reactors had an SRT of 30 d, while Cluster 2 was operated at an SRT of 25 d. The SRTs for Cluster 1 and Cluster 2 were reduced to 25 days and 15 days, respectively, in Phase 2. In Phase 3, only Cluster 1 was operated at 15 d (Figure S1). In each phase, the reactors were fed daily in a semi-continuous mode. The amount of sludge discharged from the reactors was similar to the amount of substrate fed and it depended on the SRT applied to the reactors. Lastly, SRT in this study was equal to HRT.

2.3 Anaerobic sludge and digester substrate
Seed sludge was obtained from an egg shaped anaerobic digester at a water reclamation plant (WRP 1) in Singapore. The solids retention time (SRT) for this digester was 30 ± 2 days and the working temperature was 30 ± 1°C. Reactor substrates were collected weekly from another water reclamation plant, WRP 2. After collection, substrates were immediately sieved to remove larger sized particulates like gravel or hair that could hinder the feeding and discharging process. They were kept at 4°C prior to usage. All reactors had been operating stably using WRP 2 substrates for more than six months before the study commenced.

2.4 Specific methanogenic activity tests

Serum bottles in activity tests were inoculated with sludge collected from reactors at the end of each phase. Triplicate bottles were used for each reactor in each phase except for activity test using sludge collected from Cluster 2 when it was operated at an SRT of 25 d. For bottles inoculated with this sludge, duplicate bottles per reactor were used. Basal medium was prepared following the recipe of (Angelidaki and Sanders 2004). One gram per liter of acetic acid was used as sole substrate for all activity tests.

All serum bottles were closed with butyl rubber stoppers and capped with aluminum crimped seals before they were flushed with a mixture of nitrogen and carbon dioxide gas to remove traces of oxygen in the headspace. The bottles were then stored in a shaking incubator kept at 35°C and 180 rpm.

2.5 Sample collection

Samples were collected from each of the reactors through a sampling port located at mid height of the reactors. Sampling from the reactors was once weekly; samples were immediately filtered through a 0.45-μm pore size filter, except for the samples designated for solids and total chemical oxygen demand analysis. Biogas measurements for methanogenic activity tests were taken on operational days 1, 2, 3, and 7.

2.6 Physicochemical analysis
Solids measurements were done in accordance with Standard Methods (APHA, 2012). Both total and soluble chemical oxygen demand (COD) were measured using HACH COD test kits with a detection range of 20-1500 mg/L. Volatile acids (VA) were measured using HACH VFA TNTplus 872 (HACH, Loveland, CO, USA) adopting the esterification method (Montgomery et al. 1962). The concentration of four main volatile acids (acetate, propionate, butyrate and valerate) was quantified in accordance with Standard Methods (APHA, 2012), using gas chromatography (GC 2010 plus, Shimadzu, Japan) equipped with TCD and FID detectors and a DB-FFAP capillary column from Agilent Technologies. Total alkalinity was determined using the HACH TNT 870 kit adopting the colorimetric method, while total Ammonia was quantified with HACH TNT 832 kit adopting the salicylate method (Loveland, CO, USA). The gas composition was measured using gas chromatography (GC 2010 plus, Shimadzu, Japan) equipped with TCD and ECD detectors. Total carbohydrates were measured using the Dubois method (Dubois, 1951) while total proteins were measured using DC Protein Assay kit from Bio-Rad Laboratories (Hercules, CA, USA). The assay is based on a standard method for protein quantification (Lowry et al. 1951).

2.7 DNA Extraction and Sequencing

DNA was extracted from laboratory-scale digester sludge using the FastDNA© Spin Kit for Soil and the FastPrep© Instrument (MP Biomedicals, Santa Ana, CA, USA). The manufacturer’s protocol was modified by increasing the numbers of lysing steps of cells using the FastPrep© instrument to four, each lasting 40 s. The extracted DNA was further purified using the DNA Clean and Concentrator™-10 purification kit (Zymo Research Corp., Irvine, CA, USA). The quality and concentration of the extracted and purified DNA was evaluated using NanoDrop© 2000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA), and Qbit © 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. An agarose gel stained with ethidium bromide, using a 1-kb ladder as reference, was used to ensure that the
extracted DNA was not too fragmented. The DNA sequencing was done using the Illumina HiSeq platform (Illumina, San Diego, CA, USA).

Prior to sequencing, the DNA samples' quality was checked with Bioanalyzer 2100 using a DNA 12000 Chip (Agilent, Santa Clara, CA, USA). Sample was then quantified using Invitogen’s Picogreen assay. Library was prepared according to Illumina’s TruSeq Nano DNA Sample Preparation protocol. As per manufacturer’s recommendation, DNA samples were sheared on a Covaris S220 or E220 to approximately 450 bp and uniquely tagged with Illumina barcode to allow pooling of libraries for sequencing. The quantification of the finished libraries was done using Invitogen’s Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent, Santa Clara, CA, USA). Library concentrations were normalized to 4nM and validated using qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems, CA, USA) using KAPA Illumina Library Quantification Kit (Kapa Biosystems, Roche). The libraries were pooled at equimolar concentrations and sequenced on Illumina HiSeq 2500 sequencer. Rapid mode at a read-length of 250 bp paired-end was used.

Raw reads generated by sequencing were quality checked and adaptors were removed using cutadapt version 1.9.1 with default parameters (except--overlap 10 -m 30). After quality check, the reads were processed using Ribotagger-v.8.1.r106 (Xie et al. 2016). Default parameters targeting the v4 hyper variable region was used for this analysis. These processed reads were used to calculate abundances.

2.8 Statistical Analysis

A two-sample t test assuming unequal variance was used in this study. Notwithstanding the underlying variance profiles, the t test for unequal variance has been suggested to be equally or more robust against types I and II errors than a t test for equal variance (Ruxton 2006).
Multivariate analysis of microbial community was done using PRIMER v6 with PERMANOVA+ software (Clarke and Gorley 2006). The processed reads were first standardized based on the total counts per sample. Square-root transformation was applied to the standardized data before Bray-Curtis similarity matrix was constructed. Both PERMANOVA (permutational multivariate analysis of variance) and ANOSIM (analysis of similarity) analysis were used on the similarity matrix to check the null hypotheses that there was no difference in the two clusters communities in Phase 1 and Phase 2 (Anderson and Walsh 2013). The number of permutations was set to be 9999 and substrate was used as fixed factor. PERMDISP analysis was done to determine homogeneity of variance among our samples (Anderson 2006). For comparison of Hill diversity index values, Mann-Whitney test was used in lieu of t-test due to limited amount of samples available. This test was performed using the Real Statistics Resource Pack software (Release 4.3; Copyright (2013 – 2015) Charles Zaiontz; www.real-statistics.com).

2.9 Diversity Measurement

Hill’s second diversity index ($N_2$) was used to measure the diversity in archaea and bacteria. $N_2$ is the reciprocal of Simpson’s Index and emphasizes common species or taxa, which renders it more robust against changes due to rare species (Haegeman et al. 2013). $N_2$ values were calculated using PRIMER v6 software (Clarke and Gorley 2006). The processed read counts were standardized based on their total counts prior to the diversity analysis.

3. RESULTS

3.1 Effect of SRT on process performance in replicated reactors

3.1.1 Hydrolysis

In Phase 1, Cluster 1 reactors were operated at an SRT of 30 d, while Cluster 2 reactors were operated at 25 d. Effluent volatile solids observed in all reactors did not differ
significantly, suggesting that hydrolysis was not affected by the SRT applied (Figure 1). This observation was supported by similar average VMR and total COD (TCOD) removals in both clusters, further supporting that SRT did not affect hydrolysis (p-value = 0.91 and 0.4 for VMR and TCOD removals, respectively) (Table 1). Additionally, there was no significant difference in average protein and carbohydrate removal in the two clusters (p value = 0.45 for the latter and 0.05 for the latter) (Table 1).

We acknowledged the possibility that the lack of difference observed in this phase might be caused by the small SRT difference of 5 d between the two clusters and increased the SRT difference between the two clusters to 10 d in Phase 2. Cluster 1 was operated at an SRT of 25 d and Cluster 2 at 15 d. The average VMR and TCOD in Cluster 1 remained similar to the averages in Cluster 2 (p-value = 0.4 for VMR and 0.17 for TCOD) (Table 1). Although lower TCOD removal in Cluster 2 suggested a decrease in hydrolysis efficacy when the SRT was shortened, the decrease was not statistically significant.

In Phase 3, only Cluster 1 reactors were operated at an SRT of 15 d. The average VMR was lower than the average in Cluster 2 reactors operated at 15 d in the previous phase (p-value = 0.04). This dissimilarity was most probably caused by the difference in average substrate volatile solids (VS) concentrations in the two phases. Substrates used in this study were collected weekly from a full-scale anaerobic digester and fluctuations in substrate characteristics were bound to occur. The average VS content in Phase 2 substrates was 50,956 ± 7,799 mg/L, which was higher than in Phase 3 substrates (43,500±3,745 mg/L; p-value = 0.03). It could be deduced that the VMR difference was mainly caused by the difference in substrate VS rather than the SRT, because the VMR in Clusters 1 and 2 did not differ significantly when they were operated simultaneously although their SRTs were different. This observation was corroborated by the similar TCOD removal between Phase 3 and Phase 2 (p-value = 0.58). Unlike volatile solids, the average TCOD in Phases 2 and 3 substrates did not
differ significantly (79,855±17,896 mg/L for Phase 2 and 87,500±6,570 mg/L for Phase 3; p-value = 0.96). This supported our observation that the aforementioned difference in VMR was caused mainly by a difference in substrate rather than in SRT. Results supported our hypothesis that SRT reduction would not affect hydrolysis. This was in agreement with previous studies that reported VMR to be noticeably affected only when the SRT was shorter than 10 d (Kiyohara et al. 2000, Lee et al. 2011).

3.1.2 Methanogenesis

In addition to hydrolysis, we also investigated the effect of SRT reduction on methanogenesis since hydrolysis and methanogenesis are both known to be rate limiting steps in anaerobic digestion (Batstone and Jensen 2011). Effluent volatile acids concentrations remained low in all phases although more substrate that was fed into the reactors when the SRT was reduced, suggesting that methanogenesis was enhanced at shorter SRTs (Table 1). Biogas production further supported this observation. In Phase 1, the average biogas production in Cluster 2 was 1,456 mL higher than in Cluster 1, suggesting that methanogenesis was enhanced at shorter SRTs (p-value = 0.01) (Table 1). This is because the SRT in our reactors was invariably linked and equal to HRT; hence, more substrates were added when the SRT was reduced (140 mL of substrate per feeding for an SRT of 30 d and 280 mL of substrate per feeding for an SRT of 15 d). This result supported our hypothesis that a step-wise SRT reduction affected methanogenesis more than it did hydrolysis. In Phase 2, Cluster 2 also produced significantly more biogas than Cluster 1 (p value = 0.01) (Table 1). In spite of the minimal difference in SRT between the two clusters (5 d and 10 d in Phase 1 and Phase 2, respectively), biogas production was conspicuously greater. These results were further supported by the biogas production observed in Phase 3. Biogas production in Cluster 1 during Phase 3 increased to 8,990 ± 1,205 mL/day and was similar to biogas production in Cluster 2 when it was operated at an SRT of 15 d in Phase 2 (p-value = 0.59). Lastly, the volumetric ratio
of methane to carbon dioxide in the biogas did not differ throughout the phases (60-70% CH₄ and 30-40% CO₂ in average). Hence, an SRT reduction from 30 to 15 d increased volumetric biogas production by up to 65% without jeopardizing the quality of biogas produced.

3.2 Effects of step-wise SRT reduction on microbial community

Microbial community analysis was performed on samples collected at the end of each phase to ensure that the community had reached steady state following a reduction in SRT. Comparison of the samples was done both horizontally (i.e., between phases in each cluster) and vertically (i.e., between clusters in each phase) to complement the results.

3.2.1 Bacteria

3.2.1.1 Comparison of bacteria in each cluster across different phases

Step-wise SRT reduction did not affect the relative abundance of bacteria (Table 2). In Phase 1, bacteria made up 86 ± 2 % of the community in Cluster 1 and 85 ± 3 % in Cluster 2. The average bacteria abundance in Cluster 1 did not change when the SRT was reduced to 25 d and although it decreased to 83 ± 3% in Phase 3, this decrease was not significant as discussed later on. Similarly, bacteria abundance in Cluster 2 did not change significantly following the SRT reduction to 15 d in Phase 2.

In all phases, Bacteria were dominated by members of the phyla Actinobacteria, Spirochaete, Chloroflexi, Proteobacteria, Firmicutes, and Bacteroidetes (Figures 2A and B). Of the phyla in Cluster 1, the Actinobacteria phylum abundance decreased by 3% in Phase 2, while the abundance of Bacteroidetes and Spirochaetes increased by 2% and 3%, respectively (Figure 2A). The changes observed in other phyla were less than 2%. This trend continued in Phase 3. Phyla in Cluster 2 did not change much either (Figure 2B). Actinobacteria and Spirochaete were the two phyla that underwent most noticeable changes in Phase 2, with a 6% decrease for the latter and a 7% increase for the former. These results suggested that bacteria
abundances and composition were not affected by a reduction in SRT. The lack of differences in bacteria communities across different phases also showed that other factors such as temporal drifts and substrate fluctuations did not significantly affect the community.

These observations were further supported by the results of PERMANOVA and ANOSIM analysis. PERMANOVA analysis in Cluster 1 yielded a p-value of 0.0005 with small F value (2), which indicated minimal difference between communities in Phases 1 and 2. This result was supported by ANOSIM R value (0.4), which further indicated that the difference between the two communities was not substantial. Comparison between Phase 2 and Phase 3 also yielded a non-significant PERMANOVA p-value (0.2 with F-value of 1), while the ANOSIM R value was 0.04. The PERMDISP p-value was 0.9 indicating homogenous dispersion in Phases 2 and 3 communities. These results showed there were no significant changes in Cluster 1 bacterial communities throughout all the phases.

PERMANOVA analysis for Cluster 2 bacteria suggested that SRT reduction did not cause any significant differences between Phases 1 and 2. Comparison of bacteria from the two phases yielded a PERMANOVA p value of 0.0003 with an F value of 3. The ANOSIM value was 0.5, while the PERMDISP p value was 0.0006. These results suggested that although there appeared to be a significant difference between the two communities, the differences were inconsequential. Furthermore, the difference might be caused by the heterogeneous dispersion observed in the two communities (PERMDISP p value = 0.0006). From our results so far, we could deduce that the reduction in SRT did not affect bacterial communities. Neither did other factors such as fluctuations in substrate characteristics and temporal drifts affect the bacteria community.

Lastly, PERMANOVA analysis of bacteria in Cluster 1 reactors in Phase 3 and Cluster 2 reactors in Phase 2 yielded a p-value of 0.01. Due to the small Pseudo-F value (2), it was also
concluded that the two communities were not significantly different. This interpretation was further supported by the R value from ANOSIM (0.2). Hence the trends observed in Cluster 2 for bacterial communities in Phase 2 (SRT= 15 d) were also observed in Cluster 1 when its SRT was reduced to 15 d in Phase 3.

We also observed the effects of step-wise SRT reduction on bacterial diversity. $N_2$ diversity refers to Hill’s second diversity index. A diversity index is a numerical index that could be used as quantitative estimate of biological variability within different communities (Heip et al. 1998, Hill 1973). In Cluster 1, shortening the SRT from 30 to 25 d did not seem to create any adverse effects on bacterial diversities. Average $N_2$ diversities when the SRT was 30 d and 25 d were 13 ± 3 and 14 ± 4, respectively ($U = 27 > U_{critical} = 10$, Mann-Whitney test). Similarly, $N_2$ diversities were 14 ± 4 and 15 ± 6 for the SRTs of 25 d and 15 d, respectively ($U= 24 > U_{critical} = 10$, Mann-Whitney). For Cluster 2, a reduction in SRT from 25 to 15 d caused a more significant change in the bacterial community as shown in the $N_2$ values (23 ± 3 for 25 days SRT and 19 ± 3 for 15 days SRT; $U= 6 < U_{critical} = 13$). Despite its significance, the decrease was not severe. These results suggested that step-wise SRT reduction from 30 to 15 d did not have any major adverse effects on bacterial diversity; we conclude that no significant loss of functional redundancies occurred when the SRT was decreased to 15 d.

3.2.1.2 Comparison of bacteria community in the two clusters in each phase

To further confirm our findings, we compared the communities in the two clusters within each phase where factors such as substrate fluctuations and temporal drifts could be discounted. Since we had previously showed that substrate fluctuations and temporal variations did not affect bacterial communities, we could surmise that any differences that might be observed between the two clusters were caused solely by the SRT. The difference in the phyla abundance in the two clusters was not distinct (Figures 3A and B). In Phase 1, the average
abundance of *Spirochaete* in Cluster 2 was 9% lower than in Cluster 1, while the abundance of phylum *Bacteroidetes* in the former was 5% higher than in the latter (Figure 3A). The rest of the average phylum abundances in the two clusters differed by less than 2%. In phase 2, the abundance of the phylum *Spirochaete* in Cluster 2 was 5% lower than in Cluster 1, while the abundance of phylum *Chloroflexi* was 3% higher in Cluster 2 than in Cluster 1 (Figure 3B).

PERMANOVA analysis of bacterial OTU abundance in the two clusters in Phase 1 yielded a p-value of 0.007. Although it was less than 0.05, the small Pseudo-F value (2) indicated that the p-value significance was minimal. The low PERMANOVA p-value in this phase might be caused by heterogeneous dispersion of the bacteria community within the two clusters as shown by PERMDISP p-value of 0.0001, which allowed us to reject the null hypothesis of homogenous dispersion in the bacterial communities of the two clusters. The R-value from ANOSIM analysis was small (0.3), indicating that differences in abundances of the two cluster OTUs were minimal. In conclusion, different SRTs had no perceptible effect on bacteria.

In Phase 2, no significant difference in bacterial communities was observed in the two clusters, even when the magnitude of SRT difference was increased to 10 d. The p-value from PERMANOVA was 0.06 with a Pseudo-F of 1, while the ANOSIM R-value was 0.1. The dispersion of the two communities was also homogenous as shown by PERMDISP p-value of 0.1. These results further showed that step-wise SRT reduction did not cause any noticeable effects on bacteria communities. To further confirm our observation, we also compared the diversity of the two clusters in each phase. This was done to minimize the effects that might be caused by factors other than the SRT, such as substrate fluctuations and temporal drifts. In Phase 1, the average N₂ diversity in Cluster 1 was 14 ± 4 and 24 ± 3 in Cluster 2 (p-value = 5E-9; two samples independent t-test), suggesting that shorter SRTs increased bacterial diversity. The difference might be caused by unexplained low average abundance of the
phylum Spirochaetes in Cluster 2 instead of SRT, since the SRT difference between the two clusters was only 5 d. This observation was supported by the comparison in Phase 2 where SRT difference between the two Clusters were increased to 10 d. Average N$_2$ diversities were 14 ± 4 in Cluster 1 and 19 ± 3 in Cluster 2 (U =10.5 > U$_{critical}$ = 10), suggesting that SRT did not cause any major adverse effect on bacteria community.

Lastly, we also compared the N$_2$ diversity of Cluster 1 during Phase 3 with that of Cluster 2 in Phase 2 to ensure that the trends were comparable when both were operated at the same SRT of 15 d. Indeed, in Phase 3, the average N$_2$ value in Cluster 1 was statistically similar to that of cluster 2 in Phase 2 (15 ± 6 for Cluster 1 in Phase 3 and 19 ± 3 for Cluster 2 in Phase 2, p-value = 0.15), further confirming that a reduction in SRT to 15 d did not cause any adverse effect on bacterial diversity.

To summarize, step-wise SRT reduction did not have any major effects on the relative abundance, composition, and overall diversity of bacteria in our reectors, which explains the absence of any effect of SRT reduction on hydrolysis in reactors.

3.2.2 Archaea

We hypothesized that archaea would be impacted more prominently than bacteria. The total abundance of archaea in Phase 1 was 4% for Cluster 1 and 5% for Cluster 2 (Table 2). In Phase 2, the average abundance of archaea decreased by 1% for both clusters while in the last phase, the average abundance for Cluster 1 was 4%. (Table 2). Methanosaeta dominated in all reactors in Phase 1, accounting for more than 70% of the archaea OTUs, while Methanobacterium and Methanospirillum made up an additional 23-28% of the archaea community (Figure 4).

3.2.2.1 Comparison of Archaea community in each cluster across different phases
Considering that archaea are a major player in anaerobic digestion, we further analyzed the archaeal genera with respect to overall archaea abundance only. This showed that archaea were not significantly impacted by a reduction in SRT. For Cluster 1, the PERMANOVA p-value for archaea in Phases 1 and 2 was 0.07 with a pseudo-F value of 3 while the ANOSIM R value was 0.1. The PERMDISP p value also showed a homogenous dispersion of the archaea in the two phases. A similar trend was also observed when we compared Cluster 1 archaea in Phase 2 and Phase 3 (PERMANOVA p-value of 0.05 with pseudo-F: 3 and ANOSIM R value of 0.05 while PERMDISP p-value was 0.6). Similarly, archaea in Cluster 2 did not differ significantly between Phase 1 and Phase 2, as evident from the PERMANOVA p-value of 0.3 and pseudo-F value of 1 and ANOSIM R value of 0.06. These results showed that the SRT did not affect archaea. Other factors such as variations in substrate characteristics and temporal drifts could also be ruled out.

In addition to the aforementioned analysis, we also compared the archaea communities in the two clusters within each phase. Since we have showed that substrate fluctuations and temporal variations did not affect our archaea communities, we could deduce that any differences that might be observed between the two clusters were caused solely by SRT.

Our results showed that archaea was not affected by different SRTs applied to each clusters in each phase. This observation was supported by PERMANOVA p-values from the comparison between Clusters 1 and 2 in both Phases 1 and 2 (0.1 with Pseudo-F of 2 for Phase 1 and 0.2 with Pseudo-F values of 2 for Phase 2). These results, coupled with 0.06 and 0.4 ANOSIM R-values and PERMDISP p-values of 0.9 and 0.8 for Phases 1 and 2 respectively further suggested that archaea were not adversely affected by the SRT reduction. Lastly, archaea in Cluster 1 were similar to archaea in Cluster 2 when they were both operated at an SRT of 15 d (PERMANOVA p-value: 0.7 with Pseudo-F: 0.6; ANOSIM R-value:-0.02).
Although not significant, archaea did experience some changes. In Cluster 1, the changes were more noticeable when the SRT was reduced to 15 d in Phase 3. In this phase, the average abundance of *Methanosaeta* decreased by almost 21%, suggesting that this methanogen was more susceptible to changes in physiochemical conditions due to a reduction in SRT (Figure 4A). *Methanobacterium* and *Methanospirillum* increased by 6% and 18%, respectively. This trend was similar to the trend observed in Cluster 2 when its SRT was reduced from 25 to 15 d in Phase 2 (Figure 4B). Abundance of *Methanosaeta* also decreased by 13%. This decrease coincided with 10% and 6% increases in *Methanobacterium* and *Methanospirillum* genera, respectively. Lastly, the abundances of these genera in Cluster 2 were similar to their abundances in Cluster 1 reactors when Cluster 1 was also operated at an SRT of 15 d in Phase 3.

In addition to archaea abundance and composition, we also inspected the effects of step-wise SRT reduction on archaea N\(_2\) diversity. When the SRT of Cluster 1 was reduced to 25 d, N\(_2\) diversity did not change significantly (3 ± 1 for both SRTs; \(U = 24.5 > U_{\text{critical}} = 10\), Mann-Whitney). N\(_2\) diversity for archaea also remained similar when the SRT was further reduced to 15 d in Phase 3 (average N\(_2\) = 3 ± 1; \(U = 26 > U_{\text{critical}} = 10\)). A similar N\(_2\) was also observed in Cluster 2 when its SRT was reduced from 25 d in Phase 1 to 15 d in Phase 2 (\(U = 30 > U_{\text{critical}} = 13\), Mann-Whitney). These results suggested that archaea diversity was not affected by SRT reduction and other factors such as temporal variations and substrate fluctuations.

### 3.2.2.2 Comparison of Archaea community between clusters in each phase

Likewise, we also compared archaeal genera abundances in both clusters within each phase. Average abundance of *Methanobacterium* in Cluster 2 was 8% lower than in Cluster 1, while *Methanospirillum* and *Methanobrevibacter* were 3% and 4% higher, respectively, in the former (Figure 5). In Phase 2, average abundance of *Methanosaeta* in Cluster 2 decreased to
57% of the whole archaeal community, while the average abundance in Cluster 1 remained at 74% of total Archaea, further confirming that *Methanoseta* was less tolerant of shorter SRTs. The average abundance of *Methanospirillum* was 20% higher in Cluster 2, suggesting that this genus was preferentially selected over *Methanoseta* at a shorter SRT.

Our results showed that archaea were not affected by a SRT reduction to 15 d although biogas production was enhanced (Table 1). These results seemed to contradict our hypotheses that archaea would be enhanced at shorter SRT. One possible reason that could explain this contradiction was the use of genomic DNA abundance in our study; hence, the possibility that the archaea became more active when the SRT was shortened could not be discounted. To attest to this observation we also performed activity tests, which are discussed in section 3.3.

When we compared archaeal genera diversities in the two Clusters at each phase, $N_2$ diversity in Phase 1 was $3 \pm 1$ (p-value = 0.03 independent t-test). This seemingly significant difference in $N_2$ might be caused by the small $N_2$ value because archaea was a small group of which only 3-4 genera with notable presence were identified (Figures 3 and 4). Moreover, some genera such as *Methanobacterium* and *Methanobrevibacter* were only present in some of the samples, which lead to high fluctuations in average relative abundances of these genera. The difference between the two clusters was insignificant in Phase 2 ($N_2$ diversities were $3 \pm 1$ for both clusters; p-value = 0.15). Furthermore, $N_2$ diversities in Clusters 1 and 2 were similar when SRT was 15 d in Phase 2 (for Cluster 2) and Phase 3 (for Cluster 1) (p-value: 0.24). Thus, similar to bacteria, the archaea community was not affected by a reduction in SRT.

### 3.3 Specific Methanogenic Activity

To support our observation that the methanogenic activity increased with decreasing SRT, a specific methanogenic activity test was conducted using sludge that had been
acclimatized at different SRTs. We emphasized the activity of acetoclastic methanogens due to their dominance in our reactors.

When basal medium with 1 g/L acetic acid as sole substrate was added to serum bottles inoculated with the aforementioned sludge, bottles inoculated with sludge collected from reactors that had an SRT of 15 d produced more biogas than those inoculated with sludge when the SRT was 25 d (Figure 6). For Cluster 1, bottles inoculated with 25-d old sludge produced a total of 0.02 ± 0.01 bar biogas /gram of VS$_{seed}$ on the second day compared to 0.04 ± 0.01 bar biogas /gram of VS$_{seed}$ for bottles with 15-d old sludge. Cluster 2 sludge exhibited similar trends. Hence acetoclastic methanogens in 15-d old sludge converted the substrate more actively and rapidly since all were fed with similar amounts of acetic acid in the activity test. This indicated that acetoclastic methanogens became more active at a shorter SRT, especially since the overall abundance of Methanosaeta as the sole acetoclastic methanogen in our reactor was actually lower in 15-d old sludge. To summarize, the higher biogas production confirmed an increase in the activity of the methanogens at shorter SRTs.

4. DISCUSSION

Solids retention time (SRT) has been identified as an important parameter in anaerobic digestion since it can dictate the organisms able to proliferate in anaerobic digesters and affect the quality of digested effluents (Clara et al. 2005, Lee et al. 2011, Miron et al. 2000, Tezel et al. 2011). Stable digestion at short SRT is preferred due to the higher sludge volume that can be treated, which translates into more efficient digesters (Nges and Liu 2010). Earlier studies focused mostly on microbial community dynamics at extremely short SRTs (<10 days SRT). Hence, more insights into the fate of bacteria and archaea as a function of step-wise SRT decrease would be useful to understand how AD communities adapt.

4.1 Process performance response to step-wise SRT reduction
Our results were in agreement with previous studies that reported hydrolysis as independent of SRT in the range used in this study (Elefsiniotis and Oldham 1994, Lee et al. 2011, Nges and Liu 2010). Although hydrolysis was not impacted, step-wise reduction of the SRT to 15 d enhanced biogas production, suggesting that methanogenesis was improved. The reduction in SRT also did not affect the quality of biogas produced since the volumetric ratio of methane and carbon dioxide (i.e., the average of 60-70% CH\textsubscript{4} to 30-40% CO\textsubscript{2}) remained the same in all phases. This was in agreement with previous studies that reported improvement in biogas production with reduction of SRT (Elefsiniotis and Oldham 1994, Lee et al. 2011, Nges and Liu 2010). The enhancement of methanogenesis despite the lack of change in hydrolysis might be caused by the higher amount of substrate fed to the reactors when the SRT was shortened. The substrate also contained organics in their solubilized forms (e.g., soluble COD) and volatile acids that could be utilized in the subsequent digestion steps without the need to be hydrolyzed.

4.2 Microbial community response to step-wise decrease in solids retention time

We further hypothesized that bacteria in our reactors would not be severely affected by the reduction of the SRT from 30 to 15 d. However, SRT reduction might cause more discernable effects on archaea.

4.2.1 Bacteria

In all phases, the bacteria community was dominated by the phyla Proteobacteria, Chloroflexi, Actinobacteria, Spirochaete, and Firmicutes, which are common hydrolytic, syntrophic, and fermentative bacteria in anaerobic digestion (Ariesyady et al. 2007, Guo et al. 2015, Rivière et al. 2009, Tezel et al. 2011). Our results supported the hypothesis that step-wise SRT reduction to 15 d did not affect bacteria significantly. One possible reason for this
was that the lowest SRT applied in this study was still longer than the typical generation time of the bacteria involved (Deublein and Steinhauser 2011).

The lack of noticeable changes in bacteria community following a reduction in SRT may also have been caused by the gradual nature of the reduction. In this study, the reactors were operated at each SRT for at least three complete sludge turnover times before samples were taken for microbial community analysis and the SRT was further reduced. This allowed bacteria to be acclimatized to higher organics introduced to the reactors when the SRT was reduced. The ability of anaerobic digester bacteria to acclimatize themselves to gradual changes in the environment has been reported before (Grosser 2016, Silvestre et al. 2011).

4.2.2 Archaea

Interestingly, there was no significant difference between the average archaeal abundance in the two clusters in any of the phases, possibly due to genomic DNA abundances being used in the analysis. There was a distinct possibility that existing methanogens became more active when the SRT was shortened. Some organisms might be present in a system in a dormant form while maintaining reversible low metabolic activity, especially when there are unfavorable conditions such as a lack of nutrients (Jones and Lennon 2010, Kaprelyants 1993). The dormant organisms could revert to their active stage when more favorable conditions are introduced to the system (Kaprelyants 1993). Although our reactors were not operated under starvation conditions, it is possible that the increased organics when the SRT was shortened induced the re-activation of some fraction of the dormant methanogens. This interpretation is supported by the biogas production observed in the specific methanogenic activity test, which showed that sludge that had been acclimatized to the shorter SRT produced more biogas in the first 2-3 days of the activity test.
Methanoseta and Methanospirillum were the genera that underwent most changes. Lower average abundance of Methanoseta in reactors operated at an SRT of 15 d might be caused by the increase in acetate concentrations as a result of higher substrate concentrations in the reactors operated at lower SRT. This methanogen is known to have a higher acetate affinity and hence proliferates better in lower acetate conditions (Demirel and Scherer 2008, Jetten et al. 1992). A lower SRT has also been reported as detrimental to acetoclastic methanogens (Demirel and Scherer 2008).

The increased abundance of Methanospirillum might be caused by an increase in hydrogen partial pressure since this methanogen has been shown to proliferate better at higher hydrogen partial pressure (Sakai et al. 2009). H₂ is one of the intermediates produced during the conversion of sewage sludge (Appels et al. 2008, Tezel et al. 2011). Hence, it is possible that the increase in substrate added led to an increase in H₂ partial pressure, leading to the increase in Methanospirillum abundance. The emergence of hydrogenotrophic methanogens could be beneficial for the digestion process since they were more robust against different inhibitions or variations in operating condition (Demirel and Scherer 2008, Ho et al. 2014, Vanwonterghem et al. 2015). Methanospirillum and other hydrogenotrophs could also withstand organic loading shocks better than their acetoclastic counterpart and high abundance of the former had also been reported to enhance propionate consumption and better SCOD removal in anaerobic digesters (Lerm et al. 2012, Tale et al. 2015)

5. CONCLUSIONS

- SRT was not a strong selector for the composition and diversity of a digester community within the range used in this study. However, the higher abundance of hydrogenotrophic methanogens observed at shorter SRTs could improve digester stability.
- Acetoclastic methanogens could utilize substrate more rapidly after they had been acclimatized to a shorter SRT.
- Temporal drifts and substrate fluctuations were not important factors that could affect microbial community.
- Halving the SRT to 15 days doubled the treated sludge turnover rate and increased biogas production by up to 65% without jeopardizing biogas quality.
- Digesters that are currently operated at long SRTs could apply SRT reduction as a means to improve performance, without the need for expensive and energy intensive pretreatments or bio-augmentation.

ACKNOWLEDGEMENTS

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Table 1: Process performance in replicate anaerobic reactors R1 to R4. Values represent the mean with standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Parameter (unit of measurement)</th>
<th>Phase 1</th>
<th></th>
<th>Phase 2</th>
<th></th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cluster 1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>Cluster 2&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>Cluster 1&lt;sup&gt;2,4&lt;/sup&gt;</td>
<td>Cluster 2&lt;sup&gt;2,4&lt;/sup&gt;</td>
<td>Cluster 1&lt;sup&gt;2,5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volatile matter reduction (%)</td>
<td>46 (13)</td>
<td>46 (13)</td>
<td>52 (6)</td>
<td>50 (7)</td>
<td>45 (6)</td>
</tr>
<tr>
<td>Total COD removal (%)</td>
<td>53 (11)</td>
<td>51 (13)</td>
<td>58 (8)</td>
<td>53 (9)</td>
<td>55 (5)</td>
</tr>
<tr>
<td>Biogas (mL/day)</td>
<td>5,457 (2,040)</td>
<td>6,913 (2,216)</td>
<td>5,675 (1,322)</td>
<td>8,707 (1,137)</td>
<td>8,990 (1,205)</td>
</tr>
<tr>
<td>Average volatile acids (mg/L of acetic acid)</td>
<td>112 (23)</td>
<td>115 (34)</td>
<td>103 (11)</td>
<td>99 (14)</td>
<td>119 (13)</td>
</tr>
<tr>
<td>Protein removal (%)</td>
<td>54 (19)</td>
<td>52 (18)</td>
<td>60 (7)</td>
<td>55 (10)</td>
<td>61 (8)</td>
</tr>
<tr>
<td>Carbohydrate removal (%)</td>
<td>56 (21)</td>
<td>48 (17)</td>
<td>47 (20)</td>
<td>45 (15)</td>
<td>38 (19)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Phase 1: operational days 0-188. Cluster 1 was operated at 30 days SRT and Cluster 2 was operated at 25 days

<sup>2</sup> Phase 2: operational days 282-360. Cluster 1 was operated at 25 days SRT and Cluster 2 was operated at 15 days SRT.

<sup>3</sup> Phase 3: operational days 371-436. Cluster 1 was operated at 15 days SRT.

<sup>2</sup> Cluster 1: reactors R1 and R2

<sup>2</sup> Cluster 2: reactors R3 and R4

<sup>3</sup> n: 46 per cluster

<sup>4</sup> n: 20 per cluster

<sup>5</sup> n: 12 per cluster
Table 2: Average abundance of bacteria, archaea, eukaryotes, and unclassified OTUs in replicate reactors R1 to R4. Values represent the mean abundance with standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Domain (unit of measurement)</th>
<th>Phase 1(^1)</th>
<th>Phase 2(^1)</th>
<th>Phase 3(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cluster 1(^{2,3})</td>
<td>Cluster 2(^{2,3})</td>
<td>Cluster 1(^{2,4})</td>
</tr>
<tr>
<td>Bacteria (%)</td>
<td>86 (2)</td>
<td>85 (3)</td>
<td>85 (2)</td>
</tr>
<tr>
<td>Archaea (%)</td>
<td>4 (1)</td>
<td>5 (2)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Eukaryote and unclassified (%)</td>
<td>10 (2)</td>
<td>10 (3)</td>
<td>11 (2)</td>
</tr>
</tbody>
</table>

\(^1\) Phase 1: operational days 0-188. Cluster 1 was operated at 30 days SRT and Cluster 2 was operated at 25 days.

Phase 2: operational days 282-360. Cluster 1 was operated at 25 days SRT and Cluster 2 was operated at 15 days SRT.

Phase 3: operational days 371-436. Cluster 1 was operated at 15 days SRT.

\(^2\) Cluster 1: reactors R1 and R2

Cluster 2: reactors R3 and R4

\(^3\) n: 16 per cluster

\(^4\) n: 8 per cluster

\(^5\) n: 8 per cluster
Table S1: Operational days and solids retention time applied to each cluster within each phase.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Operational day</th>
<th>SRT applied</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-188</td>
<td>30</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>282-360</td>
<td>25</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>371-436</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Mean volatile solids concentration in replicate reactors R1 to R4. The four reactors were grouped into two clusters based on their solids retention time in each phase. ●, refers to Cluster 1, which consisted of R1 and R2; ○, Cluster 2, which consisted of R3 and R4. Error bars represent the standard deviation between reactors in each cluster. In Phase 1, Cluster 1 was operated at an SRT of 30 d while Cluster 2 was operated at 25 d. In Phase 2, Cluster 1 the SRT was reduced to 25 d while for Cluster 2 the SRT was reduced to 15 d. In phase 3, only Cluster 1 was operated at an SRT of 15 d.
Figure 2: Relative abundance of phyla for each phase. (A), Cluster 1; (B), Cluster 2. ● denotes phylum abundance in Phase 1 where the SRT was 30 d in Cluster 1 and 25 d in Cluster 2; ○, Phase 2 where the SRT for Clusters 1 and 2 was 25 and 15 d, respectively. ○, Phase 3 where only Cluster 1 was operated at an SRT of 15 d.
Figure 3: Relative abundance of phyla in replicate reactors R1 to R4. •, indicated Cluster 1 which consisted of R1 and R2; ○, Cluster 2 which consisted of R3 and R4. In Phase 1, Cluster 1 was operated at an SRT of 30 d, while Cluster 2 was operated at 25 d. In Phase 2, the SRT was reduced to 25 d in Cluster 1 and to 15 d in Cluster 2. In phase 3, only Cluster 1 was operated at an SRT of 15 d. Each data point represents the mean abundance in the two replicate reactors and the error bar represents the standard deviation among the two reactors within each cluster.
Figure 4: Relative abundance of archaea genera with respect to whole archaea community. Graph (A) was for Cluster 1 while (B) was for Cluster 2. ●, denoted phyla abundance in Phase 1 where Cluster 1 SRT was 30 days while Cluster 2 SRT was 25 days. ○, Phase 2 where SRT for Clusters 1 and 2 were 25 and 15 days respectively. ⭕, Phase 3 where only Cluster 1 was operated at 15 days SRT.
Figure 5: Relative abundance of archaea genus in replicate reactors R1 to R4 with respect to overall archaea community. ●, denoted Cluster 1 which consisted of R1 and R2; ○, Cluster 2 which consisted of R3 and R4. Error bars represented standard deviation between reactors in each clusters. In Phase 1, Cluster 1 was operated at 30 days SRT while Cluster 2 was operated at 25 days SRT. In Phase 2, Cluster 1 SRT was reduced to 25 days while Cluster 2 SRT was reduced to 15 days. In phase 3, only Cluster 1 was operated at
15 days SRT. Each data point represented the average abundance in two replicate reactors in a cluster and error bar represented the standard deviation between the two reactors.
Figure 6: Relative abundance in Cluster 1 for (A) all phyla and (B) archaea genera. ●, referred to relative abundance in Phase 2; ○, Phase 3. Each data point represented the average abundance in two replicate reactors and error bar represented the standard deviation among the two reactors within each cluster.
Figure 7: Biogas production from acetoclastic methanogenic activity test. Serum bottles were inoculated with sludge taken from (A) Cluster 1 and (B) Cluster 2 reactors at the end of each phase. Biogas measured was standardized using inoculum VS concentration to account for variation in the amount of sludge added to the serum bottles. ●, SRT25, denoted bottles inoculated with sludge collected from reactors operated at 25 days SRT; ○, SRT15, 15 days SRT. Each data point represented average biogas production in two reactors within each clusters. Triplicate bottles were used for each reactor at each sampling point except of Cluster 2
SRT$_{25}$ where only duplicate bottles per reactor were used. Error bar represented standard deviation between reactors in each clusters.
Figure S1: Schematic of SRT allocation for each cluster. Days signify the operational days when sample collection for physiochemical analysis commenced in reactors. Samples for microbial community analysis were collected one to two months before the end of each phase.


Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P. and Sghir, A. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME Journal 3(6), 700-714.


CHAPTER 4

Rapid Anaerobic Process Recovery with Prolonged Community Changes
After Pulsed Temperature Decreases Under Mesophilic Conditions

ABSTRACT

Temperature variation is a common disturbance in anaerobic digestion, yet little is known about the effects of transient pulse perturbations. We hypothesized that short-term temperature changes would alter archaea community structure and function, and subsequently impact methanogenesis. We operated two laboratory-scale anaerobic reactors, labeled R1 and R2, at 35 ± 1°C for 140 d with an SRT of 30 d. On Day 49, the temperature in R1 was reduced to 25°C for 24 h before returning to 35°C. This cycle was repeated on Day 51. To confirm that changes observed in R1 were caused by temperature perturbations, a similar perturbation was applied to both R1 and R2 on Day 97.

Volatile matter reduction (VMR) did not change significantly after the perturbations, indicating that hydrolysis was not impacted. Yet, despite similar biogas production and composition, a sharp increase in acetate concentration was observed in R1 after perturbations on Days 49 and 51, and in R2 after the perturbation on Day 97, suggesting that acetoclastic methanogenesis was affected. After the first set of perturbations in R1 there was a sharp decrease in Methanoseta abundance as assessed by metagenomic analysis. Concomitantly, the abundance of hydrogenotrophic methanogens such as Methanobacterium and Methanospirillum increased. Similar trends were also observed in R2 after its first perturbation on Day 97. Lastly, the effects of the additional Day 97 perturbation in R1 were less severe suggesting increased community resistance to such perturbations.
Despite its brevity, pulsed temperature perturbations could significantly alter the methanogenic community, even in the absence of any apparent effect on biogas production and composition. The increase in the abundance of hydrogenotrophic methanogens following the perturbations could be beneficial since they were more resilient than their acetoclastic counterparts. Moreover, accumulated acetic acid could also be harvested from the digested sludge. Finally, higher archaeal diversity following repeated pulse perturbations could improve community robustness and long-term process stability. Overall, the results of this study highlight the possibility of using pulsed downward temperature cycling to enhance digestion. This could alleviate the need for additional steps designed to improve anaerobic digestion such as energy and cost intensive pretreatment.

Keywords: Anaerobic digestion, temperature, pulse perturbation, pulse disturbance, acetoclastic methanogens, hydrogenotrophic methanogens.
1. INTRODUCTION

Anaerobic digestion is recognized as one of the most attractive sludge stabilization techniques partly due to the production of energy-rich biogas (Appels et al. 2011, Batstone and Virdis 2014). Anaerobic digestion itself involves four different yet interconnected steps, which are hydrolysis, fermentation, acetogenesis, and methanogenesis. Maintaining balance among these different steps is vital in achieving successful digestion (Tezel et al. 2011).

One important consideration to achieve this balance is temperature (Nielfa et al. 2015). Based on its operating temperature, anaerobic digestion can be classified into three different groups, namely, psychrophilic digestion (10-30°C), mesophilic digestion (30-40°C), and thermophilic digestion (40-70°C) (Tchobanoglous et al. 2003). Of the aforementioned ranges, mesophilic digestion is the most ubiquitous although the use of thermophilic digestion has been gaining popularity in recent years.

Notwithstanding the temperature range selected, it is highly recommended that the operating temperature be kept as constant as possible (Tchobanoglous et al. 2003). Temperature can affect physicochemical and biochemical activities in an anaerobic digester as well as qualities of biogas and digested sludge produced (Appels et al. 2008, Batstone and Jensen 2011, Tezel et al. 2011). Some of these effects were caused by the impacts of temperature on digester microbial community composition and individual microbial growth rates, with acetoclastic methanogens especially prone to temperature disturbances (Appels et al. 2008, Chin et al. 1999, Pap et al. 2015). Thus, it is crucial that temperature changes be kept at a minimum since disturbances to any digestion stage or digester community trophic group might bring about a cascade of undesirable effects to the entire digestion process (Fernández 1999). Indeed, anaerobic digester temperature changes should not exceed 1°C/ day (Tchobanoglous et al. 2003).
Although it is important to keep the operating temperature as constant as possible, temperature fluctuation is one of the most common disturbances in anaerobic digestion (Poh and Chong 2009). Several studies on the effects of varying temperatures on different stages of anaerobic digestion have been carried out (Chin and Conrad 1995, Donoso-Bravo et al. 2009, Fey and Conrad 2000, McKeown et al. 2012, Vanwonterghem et al. 2015). Most of them confirmed that temperature affected microbial community dynamics and the resulting process performance in anaerobic digesters. However, these studies considered only long-term temperature changes, and mostly under thermophilic conditions. In contrast, little is known about the impacts of brief pulsed temperature perturbations on mesophilic anaerobic digestion. These disturbances are analogous to power failures that might affect laboratory-scale as well as full-scale anaerobic reactors in temperate regions where additional heating might be required to maintain the temperature. The impacts of these pulse perturbations on microbial community dynamics also need to be expounded.

Therefore, the objectives of this study were to understand if (i) brief temperature perturbations could significantly impact the process performance of laboratory-scale mesophilic anaerobic digesters treating sewage sludge and (ii) whether these transient temperature fluctuations were vigorous enough to alter microbial community assembly. We hypothesized that a pulsed perturbation would affect methanogenesis more than other digestion steps and that, despite its brevity, the temperature perturbation could have a lasting impact on the methanogenic community. Moreover, the methanogenic community could also develop resistance toward similar perturbations.

2. MATERIALS AND METHODS

2.1. Laboratory-Scale Anaerobic Reactor Operation

Two laboratory-scale anaerobic reactors operated at 30 days SRT and labeled as R1 and R2 were used. Each of the reactors had a working volume of 4.2 L. The reactors were mixed
with top-mounted mechanical stirrers at 180±1 rpm. Biogas produced was quantified using a U-shaped gas collection apparatus. The apparatus was filled with thermal oil as the barrier solution. For each 50 mL of biogas collected, a click was triggered and the collected biogas was released. The clicks were recorded in the computer connected to the reactors.

Normal operating temperature for both reactors was kept at 35 ± 1°C by circulating heated water in the reactors’ outer jacket. Pulse temperature perturbations occurred on operational days 49, 51, and 97. We refer to the operational phase before the perturbation (i.e., operational days 1 to 48) as Phase 1, while Phases 2 and 3 reflect operational days 52 to 96 and 98 to 140, respectively.

R1 received three pulse perturbations on Days 49, 51, and 97. On Day 49, the heating water flow to R1 was switched off and temperature dropped to 25 ± 1°C. The temperature was restored to 35 ± 1°C the next day. This was done to ensure similar starting temperatures for the next perturbation. The same cycle was repeated on operational day 51. The operating temperature was then restored and kept at 35 ± 1°C. Two cycles were applied to R1 to ensure that the perturbations were adequate. A third perturbation was applied to R1 on Day 97 to test if R1 had developed resistance toward similar perturbations. The additional perturbation was applied after one SRT had elapsed to ensure that process performance had stabilized.

The temperature in R2 was kept at 35 ± 1°C until Day 97 where it dropped to 25 ± 1°C. The temperature was restored the next day and kept at 35 ± 1°C until the end of the study. Although R2 only received one perturbation, its outcome would provide information on general effects of pulse temperature perturbations if the changes observed in R1 in Phase 2 were also observed in R2 in Phase 3.

2.2. Anaerobic sludge and digester substrate

Both reactors were seeded with anaerobic sludge obtained from a wastewater treatment plant in Singapore (WRP1) and they were fed semi-continuously with mixed thickened
primary and secondary sludge from another water reclamation plant (WRP2). Both R1 and R2 were fed from the same feed reservoir to eliminate any bias that might be introduced by using different reservoirs.

2.3. Sample Collection

Samples were collected from each of the reactors through a sampling port located halfway up the reactors. Sampling from the reactors was done twice weekly. All samples were immediately filtered through a 0.45-µm pore size filter, except for the samples designated for solids and total chemical oxygen demand analysis. Molecular samples were immediately stored at -80°C freezer prior to extraction.

2.4. Physicochemical Analysis

Solids measurements were done in accordance with Standard Methods (APHA, 2012). Both total and soluble chemical oxygen demand (COD) were measured using HACH COD test kits with a detection range of 20-1500 mg/L. Volatile acids (VA) were measured using HACH VFA TNTplus 872 (HACH, Loveland, CO, USA) adopting the esterification method (Montgomery et al. 1962). The concentration of four main volatile acids (acetate, propionate, butyrate and valerate) was quantified using gas chromatography instrumentation (GC 2010 plus, Shimadzu, Japan) equipped with TCD and FID detectors and a DB-FFAP capillary column from Agilent Technologies. Total alkalinity was quantified using the HACH TNT 870 kit adopting the colorimetric method, while total ammonia was quantified with HACH TNT 832 kit adopting the salicylate method (HACH, Loveland, CO, USA). The gas composition was quantified using gas chromatography (GC 2010 plus, Shimadzu, Japan, equipped with TCD and ECD detectors).

2.5. DNA Extraction and Sequencing

DNA was extracted from sludge samples collected from the reactors using FastDNA™ Spin Kit for Soil and FastPrep™ Instrument from MP Biomedicals (Santa Ana, CA, USA).
Numbers of cell lysing steps using the FastPrep™ instrument were increased to four times, each lasting for 40 seconds. The extracted DNA was further purified using DNA Clean and Concentrator™-10 purification kit from Zymo Research (Irvine, CA, USA). The quality and concentration of the extracted and purified DNA was determined using a NanoDrop™ 2000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA) and an Qbit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) respectively. Agarose gel stained with ethidium bromide using a 1-kb ladder as reference was used to ensure that the extracted DNA was not too fragmented.

The extracted and purified DNA was sequenced using the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Prior to sequencing, the quality of DNA samples was checked with a Bioanalyzer 2100 using a DNA 12000 Chip (Agilent, Santa Clara, CA, USA). Sample quantitation was carried out using Invitogen’s Picogreen assay. Library was prepared as per Illumina’s TruSeq Nano DNA Sample Preparation protocol. Following the manufacturer’s recommendation, DNA samples were sheared on a Covaris S220 or E220 to approximately 450 bp and uniquely tagged with Illumina barcode to allow pooling of libraries for sequencing. The finished libraries were quantified using Invitogen’s Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent, Santa Clara, CA, USA). Library concentrations were normalized to 4nM and validated using qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems, CA, USA) using KAPA Illumina Library Quantification Kit (Kapa Biosystems, Roche). The libraries were pooled at similar molar concentrations and sequenced on Illumina HiSeq 2500 sequencer. Rapid mode at a read-length of 250 bp paired-end was used. Raw reads generated from sequencing were checked for quality and adaptors were removed using cutadapt version 1.9.1 with default parameters (except --overlap 10 -m 30). After the quality check, the reads were processed using Ribotagger-v.8.1.r106 with default parameters targeting the V4 hyper variable region (Xie et al. 2016)
2.6. Statistical Analysis

Due to small sample numbers, the Wilcoxon signed rank test was used to confirm if process performance in R1 was statistically similar to R2 in each phase. In addition, we also compared process performance of individual reactors in Phase 2 to the performance in Phase 3 using Wilcoxon Signed Rank. This was done since there was no longer an unperturbed reactor in Phase 3. The test was performed using the Real Statistics Resource Pack software (Release 4.3; Copyright (2013 – 2015) Charles Zaiontz; www.real-statistics.com). Multivariate analysis of microbial community genera was done using PRIMER v6 with PERMANOVA+ software (Clarke and Gorley 2006). The processed read counts were first standardized based on the total counts per sample. Square-root transformation was applied to the standardized data to minimize effects of dominant organisms before a Bray-Curtis distance matrix was constructed. Both PERMANOVA (permutational multivariate analysis of variance) and ANOSIM (analysis of similarity) were used to check the null hypotheses that communities before and after the temperature perturbations were similar (Anderson and Walsh 2013). The number of permutations was set to be 9999 and phase was used as fixed factor. PERMDISP analysis was done to test for homogeneity of variance among samples (Anderson 2006).

Hill’s second diversity index (N_2) was used on standardized non transformed OTU data to measure the diversity in archaeal and bacterial genera. N_2 is the reciprocal of Simpson’s Index (Hill 1973). This diversity index puts more emphasis on common species, which makes it more robust against changes caused by rare species (Haegeman et al. 2013).

3. RESULTS

3.1. Effect of pulsed temperature perturbations on Days 49 and 51 on process performance
In Phase 1, the effluent volatile solids profile in R1 was similar to that in R2, although both were still in the transient or acclimation stage indicating that both reactors served as good replicates of one another (Figure 1). Effluent solids started to fluctuate more in R1 than in R2 after R1 experienced its first set of perturbations on Days 49 and 51, suggesting that the perturbations actually affected the digestion.

We hypothesized that the transient perturbations would not affect hydrolysis, a common rate-limiting step in anaerobic digestion. Similar volatile matter reduction (VMR) and total COD (TCOD) removal in R1 and R2 in Phase 2 supported this observation (Table 1). Although the average VMR in R1 decreased by 19% in Phase 2, a 17% decrease was also observed in R2, suggesting that the decrease was not caused by temperature perturbations. These results showed that temperature perturbation did not affect hydrolysis in R1.

We also looked into the possible effects of these transient perturbations on methanogenesis, which was another common rate limiting step in anaerobic digestion. Average biogas production in R1 before temperature perturbation was statistically similar to R2 (Table 1). Average biogas production in the two reactors remained statistically similar in Phase 2, suggesting that R1 was not affected by the temperature perturbations (Table 1). It should be noted that the marked increase in biogas production in this phase was caused by a different gas collection method than was used from Phase 2 onward. This change did not alter the outcome of the comparison since the change in gas collection method was done for both R1 and R2 reactors. The biogas composition remained the same (volumetric methane to carbon dioxide ratio was 60-70% to 30-40%).

Although biogas production and composition were not affected by the pulse perturbation, acetate profiles suggested that acetoclastic methanogenesis was actually affected since acetate was typically utilized in this stage (Figure 2). Following the first set of
temperature perturbations, there was a sharp increase in acetic acid concentrations in R1 from less than 10 mg/L to almost 1,800 mg/L. Hence although the pulse perturbations were short, they were strong enough to affect acetoclastic methanogenesis. This observation was confirmed by the temporal profile of archaea abundance, which will be discussed in section 3.3.

3.2. Effects of Day 97 pulse perturbation on process performance

We have shown that the first set of perturbations affected acetoclastic methanogenesis in R1. To further support observations about the effect of transient perturbations, we also applied one cycle of temperature perturbation to R2 on Day 97. The overall trends in R2 after it received its first perturbation (Phase 3) were similar to those observed in R1 in Phase 2, confirming that the changes observed in the previous phase were caused by temperature disturbances. The VMR and TCOD removal in the two reactors remained statistically similar in this phase (Table 1). Additionally, we compared average VMR and TCOD removal in R2 in Phase 3 with the averages in Phase 2 to check if the perturbation affected hydrolysis. This was done because there were no longer any unperturbed reactors to serve as control in this phase. VMR and TCOD removal in Phase 2 was statistically similar to Phase 3 (p = 1 for VMR and p = 0.2 for TCOD removal; Wilcoxon signed rank test). Moreover, fluctuations in R2 were less severe than in R1, possibly because it only received one cycle of temperature perturbation while R1 had received two cycles of the same perturbations in the previous phase (Figure 2).

We also hypothesized that after the first perturbations, the community in R1 had acquired the ability to withstand similar perturbations by selecting organisms more able to withstand such perturbations. The noticeably smaller fluctuations in effluent acetate in R1 after the perturbation on Day 97 supported this hypothesis (Figure 2). The average VMR and TCOD removals in R1 did not change significantly from Phase 2 to Phase 3, suggesting the additional
temperature perturbation did not cause any discernible additional effect on hydrolysis (p = 1 and 0.2 for VMR and TCOD removal respectively; Wilcoxon Signed ranked test). These results further supported our previous observation that pulse temperature perturbations did not affect the hydrolysis process.

Following a perturbation on Day 97 in both R1 and R2 reactors, the average biogas production in R1 and R2 was very similar (Table 1). R2 average biogas production in Phase 3 did not differ significantly from the biogas production in Phase 2 (p = 0.6; Wilcoxon Signed Rank test). Similarly, additional temperature perturbation did not significantly affect R1 biogas production in Phase 3 as compared to Phase 2 (p = 1).

Although volumetric biogas production and its composition were not affected by the temperature perturbation on Day 97, acetic acid concentration in R2 increased to 1,300 mg/L before returning to normal. This increase was similar to the increase observed in R1 in Phase 2, supporting our hypothesis that the transient temperature perturbations were substantial enough to affect acetoclastic methanogenesis.

Acetic acid concentration in R1 increased to 586 mg/L following additional perturbation on Day 97. The concentration returned to less than 10 mg/L afterwards. The smaller increase following this additional perturbation supported our hypothesis that the community had become more resistant towards similar perturbations. Moreover, the increase in acetate observed in R1 and R2 did not happen immediately suggesting that there was some delay in the community response to the perturbations. The aforementioned trends in acetic acid could be explained by the temporal profiles of archaea, which will be discussed in the next section.

3.3. Archaea response to pulse temperature perturbation.
We hypothesized that the pulse temperature perturbations affected archaea more strongly than bacteria. In Phase 1, archaea profiles in the two reactors did not differ significantly (Table 3). In Phase 2, the p and R values from PERMANOVA and ANOSIM indicated that although the archaea community in R1 started to deviate from R2, the difference was not significant (Table 3). This might be caused by the delayed response of archaea to the perturbations since both PERMANOVA and ANOSIM took into account the overall structure of the data. The notion that there was a delay in the archaeal response to the perturbations was supported by the delayed increase in acetate concentrations (Figure 2). Moreover, the PERMDISP p-value indicated lack of homogenous dispersion in the two reactors in Phase 2 (Table 3). These results showed that temperature impacted the archaea community.

After the perturbation on Day 97, the archaea community in R1 changed more significantly than in R2 (Table 3). The PERMDISP value also showed homogenous dispersion in the two archaea communities, confirming that the changes observed were mostly caused by temperature perturbation. These results confirmed our hypothesis that the temperature perturbations were strong enough to alter the archaea communities in R1 and R2 despite the briefness of the perturbations.

Due to the aforementioned delay in archaea responses to the perturbations, we also analyzed temporal relative abundances of archaea in our reactors. The phylum temporal profiles confirmed our previous observation that temperature perturbation significantly affected archaea (Figures 3A and B). Despite the changes observed, archaea only made up less than 6% of the whole digester community (Table 2). Therefore, we used relative abundance with respect to overall archaea community to avoid underestimating any changes that might be caused by temperature perturbations.
The temporal profile of the phylum *Methanomicrobia* in R1 showed a 40 % decrease in abundance 25 days after the disturbances on Days 49 and 51 (Figure 3A). This decrease coincided with the increase in acetate concentration observed in R1 and it also confirmed the observation that changes in microbial community did not happen immediately after the temperature perturbation. The aforementioned decrease coincided with an almost 40% increase in the phylum *Methanobacteria* (Figure 3A). This may explain the similar biogas production despite an increase in acetate, which was one precursor of methane. The relative abundance of *Methanomicrobia* phylum started to increase following the initial decrease, which explained the subsequent decrease in acetic acid. There was no additional change in the % relative abundance of these phyla after the perturbation on Day 97.

Similar to these trends, the abundance of the *Methanomicrobia* phylum also decreased in R2 two weeks after the reactor received its first temperature perturbation on Day 97 (Figure 3B). At the same time, *Methanobacteria* became more abundant. The changes observed in R2 in Phase 3 were less obvious than in R1 in Phase 2. One possible reason for this was the fact that only one temperature perturbation was applied to R2.

Twenty-five days after the first sets of perturbations, the relative abundance of the genus *Methanosaeta* in R1 had decreased by more than 40% (Figure 4A). This change explained the increase in acetate concentration since *Methanosaeta* is an obligate acetate user (Figure 2). Similarly, its relative abundance in R2 also decreased by almost 20% two weeks after it received its first perturbation on Day 97. The smaller decrease in R2 was expected since the reactor was only subjected to one cycle of temperature perturbation. It also translated into a smaller increase in acetate observed in R2 (Figure 2).

The decrease in *Methanosaeta* coincided with an increase in the abundance of other methanogens such as *Methanobacterium* and *Methanospirillum* (Figure 4B and C). Twenty-
five days after the initial perturbation events, *Methanobacterium* in R1 had increased by more than 20%. This genus also started to increase in R2 14 days after it was perturbed on Day 97. *Methanospirillum* in R1 increased by almost 30%. This indicated that some genera were more prone to temperature perturbations and started to be affected after only one perturbation while others were only affected after repeated perturbations. This trend was also observed in *Methanolinea* whose abundance decreased by almost 30% in R1 25 days after its first temperature perturbation, while the decrease in R2 was more subtle (Figure 4D). Moreover, the relative abundances of previously non-detected *Methanosarcina* and *Methanoculleus* in R1 increased to almost 20% and 10%, respectively (Figures 4E and F). These genera were not detected in R2, suggesting that they required more rigorous perturbations.

Since the composition was affected by the temperature perturbations, it was expected that archaea alpha diversity (as shown by Hill N₂ values) would also change. N₂ values in R1 increased from 2 in Phase 1 to within 4-5 in Phase 3 (p = 0.5 and 0.04 for comparison of Phases 1 and 2 and Phases 2 and 3, respectively; Wilcoxon sign rank test). A similar trend was also observed in R2 where N₂ diversity increased from an average of 2 to an average of 4 following its first temperature perturbation on Day 97 (p-value = 0.04; Wilcoxon ranked sign test). This result further supported our hypothesis that the pulse perturbation was strong enough to alter the archaea community.

### 3.4. Bacteria response to pulse temperature perturbation

Unlike archaea, the relative abundance of bacteria in both reactors was not affected by temperature perturbations (Table 2). PERMANOVA analysis confirmed that the bacterial community in R1 was similar to R2 in all three phases (Table 3). Although the p-value in Phase 3 was smaller than 0.05, the small pseudo-F value indicated that the differences between R1 and R2 were not significant. This was further supported by the small ANOSIM-R values (Table
3). The higher ANOSIM R observed in the last phase meant that the change observed in Phase 3 was more substantial than the change observed in Phase 2. The changes in R1 bacteria community in Phase 3 could have been exacerbated by the delayed response to perturbations on Days 49 and 51. Nevertheless, the differences were still minor. Temporal profiles of bacterial phyla further revealed the absence of an effect of temperature perturbations (Figures 5A and B).

Considering that there were more than 1000 OTUs assigned as bacteria, we used SIMPER analysis to select the top 10 bacteria with the greatest contribution to the differences between R1 and R2 in each phase. The changes observed in the average abundance of these OTUs were less than 5%, confirming that bacteria were not severely affected by the pulse perturbations (data not shown). Similarly, bacterial diversity in R1 and R2 did not change following the disturbances. In R1, the average N2 values for bacteria remained similar throughout the three operational phases (28.34 ± 3.14, 29.76 ± 5.46, and 32.05 ± 4.03 for Phases 1, 2, and 3 respectively; p values from Wilcoxon sign rank test were 0.5 for comparison between Phases 1 and 2 and 0.9 for Phases 2 and 3). The same was true for N2 values in R2 (average N2 values before and after the perturbation on Day 97 were 29.01 ± 3.47 and 34.74 ± 4.76, respectively, with p = 0.2; Wilcoxon sign rank test).

4. DISCUSSION

In this study, short pulse perturbations were used to determine if they were strong enough to affect anaerobic digestion. Results indicate no effect on the digestion process performance but perturbations were strong enough to alter the archaea community.

4.1. Effect of temperature perturbation on overall process performance

Hydrolysis is an enzyme-mediated process involving various types of temperature-sensitive catalytic enzymes (Batstone and Jensen 2011). In contrast to previous studies, which
reported different biochemical rates of reactions and solubility of many particulate organics at different temperatures (Donoso-Bravo et al. 2009, Ho et al. 2013, Lu et al. 2008, van Lier et al. 2001, Zábranská 2000), hydrolysis was not impacted by the perturbations. One possible reason was the short duration of the temperature changes. Moreover, the temperature range employed was still within the optimum range for hydrolysis of 25-35°C (Tezel et al. 2011) and reactors were also operated at a long SRT of 30 days.

The interconnected nature of anaerobic digestion steps implies that disturbance in one stage can cause cascading effects for the other steps as well. For example, a lower temperature has been reported to slow the hydrolysis rate, which would lead to a reduction in solubilized substrates for conversion into biogas by methanogens (Donoso-Bravo et al. 2009, Ho et al. 2013, Vanwonterghem et al. 2015). In our study, biogas production and composition following perturbation events remained the same, suggesting that methanogenesis was not adversely affected either by the pulse perturbations; hence no cascading effect was observed.

4.2. Effects of pulse perturbations on Archaea

Despite similar biogas production, archaea underwent some changes following the perturbations. Prior to perturbations, the most dominant archaea was the *Methanosaeta* genus, which typically dominates under low acetate conditions due to its high affinity for acetate (Demirel and Scherer 2008, Jetten et al. 1992). Another common acetoclastic methanogen, *Methanosarcina*, was not detected in either reactor prior to the perturbations. The growth of one genus to the detriment of the other has been reported before (Regueiro et al. 2014). Both of these acetoclastic methanogens use acetate as substrate and *Methanosarcina* was likely outcompeted by *Methanosaeta* due to the former’s lower affinity toward acetate. This was the reason why *Methanosarcina* could only proliferate under conditions where there was an elevated level of acetate (Ferry 2012).
The sharp decrease in *Methanosaeta* was most probably caused by the pulse temperature perturbations since acetoclastic methanogens are susceptible to temperature shocks (Ban et al. 2013). Therefore, this methanogen could only dominate in stable digesters (Ferry 2012, Nelson et al. 2011, Rivière et al. 2009). *Methanosaeta* is an obligate acetate consumer (Smith and Ingram Smith 2007). Its decrease could explain the sudden increase in acetate concentrations in our reactors following temperature perturbations since *Methanosaeta* was the only acetate consumer in our reactors prior to the perturbations.

Following the sudden reduction in its abundance in R1, this methanogen could not return to its former abundance level due to the accumulation of acetate in the reactors as reported before (Jetten et al. 1992). The emergence of *Methanosarcina* in R1 could explain the subsequent decrease in acetate as it is known as acetate consumer in reactors recovering from perturbations (Hori et al. 2006).

Methanogenesis was not adversely affected by the pulsed perturbations despite the decrease in *Methanosaeta*. This was caused by the emergence of hydrogenotrophic methanogens that could take over the role of *Methanosaeta* as the main methane producer. Hydrogenotrophic methanogens are able to survive and dominate in a wider temperature range than acetoclastic methanogens (Ho et al. 2014, McHugh et al. 2004, Rastogi et al. 2008, Vanwonterghem et al. 2015). Moreover, these methanogens have a minimum doubling rate of six hours compared to acetoclastic methanogens whose minimum doubling rate ranges from two to three days (Huang et al. 2015). Their faster doubling rates might explain their ability to maintain their dominance over the acetoclastic methanogens even after the operating temperature had been returned to normal.

The aforementioned changes in the archaea community coupled with a lack of noticeable changes in the biogas production indicated the presence of functionally redundant
taxa in our system. Functional redundancy is important to safeguard a biological system against the alteration of process rates that might be caused by changes in the microbial community (Allison and Martiny 2008).

The increase in archaea diversity following the perturbations might be due to the emergence of hydrogenotrophs, which were able to withstand the temperature fluctuations since this group consisted of more members than their acetoclastic counterpart. Members of hydrogenotrophic methanogens included orders such as *Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*, while acetoclastic methanogens consisted solely of some members of the order *Methanosarcinales* (Demirel and Scherer 2008, Sakai et al. 2009). This increase in diversity could be beneficial to the digestion process since higher diversity could serve as insurance against loss of functions due to loss of certain organisms (Naeem and Li 1997). The increase in diversity could also provide additional functional redundancy for the system.

In addition to diversity, it is also important for the microbial community to possess adequate resistance toward changes due to disturbances since these changes could affect the processes in the system (Allison and Martiny 2008). Resistance refers to the degree to which a microbial community remains unchanged following disturbances (Allison and Martiny 2008). The changes observed in archaeal communities in R1 and R2 after they experienced their first perturbations indicated that the archaea did not possess any resistance against the perturbations initially. However, it has been seen in some systems that the outcome of a perturbation in a community that has been perturbed is not the same as in their unperturbed counterpart due to the life-history adaptation of having gone through the perturbation. This observation was supported by the lack of conspicuous additional changes in archaea in R1 following additional perturbation on Day 97. Previous perturbations had likely increased the community resistance toward similar perturbations, possibly by giving more robust organisms a more competitive
advantage. Hence, pulse downward temperature perturbation could be explored as a more cost and energy-effective optimization method than conventional optimization methods such as pretreatment.

4.3. **Effects of pulse perturbation on bacteria**

The bacteria in R1 and R2 were dominated by the phyla *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Spirochaetes*, and *Firmicutes* which are hydrolytic, syntrophic, and fermentative bacteria that have been reported as common major groups in anaerobic digestion (Ariesyady et al. 2007, Guo et al. 2015, Rivière et al. 2009, Tezel et al. 2011). Unlike archaea, bacteria were not affected by temperature perturbations, possibly as a result of the higher bacterial community diversity compared to archaea. This may have led to higher redundancy in terms of activity and make the bacterial community more resistant. Such redundancy was due to the highly diverse communities in digesters. This diversity might be due to the long SRT (that is, 30 days), which was much longer than the doubling times of many organisms, including the slow-growing methanogens (Lee et al. 2011, Tezel et al. 2011). Moreover, the reactors were fed with thickened sewage sludge, a complex substrate that contains different types of organics that can support a wider range of organisms (McHugh et al. 2003).

It was interesting that the increase in hydrogenotrophic methanogens was not accompanied by an increase in the abundance of their syntrophic partners such as some members of genera *Clostridium* and *Syntrophaceticus* as well as *Spirochaetes*, as has been reported before (Lee et al. 2015, Sun et al. 2014). One possible reason for this is that although the abundance of specific OTUs did not change, the taxa involved in syntrophic relationships with hydrogenotrophs became more active. Future work should include metatranscriptomic analysis to test whether specific genes were induced after temperature changes.
5. CONCLUSIONS

- Short-term temperature pulses drove archaea community assembly in a mesophilic digester. Minimal perturbations selected for the more robust hydrogenotrophic methanogens and increased archaeal diversity – both attributes that can improve process stability in anaerobic digestion.

- Adopting intermittent pulse temperature reduction to improve digestion requires less energy than conventional pretreatment methods and potentially lowers operational costs.

- Another benefit of downward temperature cycling was the accumulation of acetic acid, which could potentially be harvested from the digester.

ACKNOWLEDGEMENTS

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Table 1: Process performance in laboratory-scale anaerobic reactors. Values represent the mean with the standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Parameter (unit of measurement)</th>
<th>Before temperature perturbations (Phase 1)(^1)</th>
<th>After perturbations on days 49 and 51 (Phase 2)(^2)</th>
<th>After perturbation on day 97 (Phase 3)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactor 1 (R1)</td>
<td>Reactor 2 (R2)</td>
<td>p(^4)</td>
</tr>
<tr>
<td>Volatile matter reduction (%)</td>
<td>65 (11)</td>
<td>65 (10)</td>
<td>0.7</td>
</tr>
<tr>
<td>Total COD removal (%)</td>
<td>52 (24)</td>
<td>51 (24)</td>
<td>0.3</td>
</tr>
<tr>
<td>Biogas production (mL/day)</td>
<td>1,023 (819)</td>
<td>1,782</td>
<td>0.01</td>
</tr>
<tr>
<td>Soluble COD removal (%)</td>
<td>91 (8)</td>
<td>91 (4)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(1 \text{ n = 11 per reactor}\)

\(2 \text{ n = 9 per reactor}\)

\(3 \text{ n = 8 per reactor}\)

\(4 \text{ p value from Wilcoxon sign rank test was used to determine if process performance in R1 was statistically similar to R2 in each phase. The null hypothesis that the process performance in R1 was similar to R2 was rejected if the p value for a two-tailed test was larger than 0.05.}\)
Table 2: Average abundance of bacteria, archaea, and unclassified OTUs in R1 and R2. The values represent the mean with standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Microbial domain (unit of measurement)</th>
<th>Before temperature perturbations (Phase 1)(^1)</th>
<th>After perturbations on days 49 and 51 (Phase 2)(^2)</th>
<th>After perturbation on Day 97 (Phase 3)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>R2</td>
<td>R1</td>
</tr>
<tr>
<td>Bacteria (%)</td>
<td>79 (2)</td>
<td>77 (2)</td>
<td>79 (3)</td>
</tr>
<tr>
<td>Archaea (%)</td>
<td>5 (1)</td>
<td>6 (1)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Unclassified (%)</td>
<td>16 (1)</td>
<td>18 (2)</td>
<td>16 (2)</td>
</tr>
</tbody>
</table>

\(^1\) n= 4 samples per reactor

\(^2\) n = 5 samples per reactor

\(^3\) n= 5 samples per reactor
Table 3: Multivariate analysis of archaeal and bacterial genera in each phase. Results were generated using PRIMER 6 software. Square root transformation was applied to the standardized data to minimize the effects of dominant OTUs. A Bray-Curtis similarity matrix was constructed prior to the analysis.

<table>
<thead>
<tr>
<th>Microbial domain</th>
<th>Phase$^1$</th>
<th>PERMANOVA$^2$</th>
<th>ANOSIM$^3$</th>
<th>PERMDISP$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$P$</td>
<td>$R$</td>
<td>$p$</td>
</tr>
<tr>
<td>Archaea</td>
<td>1</td>
<td>0.9</td>
<td>-0.02</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.007</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria</td>
<td>1</td>
<td>1</td>
<td>-0.04</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>-0.02</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.03</td>
<td>0.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^1n = 4$ samples per reactor for Phase 1; 5 samples per reactor for Phases 2 and 3.

$^2$ Null hypothesis that there was no difference in the two reactors community was rejected if $p$ value was less than 0.05.

$^3$ The closer the $R$ value to 1, the bigger the difference in the two reactors communities.

$^4p < 0.05$ indicates homogenous dispersion in the two reactors communities.
Figure 1: Effluent volatile solids concentrations from reactors R1 and R2. The operating temperature was kept at 35 ± 1°C except for Days 49, 51, and 97 when the temperature was rapidly reduced to 25 ± 1°C. Arrows indicated the days where perturbations were applied. ●, refers to R1, which experienced temperature perturbations on Days 49, 51, and 97; ○, refers to R2 which had a temperature perturbation on Day 97 only.
**Figure 2:** Acetate concentrations in R1 (●) and R2 (○). The dashed lines refer to operational Days 49, 51, and 97 when the temperature perturbations occurred. R1 received all three perturbations, while R2 was perturbed on Day 97 only.
Figure 3: Relative abundance of the archaea phylum with respect to overall archaea community as a function of time in R1 (A) and R2 (B). The dashed lines refer to operational Days 49, 51, and 97 when the temperature perturbations occurred. R1 received all three perturbations while R2 experienced only one perturbation on Day 97. ● represents Methanobacteria; ○, Methanomicrobia; and ▼, Thermoplasmata.
Figure 4: Temporal relative abundance of archaea genera with respect to overall archaea community in R1 (●) and R2 (○). The dashed lines refer to operational Days 49, 51, and 97 when the temperature perturbations occurred. R1 received all three perturbations while R2 received only the one on Day 97. The temperature was kept at 35 ± 1°C for all other days. Panel (A) Methanoseta, (B) Methanobacterium, (C) Methanospirillum, (D) Methanolinea, (E) Methanosarcina, and (F) Methanoculleus.
Figure 5: Temporal profile and heat map for bacterial phyla in R1 (A) and R2 (B). The number inside the box denotes the % relative abundance of each phylum with respect to bacteria community only.


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CHAPTER 5
CONCLUSIONS

5.1 Research objectives

Anaerobic digestion (AD) is a ubiquitous sludge stabilization method due to its ability to reduce the sludge solids content by converting organics into energy-rich biogas (Appels et al. 2008). In Chapter 1, I reviewed important aspects of AD including different steps in AD, microbial groups responsible for these steps as well as important parameters that can affect the microbial community and process performance of an anaerobic digester. Of the parameters, substrate type, solids retention time (SRT), and temperature are among the most important ones (Batstone and Jensen 2011).

Although previous research has improved our understanding of AD, there are still knowledge gaps that need to be addressed, such as the dynamics of microbial communities in digesters under a variety of conditions. Hence, the main objective of this study was to understand the microbial community dynamics and process performance of a digester as a function of substrate characteristics, solids retention time, and temperature. Information from these studies can shed light on prospective strategies that can be implemented to improve AD, especially biogas production. Outcomes will be useful to evaluate which parameters could be modified without having negative effects on microbial communities and, as a result, process performance..

5.2 Results and implication of this thesis

I have shown in Chapter 2 that proteins were the preferred substrates in our studies due to their high relative abundance compared to carbohydrates. Hence, the extent of protein and carbohydrate utilization in AD will depend on their relative concentrations with respect to one another. It is, therefore, important to take into account such substrate characteristics if substrate augmentations are to be applied to enhance AD. To solely rely on the theoretical degradation
kinetics of individual organics can lead to suboptimal results. For example, using carbohydrates to augment substrate that contains significantly higher protein levels will yield suboptimum results. This is because the additional carbohydrates will not be used efficiently, although carbohydrates could theoretically be degraded faster.

Results in chapter 3 suggest that it is not necessary to apply long SRTs to ensure the success of a digester. I have shown in Chapter 3 that step-wise SRT reduction from 30 to 15 days did not cause any significant changes in the microbial community, indicating that the methanogenic SRT (SRT > 8 days) is not the most important factor for the maintenance of the microbial community in a digester for the range of SRTs tested. I have also shown that biogas production was enhanced at a shorter SRT while other process parameters were not affected. Therefore, SRT reduction can be applied to full-scale digesters that are currently operated at a long SRT (SRT> 30 days).

In Chapter 4, I established that pulsed temperature perturbations can alter the archaeal community despite the brevity of the perturbation. Temperature perturbations, which have been been negatively perceived in the past, can actually be beneficial and can be adopted for a lower-cost improvement strategy. Implementing downward pulse temperature perturbations can enhance digester stability by promoting higher diversity and proliferation of more robust organisms such as hydrogenotrophic methanogens. Hydrogenotrophic methanogens are more able to withstand perturbations than acetoclastic methanogens. Pulsed perturbations can also increase the profitability of a digester by inducing the accumulation of acetic acid that could be harvested without jeopardizing biogas production. The benefits that can be reaped from pulse temperature cycling challenges the notion that temperature must be kept as constant as possible with changes applied not exceeding 1°C/day.
Overall, the results reported in this thesis have affirmed the importance of understanding the dynamics of a digester microbial community and process performance as function of AD parameters. This improved understanding has opened up the possibility of modifying these parameters for cheaper, less energy intensive, and more efficient strategies to optimize AD. The study also illustrates that small changes in microbial communities, or a lack thereof, might not be sufficiently reflected in tangible process parameters, such as biogas productions. Consequently, relying merely on these apparent parameters might not give a true insight into microbial communities.

The results of this study reiterate the importance of understanding the relationship between microbial diversity and ecosystem functions. Although two communities with different compositions might behave similarly under normal operating conditions, their response to perturbations can be different. For example, in Chapter 4 it was shown that a community dominated by acetoclastic methanogens was less robust against temperature perturbations than a community dominated by hydrogenotrophic methanogens, although biogas production and volatile matter reduction were similar in digesters. Based on its susceptibility to changes in parameters, *Methanosaeta* may be useful as future biological indicator for the presence of disturbances in anaerobic digesters.

5.3 Future work

Future research on the roles of proteins should include the identification of proteins that were utilized most efficiently in AD using, for example, proteomic analysis of substrates collected from the two wastewater treatment plants by mass spectrometry. The degradation of these proteins can then be followed in laboratory-scale reactors and batch degradation tests.

The SRT reduction in this study was done in a step-wise manner where SRT was reduced from 30 days to 25 days and finally to 15 days. Future work can focus on directly
reducing the SRT from 30 to 15 days. Moreover, the reactors used in this study had been operated for more than one year prior to the start of the study. Hence, future work can also be done to investigate whether microbial communities that have been freshly inoculated prior to the start of the study display similar trends as those in reactors that have been operated for an extended period of time.

Lastly, considering the short duration of the temperature study, additional work should focus on whether the changes observed in the microbial community are reversible. Microbial communities should be monitored for at least 3 SRTs following the perturbation. Moreover, due to a limited number of reactors available in this portion of the study, future studies should employ more control reactors until the conclusion of the study.

Finally, I hope the insights gained in this thesis can help to further improve our understanding of anaerobic digestion of sewage sludge, so that society may maximize its benefits.

REFERENCES


