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**NANYANG
TECHNOLOGICAL
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SINGAPORE

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

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2018

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and Temperature on Digester Process Performance
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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 WRP 2 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 *Methanosaeta* 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 $35 \pm 1^\circ\text{C}$ 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 *Methanosaeta* 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 volatile 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 pyruvic 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, pyruvic 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 Stickland 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 CO_2 , NH_3 , 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

failure 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). To produce methane, hydrogenotrophic methanogens typically work syntrophically with groups of bacteria called syntrophs. In this process, acetate is first oxidized to hydrogen by these syntrophs. 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 (Rivière et al. 2009).

1.4.1.3 *Chloroflexi*

Chloroflexi has been found to be the major phylum in many digesters (Narihiro and Sekiguchi 2007, Rivière 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 (Rivière 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 extract 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, Rivière 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 *Synergistes*

Unlike the four previously mentioned phyla, *Synergistes* 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, *Synergistes* 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 mazei*) 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 $> 100 \mu\text{M}$ (Jetten et al. 1992). Therefore, *Methanosarcina* usually thrive in a 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. 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. 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 (i.e methane production rate and methane

yield) 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 2

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 up to 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 of 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. Average of proteins and carbohydrates in WRP2 were 4 times higher than in WRP1 and protein concentrations were higher than carbohydrates. When the feed from WRP1 was switched to WRP2, there was an increase in methane production and methane yield in the reactors. While average protein removal increased by 20%, there was no significant change in average carbohydrate removal suggesting that proteins were preferred in the second phase. In additional batch tests employing basal medium and 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 when their concentrations were higher than carbohydrates. It should be noted that protein to carbohydrate (P to C) ratios in both phases were statistically similar, suggesting that ratio between the two organics was not the sole indicator of which organics would be preferentially utilized in AD. Hence, future work should include the identification of different types of proteins and carbohydrates in substrate sludge.

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. 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). Sewage sludge substrate contains complex organics that are typically grouped into proteins, carbohydrates, and lipids. In many cases, proteins and carbohydrates can make up to 50% of the sludge organics while lipids comprise the rest of the organics. The roles of these organics in anaerobic digestion have been explored (Elefsiniotis and Oldham 1994, Neyens and Baeyens 2003). Most of the previous studies reported higher removal of carbohydrates than of proteins (Elefsiniotis and Oldham 1994, 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 used proteins and carbohydrates due to their similar theoretical methane

production capabilities (0.4 L/g of VS for the former and 0.5 L/g of VS for the latter) as compared to lipids whose theoretical methane production value was 1 L/g of VS (Angelidaki and Sanders, 2004). We hypothesized that proteins in the substrate sludge could be utilized more efficiently than carbohydrates if their concentrations were higher.

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 continuously stirred (CSTR) anaerobic reactors without sludge recycle 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 $35 \pm 1^\circ\text{C}$ 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 operation 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 $30 \pm 1^\circ\text{C}$. 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. Digesters at WRP2 were operated at working temperature of $35 \pm 1^\circ\text{C}$ and SRT of 25 ± 5 days. 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 batch 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 mL of seed sludge and 3 mL of feed sludge. For the first part of the batch test, the WRP2 feed was diluted to match the solids concentration of WRP1 substrate. For the second part of the batch test, we used anaerobic basal medium as described elsewhere in lieu of sewage sludge (Angelidaki and Sanders 2004). We used bovine

serum albumin (BSA) and sucrose as representative proteins and carbohydrates at P to C ratios of 5:1 and 30:1.

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. Biogas production was measured using pressure manometer.

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- μ m 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. Carbohydrates were measured using

the Dubois method (Dubois, 1951) while 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). Samples for particulate proteins and carbohydrates were heated at 80°C for 60 minutes prior to filtration. The filtered supernatant was then tested for proteins and carbohydrates using the aforementioned assays. Total carbohydrates and proteins referred to the sum of the particulates and soluble measurements.

2.6 DNA Extraction and Sequencing

DNA was extracted from laboratory-scale digesters sludge using 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 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 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 Invitrogen'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 Invitrogen'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 COD mass balance calculation

We used chemical oxygen demand (COD) mass balance to verify the increase in methane production observed in Phase 2 and it was calculated using the equation:

$$\text{COD}_{\text{in}} = \text{COD}_{\text{out}} + \text{COD}_{\text{CH}_4} \quad (\text{Eq. 1})$$

Where COD_{in} is the particulate COD concentration in the substrate while COD_{out} referred to particulate COD in digested sludge. Methane produced was converted to COD_{CH_4} using the conversion factor of $0.35 \text{ m}^3 \text{ CH}_4$ for every kg of COD (Van Lier et al. 2008).

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. Shepard diagram was used to illustrate the goodness of fit of the

transformed data. This was done to ensure that the data was not overly skewed before I proceeded with the multivariate analysis. PERMANOVA (permutational multivariate analysis of variance) was used to check the null hypotheses that communities in Phase 1 and Phase 2 were similar (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 samples (Anderson 2006).

We corrected the p-values obtained from the aforementioned tests using Benjamini-Hochberg technique to control the false discovery rate. This was done to minimize the possibility of obtaining significant p-value ($p < 0.05$) due to chance (Benjamini and Hochberg 1995, Spector 1956). Benjamini-Hochberg method was selected over Bonferoni correction due to the latter tendency to produce very high numbers of false negative results (Spector 1956).

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, which affected reactor performance. In the two undisturbed reactors, the pooled average volatile matter reduction (VMR) increased while average TCOD removal remained statistically similar (Table 2 and S1). This implied that the calorific values per unit volatile solids removed decreased in

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

Parameter	Substrate source ¹	
	WRP1 ²	WRP2 ³
Total Solids (mg/L)	20,626 (5,630)	66,320 (16,918)
Volatile Solids (mg/L)	17,260 (4,794)	49,925 (11,314)
Total COD (mg/L)	33,895 (11,319)	92,840 (23,595)
Soluble COD (mg/L)	1,406 (380)	7,381 (5,934)
Total Protein (mg/L)	694 (701)	2,649 (1,305)
Total Carbohydrate (mg/L)	190 (142)	645 (235)
P to C ratio	3 (1)	4 (5)

¹ Mean with standard deviation in parentheses

² n= 23 samples

³ n= 20 samples

Phase 2 although both phases used sewage sludge as substrates. This emphasized the importance of intrinsic substrate characteristics.

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 methane production and methane yield in Phase 2 were significantly higher than the methane production in Phase 1 (Tables 2 and S1) suggesting that the methanogenesis was enhanced in this phase. COD balance calculation yielded an average of 3-fold increase in methane production in Phase 2. However, the actual methane production observed in our study increased by more than 3-fold possibly due to the aforementioned issue with the gas collection

apparatus used in Phase 1. Ratio of methane to carbon dioxide remained at 70% to 30 % (v/v) in average. Hydrogen was constantly detected at very low concentrations. Hence, it was difficult to distinguish the actual hydrogen peaks from the peaks that were caused by the background noise or fluctuations in baseline.

Figure 1: Mean effluent volatile solids concentrations in pooled replicate reactors R1 and R2. The reactors were fed WRP1 substrate in Phase 1 and WRP2 substrate in Phase 2.

One possible reason for the enhanced biogas production in Phase 2 was the higher proteins and carbohydrates in the substrates used in this phase. There was an average of 20% increase in protein removal in Phase 2 (Table 2 and S1). Unlike proteins, average carbohydrate removal did not change significantly when the substrate was switched to WRP2 (Table 2 and S1). The lack of increase in carbohydrate removal suggested that proteins played a bigger role than carbohydrates in enhancing the methane production in Phase 2 since carbohydrates and

proteins in the substrates had to be degraded before they can be utilized by the organisms to produce biogas.

Table 2: Process performance in replicate laboratory-scale anaerobic reactors. Values represent the pooled mean with standard deviation in parentheses.

Parameter (unit of measurement)	Substrate source	
	WRP1 ^{1,3}	WRP2 ^{2,3}
Volatile matter reduction (%)	39 (16)	46 (13)
Total COD removal (%)	49 (23)	52 (11)
Soluble COD removal (%)	82 (6)	89 (6)
Acetate (mg/L)	2 (1)	3 (3)
Propionate (mg/L)	0(0)	0.5 (1)
Methane production rate (mL/day)	533 (248)	4,144 (1,453)
Methane yield (mL/g of VS added)	240 (102)	570 (246)
Ammonia (mg/L)	563 (78)	1,467 (227)
Average protein removal (%)	36 (17)	56 (18)
Average carbohydrate removal (%)	49 (17)	56 (22)

¹ $n = 46$

² $n = 40$

³ Pooled reactors 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.

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. To exclude these alternate explanations, we conducted batch 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 and degradation of both proteins and carbohydrates under these two conditions were highly similar, indicating that location did not cause the difference observed in digestion efficiency (Figures 2 and 3).

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.*

Constituent or ratio (unit)	Substrate source	
	WRP1	WRP2
TS (mg/L)	25,900	26,000
VS (mg/L)	21,300	18,700
VS/TS	0.82	0.72
Protein (mg/L)	1,608	1,014
Carbohydrate (mg/L)	193	162
P to C	8.33	6.26
TCOD (mg/L)	37,880	31,920
SCOD (mg/L)	1360	1440
VFA (mg/L)	1240	1280
Alkalinity(mg/L CaCO ₃)	508	510
Ammonia (mg/L)	165	118
Nitrate (mg/L)	0.56	0.39

Figure 2: Methane 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. Error bars represented the standard error of the means among triplicates at each time point. Some of the error bars are contained within symbols.

Additionally, we performed batch degradation test using two different BSA (P) to Sucrose (C) ratios to verify the potential inhibitory effects of proteins on carbohydrate utilization. We used simple proteins and carbohydrates such as BSA and sucrose to minimize effects of complex carbohydrates and proteins. It should be noted that although the ratios of BSA and Sucrose added were 5:1 and 30 : 1, the actual ratio of protein and carbohydrates detected in the bottles were 4:1 for the former and 10:1 for the latter due to the presence of soluble proteins and carbohydrates from the seed sludge used to inoculate the bottles. However, it was not the objective of this study to separate the source of proteins and carbohydrates. Therefore, the removal calculations were done based on the actual proteins and carbohydrates detected in each bottles. For the bottles fed with 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, sucrose concentration only decreased by 14% and 22 % for 5:1 and 30:1 ratios

respectively (Figure 4B). These results showed that proteins were preferentially used when they were in higher concentrations. Moreover, higher P to C ratio led to the better protein removal.

(A)

(B)

(C)

(D)

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. 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. ●, bottles fed with substrate containing a P to C ratio of 5:1. ●, 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

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). Both bacteria and archaea communities in R1 were statistically similar to R2 throughout the two phases; hence, we pooled the microbial community samples from both reactors (Table S1).

In Phase 1, 74% of the OTUs were classified as bacteria while 7% of the OTUs were archaea (Table S2). In Phase 2, the average abundance of bacteria increased by 12 % while the

average abundance of archaea decreased to 4% (Table S2). The aforementioned changes suggested that the bacteria and archaea communities were affected by the change in substrates as supported by the significant p-value obtained from PERMANOVA analysis (Table S1). Moreover, PERMANOVA analysis also yielded large Pseudo-F values for both bacteria and archaea (28 for the former and 30 for the latter) confirming that substrates played a significant role in the differences observed. This observation was further corroborated by the p-value obtained from PERMDISP analysis which indicated homogenous dispersion between the two groups (Table S1); hence, the differences in the two phases were indeed caused by different substrates instead of internal variations among samples.

Some of the known and putative protein users were detected in our samples. The members of the order *W5* within the *Spirochaetes* phylum were significantly enriched when substrates were changed in Phase 2 (Figure 5 and Table S1). Members of the genus *Sedimentbacteres* also increased significantly from 0.1% to 3 % in Phase 2 (Figure 5 and Table S1). In contrast, relative abundance of *Paludibacter* genus decreased from 2% to undetectable in Phase 2. The relative abundance of genus *Mycobacterium*, a known carbohydrate user, decreased significantly from 4% to 1 %. In addition, we also detected OTUs classified as members of genera *Acinetobacter*, *Leptolinea*, and *Tetrasphaera* . These genera could use both proteins and carbohydrates (McIlroy et al. 2015). Of these genera, *Acinetobacter* and *Leptolinea* abundances decreased significantly in Phase 2 (Figure 5 and Table S1). Members of family *Anaerolineaceae* were also detected in our samples and their ability to use both proteins and carbohydrates had been postulated before. The relative abundance of this family increased by 3% in Phase 2 (Figure 5 and Table S1). The dominance of protein users could explain higher proteins uptake observed in our study.

Figure 5: Average abundance of protein and carbohydrate users in pooled reactors 1 and 2. Each data point represented the average abundance with error bars denoting the standard deviation. The letter in parentheses denoted the taxonomic ranks genus (G), family (F), and order (O). ○, samples collected in Phase 1 (WRP1 substrate); ●, samples collected in Phase 2 (WRP2 substrate).

For archaea, *Methanosaeta* dominated in both phases. However, its abundance decreased from 5% to 3% when the reactors received WRP2 substrates (Figure 6). In addition, *Methanolinea*, the second most abundant methanogen in Phase 1, disappeared in Phase 2 (Figure 6).

Figure 6: Average archaea genera abundance in pooled reactors 1 and 2 . Each data point represented the average abundance with error bars denoting the standard deviation. ○, samples collected in Phase 1 (WRP1 substrate); ●, samples collected in Phase 2 (WRP2 substrate).

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.

Similar to the profiles observed in reactors R1 and R2 at a much later stage of Phase 2, PERMANOVA analysis of reactors 3 and 4 also yielded significant p-values while PERMDISP

p-value was not significant (Table S1). 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 (3% and 0.5% in R3 and R4, respectively). This 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 1994, 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 1994, 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 than 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 possibly through catabolites repression (Breure et

al. 1986, Glenn 1976). Catabolite repression was a condition where bacteria preferentially used certain type of carbon source (typically easily degradable carbon such as glucose) in the presence of multiple carbon sources (Deutscher 2008). This mechanism led to reduced utilization of the secondary substrates and suppressed production of catabolic enzymes required for these substrates utilization (Görke and Stülke 2008). Hence the low carbohydrate concentrations in the substrates could have boosted the degradation of proteins in this study.

It should be noted that P to C ratio did not differ significantly between the two phases despite higher protein removal observed in Phase 2. Hence, similar types of substrates could lead to different utilization of these organics. From this result, it could be postulated that it was not adequate to solely rely on P to C ratio to determine the preferred organics in AD. Indeed, proteins are made of different amino acids that are joined by peptide bonds and the compositions of these amino acids affect the end products of proteins hydrolysis (Batstone and Jensen 2011). It has been reported that some amino acids can repress the production of protease (Glenn 1976). Moreover, different proteins possess different structures which can affect the extent of hydrolysis (Batstone and Jensen 2011). Therefore, future studies should incorporate the identification of different types of proteins and carbohydrates that were present in sludge to provide a more comprehensive understanding on the dynamics of these complex organics.

Batch degradation tests

Degradation tests showed that the substrate constituents were the main driver of the differences in digestion efficiencies observed. The different locations from which the substrates and the seed sludge were collected did not play an important role. 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 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). High hydrogen pressure could induce hydrogen-utilizing

organisms to produce excessive amount of amino acids (Sam-Soon 1987). 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 concentrations was caused by the secretion of hydrolytic enzymes on the surface of the bacterial cells. 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 noticeable increase in biogas production at this stage seemed to support this rationale.

Carbohydrates were not consumed as well as proteins in the batch 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). The notion that proteins could inhibit the uptake of carbohydrates were further corroborated by the results of the batch test using different P to C ratios. The sucrose in the bottles was not utilized until the BSA concentrations were noticeably lower from day 2 onward. Near the end of the activity test, particulate carbohydrates started to be solubilized possibly because proteins no longer inhibited the utilization of carbohydrates. Indeed, higher carbohydrate hydrolytic coefficients had been observed when protein concentrations were lower than carbohydrates (Elbeshbishy and Nakhla 2012). Another possible reason for this decrease was cell lysis which resulted in organic materials in being released into the bulk solution (Barker and Stuckey 1999). This observation was corroborated by the increase in soluble carbohydrate concentrations and lack of noticeable increase in biogas production observed near the end of the digestion period.

The aforementioned results coupled with the rapid increase in biogas production at the beginning of the tests 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. The low volatile fatty acids observed in reactors also implied that methanogenesis was proceeding well in all samples tested.

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

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. 2008, 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. 2008). 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. We did not detect a high abundance of carbohydrate users possibly due to higher proteins in the substrates used in this studies. Although some of the organisms detected could use both proteins and carbohydrates, it could be postulated that they had been acclimatized to use proteins instead of carbohydrates.

With regard to archaea, the dominance of *Methanosaeta* was expected since this methanogen has been reported to prevail in stably operating anaerobic digesters (McHugh et al. 2003, Nelson et al. 2011, Rivière et al. 2009). Their abundance in our reactors might be due to the low concentrations of acetate, a condition that selected for *Methanosaeta* over *Methanosarcina*, another common acetoclastic methanogens, due to the former's lower acetate threshold (less than 10 mg /L of acetate for the former and more than 500 mg/L of acetate for the latter) (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 consisted of several strains whose doubling time ranged from 0.2 to 1.5 day under varying optimum temperatures (37-50°C) (Imachi et al. 2008, Sakai et al. 2012). *Methanolinea* genus was a member of the order *Methanomicrobiales* whose high affinity toward hydrogen means that it can thrive under conditions with low partial pressure of hydrogen. Indeed, members of this order typically flourish in environment with H₂ partial pressure lower than 100 Pa or 0.001 bar (Sakai et al.

2007) Hence, it was possible that its abundance in the reactors decreased as the hydrogen partial pressure increased since 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 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*), although the magnitude of changes was less than the changes observed in the undisturbed reactor. 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.
- Despite similar P to C ratio, types of proteins and carbohydrates in the substrate also dictate the extent of their removal. Hence, it is not enough to rely solely on P to C ratio to assess the degradation potential of the different organics in the sludge.
- 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.

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Table S1: *P* values from univariate and multivariate tests. Each of the *P* values was corrected using Benjamini-Hochberg (BH) method. *P** referred to values that had been corrected using BH procedure ($P^* = Q (i/m)$; where *i* = rank and *m* = total number of tests done; and *Q* = the chosen false discovery rate (10% in our case). We rejected the null hypothesis that the parameter in Phase 1 was similar to Phase 2 if *P* is deemed significant.

Parameter	p	P*	Significant?	Parameter	p	P*	Significant?
REACTOR				PROTEIN and CARBOHYDRATE USERS			
Ammonia concentration	0.0000	0.002	Significant	Acinetobacter genus	0.0014	0.057	Significant
Volatile acids concentration	0.0000	0.003	Significant	Bradyrhizobium genus	0.0251	0.067	Significant
Methane production	0.0000	0.005	Significant	Leptolinea genus	0.0000	0.008	Significant
Methane yield	0.0000	0.010	Significant	Methanolinea genus	0.0000	0.020	Significant
Protein removal	0.0000	0.032	Significant	Methanosaeta genus	0.0000	0.033	Significant
Volatile matter reduction	0.0120	0.065	Significant	Mycobacterium genus	0.0000	0.007	Significant
TCOD removal	0.4200	0.087	Not significant	Paludibacter genus	0.0001	0.037	Significant
Carbohydrate removal	0.1128	0.080	Not significant	Sedimentibacter genus	0.0000	0.022	Significant
Acetate concentration	0.4459	0.090	Not significant	Tetrasphaera genus	0.0000	0.018	Significant
Propionate concentration	0.0642	0.075	Significant	Anaerolineaceae family	0.0005	0.052	Significant
				W5 order	0.0000	0.015	Significant
SUBSTRATE				MULTIVARIATE ANALYSIS			
Carbohydrate concentration	0.0000	0.023	Significant	<u>Reactor 1 vs Reactor 2</u>			
Volatile solids concentration	0.0000	0.012	Significant	PERMANOVA bacteria	0.4453	0.088	Not significant
Total solids concentration	0.0000	0.013	Significant	PERMDISP bacteria	0.0909	0.077	Not significant
Protein concentration	0.0001	0.048	Significant	PERMANOVA archaea	0.1253	0.082	Not significant
sCOD concentration	0.0010	0.055	Significant	PERMDISP archaea	0.6996	0.095	Not significant
TCOD concentration	0.0030	0.060	Significant	<u>Phase 1 vs Phase 2 (pooled reactors 1 and 2)</u>			
Butyrate concentration	0.0033	0.062	Significant	PERMANOVA bacteria	0.0001	0.043	Significant
Acetate concentration	0.0081	0.063	Significant	PERMDISP bacteria	0.6037	0.092	Not significant
Priopionate concentration	0.0271	0.068†	Significant	PERMANOVA archaea	0.0001	0.042	Significant
P to C ratio	0.1108	0.078	Not significant	PERMDISP archaea	0.3632	0.085	Not significant
				<u>Reactor 3</u>			
				PERMANOVA	0.0470	0.073	Significant
				PERMDISP	0.7700	0.097	Not significant
				<u>Reactor 4</u>			
				PERMANOVA	0.0001	0.045	Significant
				PERMDISP	0.8800	0.098	Not significant

† Critical P value (i.e largest P where $P < P^*$). P value that was smaller than this critical P value was deemed significant

Table S2: Average abundance of bacteria, archaea, unclassified OTUs in pooled replicate reactors R1 and R2. The reactors received WRP1 substrate in Phase 1 and WRP2 substrate in Phase 2. The values represented the mean with standard deviation in parentheses.

Group (unit of measurement)	Phase 1 ¹	Phase 2 ²
Bacteria (%)	74 (7)	86 (2)
Archaea (%)	7 (2)	4 (1)
Unclassified (%)	19 (2)	10 (2)

¹ n: 16 samples

² n: 16 samples

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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 could be enhanced because a reduction in SRT would increase archaeal 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 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 in each phase neither hydrolytic activity nor the hydrolytic bacterial community differed significantly. Step-wise reduction of SRT did enhance volumetric methane production, although the archaeal community in general was largely unaffected. In conjunction with the increase in the quantity of the methane produced,

there was a decrease in the methane yield. However, the decrease was not statistically significant. The abundance of *Methanosaeta* decreased more noticeably at shorter SRTs, suggesting that it was more susceptible to changes in SRT. Overall, methanogenesis was not negatively impacted by the SRT reduction within the range used in this study. On the contrary, the increase in the amount of methane produced could be beneficial for real life application.

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 ($SRT \leq 8$ 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 $35 \pm 1^\circ\text{C}$ 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. For SRT 30 days, the amount of sludge discharged and substrate fed into the reactors were 140 mL and the average organic loading rate (OLR) was

1,589 $\pm$ 453 mg VS/L of reactor per day; for SRT 25 days, we discharged and fed 168 mL of sludge daily and the average OLR was 2,011 $\pm$ 286 mg VS/ L of reactor per day. Lastly, for SRT 15 days, we discharged 280 mL of sludge daily and the average OLR was 2,835 $\pm$ 214 mg VS/ L of reactor per day. SRT in this was 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 $\pm$ 2 days and the working temperature was 30 $\pm$ 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.

We operated the reactors using WRP 2 substrate for at least 6 months prior to the start of the study to ensure that the organisms in the reactors had been acclimatized to the substrate sludge since the seed and substrates originated from different WRPs.

2.4 Specific methanogenic activity tests

Serum bottles in activity tests were inoculated with sludge collected from reactors at the end of each phase. We did not supply any substrate to the sludge for up to 3 days prior to the start of the activity test. 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. Methane production was measured using pressure manometer with bar as unit. The pressure reading was normalized with inoculum volatile solids concentration.

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. Starting pressure in the serum bottles was maintained at 0 bar after the flushing to avoid presence of CO₂ that could also be used as substrate. Hence, although residual CO₂ might still be present in the bottles, the amount was insubstantial compared to the amount of acetate added. 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 Invitrogen'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 Invitrogen'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. PERMANOVA (permutational multivariate analysis of variance) analysis was 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 SRT was used as fixed factor. PERMDISP analysis was done to determine homogeneity of variance among our samples (Anderson 2006).

2.9 Diversity Measurement

Hill's first and second indexes (N_1 and N_2), were used to measure the diversity of archaea and bacteria species. Hill index emphasizes common species or taxa, which renders it

more robust against changes due to rare species (Haegeman et al. 2013). Although rare species often occupied a large portion of the community, appropriate measurement of these species has remained a challenge due to over and under sampling issues. Therefore, Hill number has been proposed as an appropriate diversity measure (Kang et al, 2016). Despite the aforementioned challenge, it has been suggested that rare taxa could indirectly affect bacteria community (Boeken and Shachak, 2006). Hence, I also used Shannon diversity index which is equally sensitive to common and rare species (Morris et al, 2014).

The diversity indices 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

Prior to the start of the analysis, I evaluated the performance of the two reactors in each cluster. Results showed that the performance in reactor 1 was statistically similar to reactor 2 (Table S2). Similarly, reactor 3 performance was statistically similar to reactor 4 (Table S2).

3.1 Effect of SRT on process performance in replicated reactors

3.1.1 Hydrolysis, acidogenesis/fermentation, and acetogenesis

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).

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.

This observation was supported by similar average VMR and total COD (TCOD) removals in both clusters (Table 1 and Table S1). Moreover, there was no significant difference in average protein and carbohydrate removals in the two clusters. These results suggested that SRT difference did not affect hydrolysis significantly.

Table 1: Process performance in replicate anaerobic reactors R1 to R4. R1 and R2 were grouped into Cluster 1 while R3 and R4 were grouped into Cluster 2. Values represent the mean with standard deviation in parentheses.

Parameter (unit of measurement)	Phase 1 ¹		Phase 2 ¹		Phase 3 ¹
	Cluster 1 ²	Cluster 2 ²	Cluster 1 ³	Cluster 2 ³	Cluster 1 ⁴
Volatile matter reduction (%)	46 (13)	46 (13)	52 (6)	50 (7)	45 (6)
Total COD removal (%)	53 (11)	51 (13)	58 (8)	53 (9)	55 (5)
Volumetric methane production (mL/day)	4,171 (1,428)	4,839 (1,551)	3,920 (925)	6,095 (796)	5,919 (843)
Methane yield (mL /g of VS added)	602 (212)	564 (210)	474 (137)	441 (89)	502 (100)
Effluent volatile acids (mg/L)	112 (23)	115 (34)	103 (11)	99 (14)	119 (13)
Effluent acetate (mg/L)	4 (4)	5 (6)	10 (4)	9(3)	9(6)
Effluent propionate (mg/L)	1 (1)	1(4)	0 (0)	0 (0)	0(0)
Protein removal (%)	54 (19)	52 (18)	60 (7)	55 (10)	62 (8)
Carbohydrate removal (%)	56 (21)	48 (17)	47 (20)	45 (15)	38(19)

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 n: 46 per cluster; ³ n: 20 per cluster; ⁴ n: 12 per cluster

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 Cluster 2 (Tables 1 and S1). Although lower TCOD removal in Cluster 2 might suggested a decrease in hydrolysis efficacy when the SRT was shortened, the decrease was not statistically significant.

In Phase 3, only Cluster 1 was operated at an SRT of 15 d. The process performance observed in this phase did not differ significantly from the process performance observed in Cluster 2 when it was operated at SRT 15 d suggesting that the result observed in Cluster 2 in Phase 2 was replicated in Cluster 1 in Phase 3 (Tables 1 and S1). 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).

Besides the effects on hydrolysis, we further investigated the possible effects of SRT reduction on the subsequent digestion steps namely fermentation and acetogenesis. In each phase, we did not observe conspicuous difference in the volatile acids concentration between the two clusters despite the different SRT applied (Table 1). Hence, we could conclude that SRT reduction in the range used in this study did not severely affect fermentation and acetogenesis.

3.1.2 Methanogenesis

We further 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). At each phase, effluent volatile acid concentrations in the two clusters remained similar although cluster that was operated at shorter SRT received more substrate than the cluster operated at longer SRT. This because SRT in our study was invariably linked

and equal to HRT. Therefore, more substrates were added when SRT was reduced. It could then be surmised that methanogenesis was not negatively impacted by the SRT reduction (Table 1 and S1). This observation was further supported by the lack of significant decrease in methane yield following the SRT reduction. Indeed, cluster that was operated at shorter SRT consistently produced more methane. 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). These results underlined the advantage of SRT reduction. It should be noted that hydrogen concentration remained very low throughout the study; hence, it was difficult to separate the hydrogen peaks from the peaks caused by the background noise or baseline fluctuations.

Lastly, Cluster 1 average methane production in Phase 3 was comparable to average methane production observed in Cluster 2 in Phase 2 showing that the results were replicable. Hence, an SRT reduction from 30 to 15 d significantly increased volumetric methane production without jeopardizing methane yield. This result also supported our hypothesis that a step-wise SRT reduction affected methanogenesis more than it did hydrolysis.

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 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. Similarly,

bacteria abundance in Cluster 2 did not change significantly following the SRT reduction to 15 d in Phase 2.

Table 2: Average abundance of bacteria, archaea, eukaryotes, and unclassified OTUs in replicate Clusters 1 and 2. Values represent the mean abundance with standard deviation in parentheses.

Domain (unit of measurement)	Phase 1 ¹		Phase 2 ¹		Phase 3 ¹
	Cluster 1 ²	Cluster 2 ²	Cluster 1 ³	Cluster 2 ³	Cluster 1 ⁴
Bacteria (%)	86 (2)	85 (3)	85 (2)	85 (3)	83 (3)
Archaea (%)	4 (1)	5 (2)	3 (2)	4 (1)	4 (2)
Eukaryote and unclassified (%)	10 (2)	10 (3)	11 (2)	12 (2)	13 (3)

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 n: 16 per cluster; ³ n: 8 per cluster; ⁴ n: 8 per cluster

In all phases, bacteria community 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 distinctly affected by a reduction in SRT.

Figure 2: Relative abundance of phyla in each cluster. (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. Error bars represent standard deviation of the mean.

The notion that SRT was not a strong selector for bacteria communities' assembly was supported by the results of PERMANOVA analysis of the bacteria species in the two clusters (Table 3 and S1). Although corrected p-values indicated significant difference between bacteria communities in Phases 1 and 2, small F values indicated that the effects of the factor (i.e SRT) were weak. Moreover, the difference in Cluster 2 might also be caused by the heterogeneous dispersion in this cluster communities in Phases 1 and 2 as shown by the significant p-value from PERMDISP analysis (Table 3 and S1). From our results so far, we could deduce that neither the reduction in SRT nor other factors such as fluctuations in substrate characteristics and temporal drifts severely affected the reactors bacterial communities.

Lastly, PERMANOVA analysis of bacteria in Cluster 1 in Phase 3 and Cluster 2 in Phase 2 also yielded small Pseudo-F value (Table 3). Hence, the two communities were not significantly different and it could be surmised that the trends observed in the bacterial community in Cluster 2 when it was operated at 15 d SRT (Phase 2) 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 species diversity. In Cluster 1, shortening the SRT from 30 to 15 d did not seem to significantly affect the bacterial diversities (Table 4). For Cluster 2, SRT reduction from 25 to 15 d caused a more significant change in the bacterial diversity. However, the difference did not seem to be too conspicuous. These results suggested that step-wise SRT reduction from 30 to 15 d did not severely affect bacterial diversity.

Table 3: Multivariate analysis of archaeal and bacterial species. Top section referred to the analysis in each Cluster across different Phases while bottom section was for the analysis between the two Clusters 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. *P* values listed in this table were uncorrected.

Within each cluster across different Phase						
Group	Cluster	Phase	PERMANOVA		PERMIDSP	
			P	Pseudo-F	P	F
Bacteria	1	1 and 2	0.0005	2	0.6	0.3
		2 and 3	0.2	1	0.9	0.007
	2	1 and 2	0.0003	2	0.0006	10
Archaea	1	1 and 2	0.07	3	0.2	0.7
		2 and 3	0.05	2	0.6	0.8
	2	1 and 2	0.3	1	0.4	0.8
Between clusters in each phase						
	1 and 2	1	0.0007	2	0.0001	19
Bacteria	1 and 2	2	0.06	1	0.1	4
	1 and 2	3 ¹	0.01	1	0.2	2
	1 and 2	1	0.1	2	0.9	0.007
Archaea	1 and 2	2	0.2	2	0.8	0.04
	1 and 2	3 ¹	0.7	0.5	0.6	0.6

¹ Comparison was done between Cluster 1 in Phase 3 and Cluster 2 in Phase 2.

Table 4: Average diversity indices values for archaeal and bacterial OTUs. Results were generated using PRIMER 6 software. The raw count data was standardized based on total counts per sample prior to diversity analysis. The values was mean with standard deviation in parentheses

Group	Cluster	Phase 1 ^{1,2}			Phase 2 ^{1,3}			Phase 3 ^{1,4}		
		N1	N2	H'	N1	N2	H'	N1	N2	H'
Bacteria	1	28 (1)	14 (1)	3(0.2)	28 (1)	14 (1)	3(0.2)	30 (5)	15(6)	3(0.1)
	2	38 (5)	24 (3)	4 (0.1)	33 (7)	19 (3)	3 (0.2)			
Archaea	1	3 (1)	3 (1)	1 (0.4)	3 (1)	3(1)	1 (1)	3 (1)	3 (1)	1(0.5)
	2	4 (1)	3 (1)	1 (0.2)	4 (1)	3 (1)	1 (0.4)			

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 n: 16 per cluster; ³ n: 8 per cluster; ⁴ n: 8 per cluster

3.2.2 Comparison of bacteria 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).

Figure 3: Relative abundance of phyla in each phase. ●, indicated Cluster 1 which consisted of R1 and R2; ○, Cluster 2 which consisted of R3 and R4. Graph (A) was Phase 1 where Cluster 1 was

operated at an SRT of 30 d, while Cluster 2 was operated at 25 d. Graph (B), Phase 2 where the SRT was reduced to 25 d in Cluster 1 and to 15 d in Cluster 2. Error bars represent standard deviation of the mean.

PERMANOVA analysis of bacterial abundance in the two clusters in Phase 1 yielded a significant p-value (Table S1). However, small Pseudo-F value (2) indicated that the p-value significance was minimal (Table 3). The significant 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 (Table S1), which allowed us to reject the null hypothesis of homogenous dispersion in the bacterial communities of the two clusters. In conclusion, different SRTs had no perceptible effect on bacteria.

In Phase 2, no significant effect of SRT difference was observed in microbial communities in the two clusters, even when the magnitude of SRT difference was increased to 10 d. The p-value from PERMANOVA was not profoundly significant since the p value is the same as the critical value after B-H correction (Table S1). Moreover, small Pseudo-F value (2) again suggested that the effects of the factor (i.e SRT difference) was minimal (Table 3). The dispersion of the two communities was also homogenous as shown by PERMDISP p value (Table S1). These results further showed that step-wise SRT reduction did not cause any noticeable effects on bacterial communities.

Looking into different bacteria that were present in the sample, we detected some that were known to be important in AD (Table 5). The bacteria community was dominated by family *Anaerolineaceae* from the phylum *Chloroflexi*. Members of this family have been identified as hydrolytic bacteria. Another dominant bacteria group was W5 order within the *Spirochaetes* phylum. Members of this order included putative protein degrader (Pelletier et al. 2008). We also observed proteolytic genera *Sedimenbacteres*, *Bacillus*, and *Clostridium* from the *Firmicutes* phylum in our samples (Nelson et al. 2011). *Cloacibacillus* (from *Synergistetes*)

was another hydrolytic genus that used protein. Besides protein users, some carbohydrate users were also present in our samples such as members of the genus *Rhizobiales* from *Proteobacteria* phylum (Xia et al. 2015). Some genera were also involved in the degradation of both proteins and carbohydrates such as *Leptolinea* (*Chloroflexi*) and *Clostridium* (*Firmicutes*) (Narihiro and Sekiguchi 2007, Rui et al. 2015).

Table 5: Average relative abundance of selected bacteria in replicated Clusters 1 and 2. The values represent mean with standard deviation in parentheses.

Group (Taxonomic classification)	Phase 1 ^{1,2}		Phase 2 ^{1,3}		Phase 3 ^{1,4}
	Cluster 1	Cluster 2	Cluster 1	Cluster 2	Cluster 1
Hydrolytic/Fermentative/Acetogenic					
Anaerolineaceae (Family)	14 (2)	12 (3)	13(2)	16(3)	13 (2)
Bacillus(Genus)	0.4 (1)	0.3 (0.4)	0.1 (0.3)	0.3 (0.5)	0 (0)
Bifidobacterium(Genus)	0.1 (0.2)	0.1 (0.3)	0.1 (0.2)	0 (0.1)	0.1 (0.2)
Chloroflexi (Phylum)	0.2 (0.3)	0.4 (1)	0.3 (0.3)	0.4 (1)	0.2 (0.3)
Cloacibacillus (Genus)	0.1 (0.2)	0.2 (0.3)	0.3 (0.3)	0 (0)	0 (0)
Clostridium (Genus)	0.2 (0.5)	0.3 (0.4)	0.1 (0.3)	0.1 (0.3)	0.2 (0.4)
Leptolinea (Genus)	0.2 (0.3)	1 (1)	2 (1)	1 (1)	1 (1)
Rhizobiales(Order)	1 (1)	1 (1)	1 (0.5)	1 (1)	1 (1)
Sedimentibacter(Genus)	2 (1)	1 (1)	1(1)	1 (1)	1 (1)
Spirochaetaceae (Family)	1 (1)	1 (1)	2 (1)	2 (1)	2 (1)
Streptococcus(Genus)	1(1)	1 (1)	1 (0.4)	1 (1)	1 (0.4)
W5(Order)	19 (5)	8 (3)	21 (6)	16 (4)	21 (6)
Syntrophs					
Smithella(Genus)	1 (1)	1 (2)	1 (1)	1 (1)	0.2 (0.4)
Synergistaceae (Family)	2 (1)	1 (1)	1 (1)	1 (1)	1 (1)

Syntrophaceae(Family)	0.3 (0.5)	0.2 (0.4)	1 (0.4)	0.3 (0.3)	0.4(1)
Syntrophobacter(Genus)	0.3 (1)	0.4 (1)	0.1 (0.3)	0.3 (0.3)	0.2 (0.4)
Syntrophomonas(Genus)	1 (1)	1 (1)	1 (1)	0.4 (1)	1 (1)
Syntrophorhabdus(Genus)	0.4 (1)	1 (1)	0.3 (1)	0.2 (0.2)	0.3 (0.4)

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 n: 16 per cluster; ³ n: 8 per cluster; ⁴ n: 8 per cluster

Many of the aforementioned bacteria were simultaneously involved in fermentation and acetogenesis processes. *Cloacibacillus* (from *Synergistetes*) and *Clostridium* (from *Firmicutes*) were examples of hydrolytic genera present in our sludge that were also involved in fermentation and acetogenesis (Ganesan et al. 2008, Rui et al. 2015, Ryan et al. 2010). Other fermentative and acetogenic genera in our samples were *Bifidobacterium* (*Actinobacteria*) and *Streptococcus* (*Firmicutes*) (Kosaric and Blaszczyk 1992).

Moreover, we also observed the presence of different groups of syntrophic bacteria in our reactors. *Syntrophobacter* genus and *Synergistaceae* family which included syntrophic genus *Synergistia* were detected (Werner et al. 2011). Other syntrophs detected included members of genera *Smithella*, *Syntrophorabdus*, *Syntrophobacter* (*Proteobacteria*), and *Syntrophomonas* (*Firmicutes*). These genera were capable of degrading various fatty acids (Ariesyady et al. 2007, McInerney et al. 1981, Qiu et al. 2008).

Despite the varied groups of bacteria detected, their abundances were not severely affected by the reduction in SRT (Table 5). This could explain the lack of conspicuous effects of SRT reduction on the bacteria-driven processes such as hydrolysis.

Moreover, we also compared the diversity of the two clusters in each phase. In Phase 1, the diversities of bacterial species in Cluster 1 was significantly different than Cluster 2 (Table 4 and S1). However, the difference might be caused by the heterogenous dispersion in the two communities as shown by the significant p-value yielded from PERMDISP analysis (Table 3) since SRT difference between the two clusters was only 5 d. This observation was supported by the lack of significant difference observed in Phase 2 where SRT difference between the two Clusters were increased to 10 d, confirming that SRT did not cause any major adverse effect on bacteria community (Table 4).

3.2.3 Comparison of Archaea community in each cluster across different phases

We hypothesized that archaea would be impacted more prominently than bacteria. However, the total abundance of archaea in different phases indicated that the changes when SRT was reduced were not significant (Table 2). *Methanosaeta* genus 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).

Figure 4: Relative abundance of archaea genus with respect to whole archaea community in each cluster. 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.

We further analyzed the abundance of archaeal species with respect to overall archaea only. Archaea communities remain largely unaffected by the SRT reduction across different phases in both Clusters 1 and 2 as shown by the results of PERMANOVA and PERMDISP analysis (Table 3 and S1). This indicated that archaea were not significantly impacted by a reduction in SRT. Other factors such as variations in substrate characteristics and temporal drifts could also be ruled out.

Although not significant, *Methanosaeta* genus abundance in Cluster 1 decreased from $73 \pm 25\%$ to $53 \pm 30\%$ when its SRT was reduced to 15 d in Phase 3, suggesting that this methanogen was more susceptible to changes in physiochemical conditions due to a reduction in SRT (Figure 4A). Decreased abundance in *Methanosaeta* genus from $70 \pm 15\%$ to $57 \pm 9\%$ was also observed in Cluster 2 when its SRT was reduced from 25 to 15 d in Phase 2 (Figure 4B). Lack of significant difference observed across different phases showed that factors such as temporal drifts and substrate fluctuations did not significantly affect the community.

In addition to archaea abundance and composition, we also inspected the effects of step-wise SRT reduction on archaea diversity (Table 4). When the SRT of Cluster 1 was reduced to 25 d, the average diversity indices did not change significantly (Table 4 and S1). These values remained similar when the SRT was further reduced to 15 d in Phase 3. Similar diversity indices values were observed in Cluster 2 when its SRT was reduced from 25 d in Phase 1 to 15 d in Phase 2 (Table 4). These results suggested that archaea diversity was not affected by SRT reduction and other factors such as temporal variations and substrate fluctuations.

3.2.4 Comparison of Archaea community between clusters in each phase

Likewise, we also compared archaeal 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, the average abundance of *Methanospirillum* was 20% higher in Cluster 2, suggesting that this genus was preferentially selected over *Methanosaeta* at a shorter SRT. However, PERMANOVA analysis showed that archaea communities in the two clusters remained statistically similar despite the difference in the SRT applied (Table 3 and S1). PERMANOVA analysis of archaea community in Cluster 1 reactors in Phase 3 and Cluster 2 reactors in Phase 2 yielded an insignificant p value (Table 3 and S1). Hence, the trends observed in Cluster 2 for archaea communities in Phase 2 (SRT= 15 d) were also observed in Cluster 1 when its SRT was reduced to 15 d in Phase 3.

Figure 5: Relative abundance of archaea genus with respect to overall archaea community in each phase. Graph (A) referred to archaea in Phase 1 and (B) Phase 2. ●, 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.

When we compared archaeal species diversity in the two Clusters at each phase, diversity in Cluster 1 was significantly different than diversity in Cluster 2 in Phase 1 (Tables 4 and S1). This significant difference might be because archaea was a small group of which only 3-4 genera were notably identified and some of these genera such as *Methanobacterium* and *Methanobrevibacter* were only present in some of the samples. This led to high fluctuations in average relative abundances of these genera. The difference between the two clusters was insignificant in Phase 2 when the SRT difference between the two Clusters were increased to 10 d (Table 4). Thus, similar to bacteria, archaea community was not severely affected by a reduction in SRT.

Our results showed that archaea were not affected by a SRT reduction to 15 d although volumetric methane 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 were discussed in section 3.3.

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 0.01 ± 0.007 bar methane /gram of VS_{seed} on the second day compared to 0.03 ± 0.007 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.

Figure 6: Biogas production from acetoclastic methanogenic activity test. Serum bottles were inoculated with sludge taken from (A) Cluster 1 at the end of Phases 2 and 3 and (B) Cluster 2 at the end of Phase 2. 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 SRT₂₅ where only duplicate bottles per reactor were used. Error bar represented standard deviation of the mean between reactors in each clusters.

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% CH₄ to 30-40% CO₂) 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.

Methanosaeta and *Methanospirillum* were the genera that underwent most changes. A lower SRT has been reported as detrimental to acetoclastic methanogens such as *Methanosaeta* (Demirel and Scherer 2008). However, *Methanosaeta* remained the most dominant methanogen due to the low acetate concentration. This methanogen is known to have a higher acetate affinity and hence proliferates better in lower acetate conditions (i.e less than 10 mg/L of acetate) (Demirel and Scherer 2008, Jetten et al. 1992).

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). Concentration of H₂ in our samples were consistently low; hence, it was difficult to separate the actual peaks from the peaks caused by the baseline fluctuations or background noise. However, 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. However, it is important to conduct a thorough pilot-scale study prior to applying this method to full-scale digesters.

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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 $35 \pm 1^\circ\text{C}$ 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, an observation that was further supported by lack of noticeable changes on hydrolytic bacteria profiles. 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 *Methanosaeta* 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 than R2 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. Overall, the results of this study highlight the possibility of using pulsed downward temperature cycling to enhance digestion methanogenic community. 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). Indeed, it had been suggested that anaerobic digester temperature changes should not exceed 1°C/ day (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).

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 $35 \pm 1^\circ\text{C}$ 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. We planned the perturbations on Days 49 and 51 to coincide with the need to switch off the heated water circulation to R1 for maintenance purpose. On Day 49, the heating water flow to R1 was switched off and temperature dropped to $25 \pm 1^\circ\text{C}$. The temperature was restored to $35 \pm 1^\circ\text{C}$ 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 $35 \pm 1^\circ\text{C}$. After one SRT had elapsed, an unplanned perturbation occurred to both R1 and R2 on Day 97. Since this perturbation was similar to the perturbation applied to R1 on Day 49, we incorporated this event to investigate if R1 had developed resistance toward similar perturbations.

The temperature in R2 was kept at $35 \pm 1^\circ\text{C}$ until Day 97 where it dropped to $25 \pm 1^\circ\text{C}$. The temperature was restored the next day and kept at $35 \pm 1^\circ\text{C}$ 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- μ m 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 FastDNATM Spin Kit for Soil and FastPrepTM Instrument from MP Biomedicals (Santa Ana, CA, USA). Numbers of cell lysing steps using the FastPrepTM instrument were increased to four times, each lasting for 40 seconds. The extracted DNA was further purified using DNA Clean and ConcentratorTM-10 purification kit from Zymo Research (Irvine, CA, USA). The quality and concentration of the extracted and purified DNA was determined using a NanoDropTM 2000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA) and an QbitTM 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 Invitrogen'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 Invitrogen'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, Mann-Whitney 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 Mann-Whitney test. 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. PERMANOVA (permutational multivariate analysis of variance) was 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).

We corrected the p-values obtained from the aforementioned tests using Benjamini-Hochberg technique to control the false discovery rate. This was done to minimize the possibility of obtaining significant p-value ($p < 0.05$) due to chance (Benjamini and Hochberg 1995, Spector 1956). Benjamini-Hochberg method was selected over Bonferoni correction due to the latter tendency to produce very high numbers of false negative results (Spector 1956).

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.

Figure 1: Effluent volatile solids concentrations from reactors R1 and R2. The operating temperature was kept at $35 \pm 1^\circ\text{C}$ except for Days 49, 51, and 97 when the temperature was rapidly reduced to $25 \pm 1^\circ\text{C}$. 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.

We hypothesized that the transient perturbations would not affect hydrolysis, a common rate-limiting step in anaerobic digestion. Prior to the temperature perturbation, averages

volatile matter reduction (VMR) calculated from mass balance equation and total COD (TCOD) removal of the two reactors were highly similar (Tables 1 and S1). These averages remained similar in Phase 2, supporting the observation that hydrolysis was not affected by the temperature perturbation (Tables 1 and S1). 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.

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

Parameter (unit of measurement)	Before temperature perturbations (Phase 1) ¹		After perturbations on days 49 and 51 (Phase 2) ²		After perturbation on day 97 (Phase 3) ³	
	Reactor 1 (R1)	Reactor 2 (R2)	Reactor 1 (R1)	Reactor 2 (R2)	Reactor 1 (R1)	Reactor 2 (R2)
Volatile matter reduction (%)	65 (11)	65 (10)	47 (18)	46(15)	48(8)	47 (10)
Total COD removal (%)	52 (24)	51 (24)	61 (14)	62(17)	67 (12)	65 (14)
Methane production (mL/day)	716 (573)	1,247 (640)	3,663 (1,342)	3,547 (1,448)	3,259 (1,154)	3,150 (699)

¹ n = 11 per reactor; ² n = 9 per reactor;

The concentrations of propionate remained similar between the two reactors throughout the test period suggesting that temperature perturbations did not significantly affect fermentation and acetogenesis processes (Figure 2). The concentrations of butyrate were less than 10 mg/L throughout the test period. It should be noted that the increase in propionate in Days 77-92 could not be attributed to temperature perturbations since this increase occurred in both the disturbed (R1) and undisturbed (R2) reactors. These observations were supported by

the profiles of the known hydrolytic, fermentative and acetogenic bacteria which would be discussed at the later section.

Figure 2: Volatile acids 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. ● denoted acetate concentration in R1; ○, propionate concentration in R1; ▼, acetate in R2; △, propionate in R2

We also looked into the possible effects of these transient perturbations on methanogenesis, another common rate limiting step in anaerobic digestion. Average methane production in R1 before temperature perturbation was statistically similar to R2 (Table 1 and S1). It should be noted that the marked increase in methane production in Phase 2 was caused by a different gas collection method that was used from this phase 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 throughout the three phases (volumetric methane to carbon dioxide ratio was 60-70% to 30-40%).

Although methane production was 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 an increase in acetate concentrations in R1 from less than 10 mg/L to almost 1,800 mg/L. The elevated acetate concentrations persisted from Day 58 to Day 99. Hence although the pulse perturbations were short, they were strong enough to affect acetoclastic methanogenesis despite similar volumetric methane production. This observation was confirmed by the temporal profile of archaea abundance, which would 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 compared the performance of R2 after it received one cycle of temperature perturbation 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 and Table S1). 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 because there were no longer unperturbed reactors to serve as control in this phase. VMR and TCOD removal in Phase 2 did not differ significantly than in Phase 3. Moreover, fluctuations in R2 in Phase 3 were less severe than in R1 in Phase 2, possibly because it only received one cycle of temperature perturbation while R1 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 effluent solids fluctuations in R1 than in R2 after R1 received additional 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 (Table 1 and S1). These results further supported our previous observation that pulse temperature perturbations did not affect the hydrolysis process.

Following perturbation on Day 97 in both reactors, the average methane 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 (Table 1 and S1). Similarly, additional temperature perturbation did not significantly affect R1 biogas production in Phase 3.

Although volumetric methane production and biogas composition were not affected by the temperature perturbation on Day 97, acetate 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.

Acetate concentration in R1 transiently increased to 586 mg/L following additional perturbation on Day 97, smaller than the increase observed in R2 despite both being subjected to same perturbation. It was possible that the community in R1 had become more resistant towards similar perturbations; hence, the smaller increase than R2. 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 would be discussed in the next section.

We acknowledged the short duration of our study. However, the performance of the two reactors was similar before temperature perturbations were applied. Additionally, prior to the start of the study, the reactors had been operated stably for 6 months using feed collected from a different WRP. The performance of the two reactors was also highly similar throughout these 6 months period (Figure S3). Differences in some of the physiochemical properties of the two reactors were only observed after the pulse temperature perturbations. Hence, this could lend support to our observation that temperature perturbations did affect anaerobic digestion in our study.

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 2). The p value from PERMANOVA in Phase 2 indicated that although the archaea community in R1 started to deviate from R2, the difference was not significant (Table S1). This might be caused by the delayed response of archaea to the perturbations since PERMANOVA 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). These results showed that temperature impacted the archaea community. After the perturbation on Day 97, the archaea community in R1 diverged more noticeably from R2 (Table S1). PERMDISP p-value showed homogenous dispersion in the two archaea communities, confirming that the differences 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 S1A 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.

Table 2: Average abundance of bacteria, archaea, and unclassified OTUs in R1 and R2. The values represent the mean with standard deviation in parentheses.

Microbial domain (unit of measurement)	Before temperature perturbations (Phase 1) ¹		After perturbations on days 49 and 51 (Phase 2) ²		After perturbation on Day 97 (Phase 3) ³	
	R1	R2	R1	R2	R1	R2
Bacteria (%)	79 (2)	77 (2)	79 (3)	77 (2)	82 (2)	77 (3)
Archaea (%)	5 (1)	6 (1)	4 (2)	5 (2)	3 (1)	3 (0)
Unclassified (%)	16 (1)	18 (2)	16 (2)	18 (2)	15 (2)	20 (3)

¹n= 4 samples per reactor; ²n = 5 samples per reactor; ³ n= 5 samples per reactor

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 S1A). 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 S1A). 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 S1B). 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 3A). 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).

Figure 3: 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 $35 \pm 1^\circ\text{C}$ for all other days.

Panel (A) *Methanosaeta*, (B) *Methanobacterium*, (C) *Methanospirillum*, (D) *Methanolinea*, (E) *Methanosarcina*, and (F) *Methanoculleus*.

The decrease in *Methanosaeta* coincided with an increase in the abundance of other methanogens such as *Methanobacterium* and *Methanospirillum* (Figure 3B 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 3D). Moreover, the relative abundances of previously non-detected *Methanosarcina* and *Methanoculleus* in R1 increased to almost 20% and 10%, respectively (Figures 3E and F). These genera were not detected in R2, suggesting that they required more rigorous perturbations.

Due to the short duration of the study, we could not discount the possibility that the reactors were still in the dynamic stage. However, the communities in the reactors were similar prior to the perturbations. Differences in some of the methanogens were only observed after temperature perturbations were applied. Hence, this could lend support to our observation that pulse temperature perturbations could significantly affect archaea.

3.4. Bacteria response to pulse temperature perturbation

Since bacteria played important roles in the first three steps of digestion, we also investigated the effects of the perturbations on bacteria. Our analysis showed that the overall bacteria abundance was not affected as severely by temperature perturbations as archaea (Table 2). Temporal relative abundance of bacteria phyla further showed lack of effects from

temperature perturbations (Figures S2A and B). This observation was supported by the results of PERMANOVA analysis of bacteria OTUs throughout all three phases (Table S1).

From the phylum *Firmicutes*, some important bacteria detected in our samples were *Acetivibrio*, *Acetobacterium*, *Clostridium*, *Corpothemobacter*, *Ruminococcus*, and *Sedimenbacter*. These bacteria were involved in hydrolysis of different organics materials in anaerobic digestion (Nelson et al. 2011, Tezel et al. 2011). Genera *Bacteroidetes*, *Paludibacter*, and *Parabacteroides* were the hydrolytic and fermentative bacteria from phylum *Bacteroidetes* detected in our samples (Nelson et al. 2011). One of the hydrolytic bacteria genus detected in the *Proteobacteria* phylum was *Acinetobacter*, a facultative bacteria who could consume the residual oxygen to maintain anoxic condition (Rui et al. 2015). We also detected genus *Bifidobacterium* from the phylum *Actinobacteria* and genera *Aminobacterium* and *Cloacibacillus* from phylum *Synergistetes* which included protein consumers (Ganesan et al. 2008, Nelson et al. 2011). Most of the aforementioned bacteria were also involved in the subsequent fermentation step. The relative abundance of these genera did not change noticeably throughout the phases suggesting that they were not strongly affected by the pulse perturbations (Table 4). Similarly, no significant change was observed in the relative abundances of families *Anaerolineaceae* (from the phylum *Chloroflexi*), *Eubacteriaceae* (*Firmicutes*) and *Propionibacteriaceae* (*Actinobacteria*) after temperature perturbations. Some members of these families have also been identified as hydrolytic and fermentative bacteria. Our result thus far confirmed our previous observation that hydrolysis and fermentation were not affected by the pulse temperature perturbations.

Table 4: Average relative abundances (in percentage) of selected bacteria genera in reactors R1 and R2. The values represent the mean with standard deviation in parentheses.

Genus (unless otherwise stated)	R1			R2		
	Phase 1 ¹	Phase 2 ²	Phase 3 ³	Phase 1 ¹	Phase 2 ²	Phase 3 ³
Propionibacteriaceae (Family)	0.7(0.6)	0.9 (0.5)	0.4 (0.3)	0.3 (0.7)	0.6 (0.4)	0.9 (0.3)
Bacteroides	0	0.03 (0.1)	0.1 (0.1)	0	0	0.1 (0.1)
Paludibacter	0	0.1 (0.1)	0.2 (0.2)	0	0.03 (0.1)	0.2 (0.2)
Parabacteroides	0	0.1 (0.2)	0.03 (0.1)	0.0	0.02 (0.1)	0
Anaerolineaceae (Family)	16 (2)	16 (2)	17 (1)	16 (2)	19 (2)	18(2)
Leptolinea	1.(0.8)	0.8 (1)	0.3 (0.2)	1 (0.8)	1 (0.7)	0.6 (0.3)
Levilinea	0.1 (0.2)	0	0	0	0	0
Clostridiales (Order)	0	0.4 (0.5)	0.7 (0.4)	0	0.3 (0.3)	0.6 (0.4)
Clostridium sensu stricto 1	0.1 (0.2)	0.1 (0.2)	0.3(0.2)	0.1 (0.2)	0.1 (0.1)	0.4 (0.2)
Clostridium sensu stricto 11	0	0.04(0.1)	0.03 (0.1)	0	0	0
Coprothermobacter	0	0	0	0	0	0.03 (0.1)
Ruminococcus	0.2 (0.4)	0.2 (0.3)	0	0.3 (0.5)	0.1 (0.1)	0
Sedimentibacter	0	0.7 (0.5)	0.9 (0.5)	0.1 (0.2)	0.3 (0.3)	0.6 (0.5)
Planctomyce	0	0	0.2(0.2)	0.2 (0.3)	0.0	0.1 (0.1)
Planctomycetaceae (Family)	0.1 (0.3)	0	0.03(0.1)	0.2 (0.5)	0.03 (0.1)	0.1 (0.1)
Acinetobacter	0.1 (0.2)	0.07 (0.1)	0.0	0	0.03 (0.1)	0.03 (0.1)
Aminobacterium	0	0.1 (0.1)	0.2 (0.2)	0	0	0.04 (0.1)
Cloacibacillus	0	0.1 (0.2)	0.0	0.1 (0.2)	0.1 (0.2)	0.1 (0.2)

¹ n = 4 samples per reactor; ² n = 5 samples per reactor; ³ n = 5 samples per reactor

Another important step in anaerobic digestion is acetogenesis where different fatty acids were converted into acetate by acetogens which include the syntrophic partners of hydrogenotrophic methanogens. Some of the known syntrophs detected in our samples were members of the genera *Smithella*, *Syntrophomonas*, *Desulfovibrio*, *Syntrophorabdos*, and *Syntrophus* (Ariesyady et al. 2007, McInerney et al. 1981, Mountfort et al. 1984, Qiu et al. 2008, Zhang et al. 2004). Prior to the temperature perturbations, relative abundances of these genera were similar in the two reactors (Table 5). Although relative abundance of these genera did not differ significantly in Phase 2, abundance of *Smithella* in the disturbed reactor R1 was lower than in R2, suggesting that this genus was more prone to temperature disturbance. The

abundance of this genus decreased further in R1 after R1 received additional perturbation in Phase 3. A decrease in *Smithella* genus abundance was also observed in R2 after it received its first perturbation in Phase 3, further suggesting that this genus was more susceptible to temperature fluctuations (Table 5). The decrease in *Smithella* genus coincided with the increase in the *Syntrophomonas* genus, indicating the latter lower susceptibility to temperature perturbations.

Table 5: Average abundances of syntrophic bacteria genera in reactors R1 and R2. The values represent the mean with standard deviation in parentheses

Genus (unless stated otherwise)	Reactor R1			Reactor R2		
	Phase 1 ¹	Phase 2 ²	Phase 3 ³	Phase 1 ¹	Phase 2 ²	Phase 3 ³
<i>Smithella</i>	3 (1)	2 (0.8)	0.6 (1)	2 (0.8)	3 (0.5)	2 (0.8)
<i>Syntrophomonas</i>	1 (0.7)	1 (0.8)	3 (1)	0.4(0.3)	0.7 (0.4)	2 (0.9)
<i>Desulfovibrio</i>	0.1 (0.2)	0.1 (0.1)	0.1 (0.1)	0 (0)	0 (0.1)	0.2 (0.1)
<i>Syntrophobacter</i>	0.1 (0.2)	0.3 (0.3)	0.4 (0.2)	0.3 (0.3)	0.3(0.2)	0.2 (0.2)
<i>Syntrophorhabdus</i>	0.8 (1)	0.5 (0)	0 (0)	1 (0.2)	0.3 (0.3)	0.2 (0.2)
<i>Syntrophus</i>	0.2 (0.3)	0 (0)	0 (0)	0.2 (0.4)	0.2 (0.3)	0 (0.1)

¹ n = 4 samples per reactor; ² n= 5 samples per reactor; ³ n = 5 samples per reactor

4. DISCUSSION

In this study, short pulse perturbations were used to determine if they were strong enough to affect anaerobic digestion. Results indicated 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, Záborská 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, methane production and methane yield following perturbation events remained the same, suggesting that methanogenesis was not adversely affected by the pulse perturbations either; hence no cascading effect was observed.

Additionally, the accumulation of acetic acids could be an added benefit of subjecting the reactor to transient pulse perturbation since no discernable effects on methane production was observed. The practice of harvesting organics such as volatile acids from digested sludge has been gaining traction due to these organics high monetary value (Agler et al. 2011). However, this practice is typically done in expense of methane production since methanogenesis needs to be suppressed to allow for the accumulation of the volatile acids (Vanwonterghem et al. 2015). Our results showed that by subjecting the reactors to a gentler perturbation (i.e intermittent short temperature cycling), we might be able to harvest the volatile acids without sacrificing the methane production. A more detailed study to explore the feasibility of applying temperature cycling to harvest organic acids without jeopardizing methane production is still needed.

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 genus was the *Methanosaeta* 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.

Delayed response by some organisms following disturbances to their environment has been widely reported in ecological study. These delays could lead to local extinction, a deterministic phenomenon often referred to as “extinction debt” which often occurred in species with long generation time (Kuussaari et al. 2009, Tilman et al. 1994). This could explain the delay in the decrease of *Methanosaeta*, a slow-growing methanogen, in our study. However, decrease in our study was directly compensated by the proliferation of other methanogens which ensured that the overall biogas and methane production were not affected. These results 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).

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 such as hydrogenotrophic methanogens 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*, 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). These bacteria were not significantly impacted by the temperature perturbation, and this could explain the lack of changes observed in the hydrolysis, fermentation, and acetogenesis after temperature perturbation. Bacteria were not affected as severely by temperature perturbations as archaea, possibly because there were considerably more bacteria than archaea. Moreover, multiple bacteria genera from different phyla could perform same functions, and this could lead to higher redundancy in terms of activity and make the bacteria community more resistant.

The bacteria community was highly diverse possibly due to the long SRT (that is, 30 days), which was much longer than the doubling times of many organisms (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 significant difference in the abundance of their syntrophic partners such as members of genera *Syntrophomonas*, *Syntrophobacter*, *Syntrophoradbus*, *Syntrophus*, and *Desulfovibrio* (Bryant et al. 1977). Of the syntrophic partners detected, only genera *Smithella* and *Syntrophomonas* showed more noticeable changes. Some members of genus *Smithella* were syntrophic propionate oxidizing bacteria and the decrease in this genus abundance after temperature perturbations in our study might be caused by the elevated amount of acetate in our sludge since presence of acetate could be detrimental to this genus (Liu et al. 1999). It had also been suggested that this genus was less tolerant to lower temperature (Gan et al. 2012). This genus decrease coincided with slight increase in the average abundance of *Syntrophobacter*, a genus which contained different syntrophic species such as *Syntrophobacter sulfatireducens* which had been reported to negatively correlate with *Smithella* (Ban et al. 2015). Previous study had also suggested that this syntrophic propionate user was low-temperature tolerant (Ban et al. 2013). Abundance of *Syntrophomonas* genus also increased noticeably. This genus could proliferate in wider range of optimum temperature (Lin et al. 2016), an advantage that could render it less susceptible to temperature fluctuations. Despite these slight changes, we did not observe major changes observed following the temperature perturbation possibly due to the use of metagenomics data in this study. Hence, although the abundance of specific OTUs did not change, the taxa involved in syntrophic relationships with hydrogenotrophs could have become more active. Future work should include metatranscriptomic analysis to attest to this observation.

5. CONCLUSIONS

- Microbial community developed resistance toward similar perturbation.

- Short-term temperature pulses drove archaea community assembly in a mesophilic digester. Minimal perturbations selected for the more robust hydrogenotrophic methanogens, an attribute that can improve process stability in anaerobic digestion.
- Pulse temperature cycle opened up the possibility to harvest both methane and valuable intermediates such as VA from an anaerobic digestion
- Adopting intermittent pulse temperature reduction to improve digestion requires less energy than conventional pretreatment methods and potentially lowers operational costs.

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Figure S1: 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 S2: 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.

Figure S3: Effluent volatile solids concentrations in reactors R1 and R2. Day 0 referred to the start of this study. Prior to the start of the study, the reactors were fed with sewage sludge collected from different WRP. ● referred to reactor R1; ○, R2.

Table S1: *P* values from univariate and multivariate tests. Each of the *P* values was corrected using Benjamini-Hochberg (BH) method. For univariate test, *P* was originally calculated using Mann-Whitney test prior to correction. For multivariate analysis (PERMANOVA and PERMDISP), *P* values were generated using PRIMER 6 software with PERMANOVA +. *P** referred to values that had been corrected using BH procedure ($P^* = Q (i/m)$; where *i* = rank and *m* = total number of tests done; and *Q* = the chosen false discovery rate (10% in our case). We rejected the null hypothesis that the parameters in the two phases were similar when *P* was deemed significant.

Parameter	P	P* ³	Significance
Methane Production Phase 1 ¹	0.07	0.014	Not significant
Methane Production Phase 2 ²	0.9	0.076	Not significant
Methane Production Phase 3 ²	0.9	0.081	Not significant
TCOD Removal Phase 1 ²	0.8	0.057	Not significant
TCOD Removal Phase 2 ¹	0.9	0.071	Not significant
TCOD Removal Phase 3 ²	0.7	0.052	Not significant
VMR Phase 1 ²	0.9	0.062	Not significant
VMR Phase 2 ²	0.9	0.067	Not significant
VMR Phase 3 ²	0.7	0.048	Not significant
Microbial community analysis			
Archaea Permanova Phase 1 ¹	0.9	0.086	Not significant
Archaea Permanova Phase 2 ¹	0.2	0.029	Not significant
Archaea Permanova Phase 3 ¹	0.07	0.019	Not significant
Archaea Permdisp Phase 1 ¹	0.9	0.09	Not significant
Archaea Permdisp Phase 2 ¹	0.01	0.005	Not significant
Archaea Permdisp Phase 3 ¹	0.1	0.024	Not significant
Bacteria Permanova Phase 1 ¹	1	0.095	Not significant
Bacteria Permanova Phase 2 ¹	1	0.1	Not significant
Bacteria Permanova Phase 3 ¹	0.03	0.01	Not significant
Bacteria Permidsp Phase 1 ¹	0.6	0.043	Not significant
Bacteria Permidsp Phase 2 ¹	0.3	0.033	Not significant
Bacteria Permidsp Phase 3 ¹	0.5	0.038	Not significant

¹ Multivariate analysis; *n* = 4 samples per reactor for Phase 1; 5 samples per reactor for Phases 2 and 3.

² Univariate analysis; *n* = 11 samples per reactor for Phase 1; 9 samples per reactor for Phase 2; 8 samples per reactor for Phase 3

³ Null hypothesis that the two groups were similar was rejected when $P < P^*$ (i.e when P was significant)

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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 ($\text{SRT} > 8$ 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 ($\text{SRT} > 30$ 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 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 $1^{\circ}\text{C}/\text{day}$.

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.

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