Transitions of microbial communities in high-solids anaerobic digestion with percolate recirculation

By

Hok Nam Joey Ting

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Abstract

High-solids anaerobic digestion (HSAD) is a growingly popular strategy for recovery of biomethane from the organic fraction of municipal solid waste (OFMSW). The focus of this thesis study is to advance fundamental understanding and engineering performance of high-solids anaerobic digestion process with percolate recirculation.

The study presents a microbiological diagnosis of a mesophilic HSAD system with percolate recirculation. The results demonstrated a significant decrease in microbial diversity in both the solid digestate and the liquid percolate. Also, the digestate from the top and middle sections of the digester had similar diversity, whereas the digestate from the bottom of the tank had a slightly lower diversity. These results suggest that despite percolate recirculation, substrate gradients might have developed across the system. Archaeal communities showed shifts towards known hydrogenotrophic and ammonia-tolerant methanogens (genera *Methanocelleus*, *Methanolinea*, *Methanosarcina,* vadin CA11, etc.), which was a consequence of changing volatile fatty acids and increased ammonia-nitrogen levels over time. Compared to initial solid and liquid inoculum, the relative abundances of some bacteria (phyla *Proteobacteria and Firmicutes*) and archaea of the genus *Methanosarcina* changed between two phases in the opposite direction, indicating a shift of microbes between two phases.

Preface

Some of the findings presented in this thesis (Chapter 3) has been published as Ting, H.N.J.; Lin, L.; Cruz, R.B.; Chowdhury, B.; Karidio, I.; Zaman, H.; Dhar, B.R. (2020). Transitions of microbial communities in the solid and liquid phases during high-solids anaerobic digestion of organic fraction of municipal solid waste, Bioresource Technology, 317, 123951. Hok Nam Joey Ting was responsible for running the experiment, and data analysis. Long Lin was also responsible for aiding in the experiment and data analysis. Raul Bello Cruz and Bappi Chowdhurry both aided in the experiment and data analysis as well. Dr. Ibrahim Karidio and Hamid Zaman were responsible for project administration, while Bipro Ranjan Dhar was responsible for the conceptualization, funding and supervising the project. All the authors contributed to the preparation of the manuscript.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Anaerobic Digestion (AD)

Chemical oxygen demand (COD)

Continuously stirred tank reactor (CSTR)

Edmonton Waste Management Centre (EWMC)

Free ammonia nitrogen (FAN)

Food waste (FW)

High-solids anaerobic digestion (HSAD)

Municipal solid waste (MSW)

Operational taxonomical unit (OTU)

Organic fraction of municipal solid waste (OFMSW)

Organic loading rate (OLR)

Particulate chemical oxygen demand (PCOD)

Principal coordinate analysis (PCoA)

Quantitative polymerase chain reaction (qPCR)

Soluble chemical oxygen demand (SCOD)

Syntrophic acetate oxidation (SAO)

Syntrophic acetate oxidation with hydrogenotrophic methanogenesis (SAO-HM)

Syntrophic acetate oxidizing bacteria (SAOB)

Substrate to inoculum ratio (S/I)

Total ammonia nitrogen (TAN)

Total chemical oxygen demand (TCOD)

Total solids (TS)

Volatile fatty acids (VFAs)

Volatile solids (VS)

Chapter 1 Introduction

1.1 Background

Solid waste management is an increasingly important topic in society. From 2002 to 2016, the amount of solid waste collected in Canada increased by 11%, and in 2018 the amount of residential waste collected for disposal totaled 10.8 million tonnes (Environment and Climate Change Canada, 2018; Statistics Canada, n.d.). Landfilling is one of the most common waste management strategies. However, there are many disadvantages to them. One drawback of landfilling is the need to provide aftercare once the landfill is closed (Laner et al., 2012). This would mean additional costs. In addition to landfilling, there are alternate strategies for waste management, such as incineration or anaerobic digestion (AD) (Tan et al., 2014). However, many challenges also exist with these alternatives such as anaerobic digestion (Y. Chen et al., 2008; Fagbohungbe et al., 2017; Richard et al., 2019). Thus, the improvement in process efficiency and operational stability in alternative options such as anaerobic digestion could make these options more attractive.

As previously mentioned, anaerobic digestion is a promising alternative strategy in the waste management practice. It can handle various feedstocks, including municipal solid waste (Jain et al., 2015; M. Y. Qian et al., 2016). AD is the breakdown of organic matter with microbes in an oxygen free environment (Jain et al., 2015). The process is often broken down into 4 steps. They are hydrolysis, acetogenesis, acidogenesis, and methanogenesis (Richard et al., 2019). The process is capable of degrading organics in the waste while generating products such as methane gas at the same time (Jain et al., 2015). Additionally, the digestate from the process could also be used as fertilizer (Richard et al., 2019). However, the cost of the AD process may be a barrier to

its success; additionally, it is a complex process with many influencing factors (Y. Chen et al., 2008; Mata-Alvarez et al., 2000; Park et al., 2018). Further research into improving the process efficiency can make AD a more attractive process.

The process can be separated into wet or dry AD, depending on the solids content of the reactor (Rocamora et al., 2020). Although there has recently been an increased number of publications on dry AD (Ge et al., 2016), there are many challenges that are unique to dry AD (Rocamora et al., 2020). For example, one common limitation to dry AD is the difficulties of mixing (Abbassi-Guendouz et al., 2012; Garcia-Bernet et al., 2011). This lack of mixing could lead to localized inhibition (Chanakya et al., 1993, 1997; Yebo Li et al., 2011; Rapport et al., 2008; A. H. M. Veeken & Hamelers, 2000), which would inhibit the microbes.

One area of interest is the investigation of the microbial population. This is as understanding of microbial community could better aid in the optimization of the AD (De Vrieze et al., 2012; Mata-Alvarez et al., 2000). Different microbes are associated with each of the four steps of AD (Jain et al., 2015). These microbes work together to transform the organic matter in municipal solid waste (MSW) into the desired methane gas. Varying the process conditions would therefore change the microbial community (Yan et al., 2019; Yi et al., 2014). For example, changing conditions such as the rate of percolation or ratio of feedstock added could affect the accumulation of inhibitors such as volatile fatty acids (M. Qian et al., 2017; Sun et al., 2016). The accumulation of inhibitors would then inhibit the microbial community and thus affecting the overall methane yield (Jiang et al., 2018; Yan et al., 2019). Therefore, it is important to understand the microbial community of the AD process. Additionally, although the wet and dry process operate on similar biological process, there are still differences between them (Rocamora et al., 2020). Although there has recently been an increased amount of publications on dry AD

(Ge et al., 2016), there is limited amount of research on dry AD with percolate recirculation. Therefore, additional research into the microbial community should be investigated to better understand the dry AD process. Additionally, a further understanding in microbial communities could aid in the challenge of process instability.

1.2 Objectives

Based on the research gaps highlighted in the background section, the focus of this study would be on the high-solids anaerobic digestion (HSAD) of the organic fraction of municipal solid waste (OFMSW) with percolate recirculation. The specific goals of the thesis are highlighted below:

1. To observe the temporal trends in microbial communities and various indicator parameters in liquid percolate.
2. To identify spatial trends in the microbial communities in the digestate.

1.3 Thesis organization

The thesis will be subdivided into 4 chapters. Chapter 1 will provide an introduction to anaerobic digestion, highlight research gaps and summarize the goals and objectives of this thesis. Chapter 2 will provide an extensive literature review on the topic of anaerobic digestion. It will look at process parameters in the anaerobic digestion process as well as highlight key points in the microbial community of a digester. Chapters 3 will present the results of the experiment as well as the discussion. Lastly, Chapter 4 will summarize the key points of the thesis, provide recommendation for future studies as well as discuss the limitations of the experiments.

Chapter 2 Literature review

2.1 Anaerobic digestion

As society becomes more environmentally conscious of their actions, cities around the world are adopting strategies to lessen their impact. For example, The City of Edmonton has implemented a 25-year waste management strategy. One of the goals is to divert 90% of their single unit residential waste from landfills (The City of Edmonton, 2019). This is likely due to the negative environmental impacts of the landfilling, such as the release of greenhouse gases into the atmosphere (Rocamora et al., 2020; Tan et al., 2014). One attractive alternative to divert organic waste from landfill is anaerobic digestion (AD). AD is a multi-step process involving a consortium of microbes working to convert organic waste into products such as methane gas in the absence of oxygen (Anukam et al., 2019; Meegoda et al., 2018).

AD consists of four main steps; they are hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Anukam et al., 2019; Richard et al., 2019). Hydrolysis is usually considered the rate-limiting step (Yebo Li et al., 2011; Richard et al., 2019). It involves the breakdown of complex organics. The acidogenesis step then follows, converting the products from hydrolysis into products such as volatile fatty acids (VFAs), acetic acid, and H_2/CO_2. Acetogenesis then further converts things into H_2/CO_2, and acetate. Finally, the methanogens, which could only consume simple substrates, converts materials such as acetic acid and H_2/CO_2 into CH_4 and CO_2 in the methanogenesis step (Meegoda et al., 2018; Richard et al., 2019). The exact composition of biogas produced from the process varies depending on various parameters. However, the typical CH_4 content in biogas is 50-75%, while typical CO_2 content is 25-50% (Anukam et al., 2019).

A wide variety of bacteria and archaea are needed for each of the four steps mentioned above in the AD process, with the archaea mainly responsible for the methanogenesis step (Meegoda et al., 2018). There are many different types of species that make up the methanogens, each with their own unique pathway. For example, the acetoclastic pathway usually accounts for approximately 70% of the methane produced, while hydrogenotrophic pathway accounts for 30% of the methane produced (Jain et al., 2015). It is well known that these methanogens are often more sensitive to environmental parameters compared to their bacteria counterparts (Masoud Kayhanian, 1994; Nakakubo et al., 2008; Rocamora et al., 2020). Thus, inhibition in one step of the reaction could cause a chain reaction of inhibition and causing other processes to fail as well. One example is inhibition due to ammonia. The increase of ammonia concentrations in a reactor could inhibit methanogens. This would not only lower the overall biogas production, but could also result in further inhibition from the accumulation of VFAs (Angelidaki & Ahring, 1993; Sun et al., 2016). Thus, ammonia and VFAs are often monitored in the anaerobic digestion process (Duan et al., 2012; Ryue et al., 2019; Sun et al., 2016).

Anaerobic digestion could often be categorized into wet type anaerobic digestion and dry type anaerobic digestion. The difference between wet type and dry type, otherwise known as high-solids anaerobic digestion (HSAD) is the percentage of solids in the reactor. Typically, a reactor with >15% total solids (TS) will be considered a HSAD (Jain et al., 2015; M. Qian et al., 2017; M. Y. Qian et al., 2016). Both HSAD and wet-type AD operate on similar principles and thus have many similarities, however the uniqueness of the high solid content will result in slight variations between the two processes (Rocamora et al., 2020). Due to the high solid content usually associated with municipal solid waste (MSW), HSAD could be attractive for MSW type waste (Rocamora et al., 2020; Schievano et al., 2010; Wilson et al., 2016; Zhang et al., 2018).

There are many advantages and limitations of HSAD systems. Advantages include lower water usage, simpler operation compared to wet-type digester, smaller reactor volumes, as well as easier handling of residuals (De Baere, 2006; Jha et al., 2013, 2010; Rocamora et al., 2020; Zhang et al., 2018). However, there are also limitations to this as well. Limitations includes localized inhibition (Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). The localized inhibition is caused by the high solid content (Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). Other limitations include a generally lower methane yield, however, there have been conflicting literature on this point (Abbassi-Guendouz et al., 2012; Rocamora et al., 2020; Yi et al., 2014).

One solution to the localization problem is the use of percolate recirculation. The percolate recirculation would transport media within the liquid (Yebo Li et al., 2011; M. Qian et al., 2017; A. Veeken & Hamelers, 1999). Additionally, the percolate/leachate could also act as an inoculum (Yebo Li et al., 2011; Wilson et al., 2016). However, many challenges still exist with the operation of HSAD systems. There are limited studies that investigate HSAD with percolate recirculation. Although Rocamora et al., 2020 suggested that extrapolation of results between wet and dry type digester is possible, there are many challenges unique to HSAD operation. Thus, the focus of this thesis is to investigate dry type anaerobic digestion.

2.2 High-Solids anaerobic digestion (HSAD)

One challenge often associated with HSAD is the mass transfer limitations (Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). The high amount of solids will make the reactor difficult to mix when compared to wet type digesters (Abbassi-Guendouz et al., 2012; Garcia-Bernet et al., 2011; Ge et al., 2016). This will result in pockets of localized inhibition in the

reactor, which would greatly affect the process performance (Chanakya et al., 1997; Yebo Li et al., 2011; A. H. M. Veeken & Hamelers, 2000). One solution to alleviate localization and uneven distribution is the use of leachate/percolate recirculation (Ge et al., 2016; Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). This will be further discussed in a later section.

Similar to wet-type AD, HSAD could also suffer from VFA and ammonia inhibition. The process of VFA and ammonia inhibition will be covered in a later section. Other limitations of higher solid concentrations include a longer retention time and lower methane yield compared to lower solid content digesters (Abbassi-Guendouz et al., 2012; X. Chen et al., 2014; Fernández et al., 2008; Yebo Li et al., 2011). For example, Fernández et al., 2008 compared reactors with 20% and 30% TS content. The reactor with 20% TS began methane generation earlier when compared to the reactor with 30% TS. It also produced more methane when compared to the 30% TS content reactor. Similarly, Abbassi-Guendouz et al., 2012 also observed a similar relationship between the total solids content of a reactor and the cumulative specific methane yield. The cumulative methane yield for 10% to 25% TS was ~180 mL/g VS while the yield for TS 30% and higher was less than ~150 mL/g VS. However, other studies such as Yi et al, 2014 noted a higher methane yield for reactors operating at higher solid contents.

One of the advantages of HSAD over wet-type systems is the higher TS content, which may result in smaller reactors, thus potentially lower costs (Ge et al., 2016). Additionally, the solid digestate from HSAD systems is much easier to handle compared to the residual from wet type digesters (Yebo Li et al., 2011). As well, HSAD systems have lower heating/energy requirements (Jha et al., 2013). It also has lower water requirements as HSAD does not require the dilution of the substrate with water to decrease the solids content (Jha et al., 2013; Karthikeyan & Visvanathan, 2013). This reduction could also result in cost savings. Lastly,

HSAD systems could be more suitable for low moisture content feedstocks, such as lignocellulose biomass (Ge et al., 2016).

2.3 HSAD process configurations

There are many different technologies and process configurations in HSAD. HSAD reactors are often separated into two categories, which are batch and continuous reactors (Fu et al., 2018; Rocamora et al., 2020). Each of these processes has its own advantages and disadvantages.

Batch reactors have the advantage of simplicity (Fu et al., 2018; Rocamora et al., 2020). Substrate and inoculum are often premixed before being loaded into a digester vessel. The digestion process then proceeds in the vessel, and after the reaction is completed, the digestate is unloaded (M. Y. Qian et al., 2016). The garage type batch system is often used for non-free flowing materials such as MSW (M. Y. Qian et al., 2016). One drawback of the batch system is that it could lack mixing during the process (Fu et al, 2018). This leads to localization and mass transfer limitations that are common with HSAD systems that were previously mentioned. This localization due to high solid content may also affect the microbial community (Yebo Li et al., 2011; Rapport et al., 2008).

One solution to this is the incorporation of percolate recirculation (Rocamora et al., 2020). The percolate/leachate recirculation not only could provide an additional source of inoculum, but it could also transport media and provide a mixing effect that would reduce the localization issue (Ge et al., 2016; Michele et al., 2015; M. Qian et al., 2017; Wilson et al., 2016). The use of leachate/percolate as an inoculum source could reduce the substrate to inoculum (S/I) ratio and thus improve process efficiency (Yebo Li et al., 2011; Wilson et al., 2016). One example of batch type reactor is the BEKON system, which is a garage type reactor with a percolation

system (Fu et al., 2018; M. Y. Qian et al., 2016). The BEKON system is a single staged process where the garage-type vessel is sealed after feedstock is loaded. No additional material or mixing is needed after this, and percolation and heating are provided (BEKON, 2015). Advantages of the batch system include ease of operation, simplicity, and lower operating costs (Ge et al., 2016). However, biogas generation fluctuates during the entire process as the reaction proceeds through the different phases of AD (Fu et al., 2018). This would lead to inconsistent biogas generation. Additionally, the batch system also suffers from operational downtimes from the loading and unloading of the reactor (Jain et al., 2015; M. Y. Qian et al., 2016).

The continuous process operates slightly differently when compared to batch systems. Unlike batch reactors, which are loaded once at the beginning, a continuous reactor is continuously loaded at regular intervals and thus results in continuous biogas production (Fu et al., 2018; Hitachi Zosen Inova, n.d.; Yebo Li et al., 2011). One example of a continuous system is the Kompogas digester (Hitachi Zosen Inova, n.d.; Yebo Li et al., 2011). In the Kompogas digester, feedstock is pushed through the reactor and mixed with agitators as the AD process commences (Hitachi Zosen Inova, n.d.; Yebo Li et al., 2011; Rocamora et al., 2020). This process allows for continuous gas generation (Fu et al., 2018). Other examples include the Valorga, and Dranco systems (Yebo Li et al., 2011). Advantages of the continuous system include more stable and consistent gas generation (Fu et al., 2018). Additionally, the only operational downtime occurs during maintenance and repair (Jain et al., 2015). However, the continuous process is generally more costly and complicated compared to the batch process (Ge et al., 2016).

2.4 Important process parameters in HSAD operation

As mentioned before, different inhibitors can adversely affect the AD process. Although the presence of these inhibitors may stifle the process, these parameters can be used as an indication of the process conditions. In this section, the discussion will focus on the role of VFAs and ammonia in the anaerobic digestion process.

2.4.1 VFAs

As previously mentioned, VFAs are generated as part of the acidogenesis process, where fermentative bacteria convert products into VFAs such as acetate, propionate, and butyrate.

High concentrations of VFAs in the digester could be an indication of inhibition (Sun et al., 2016; Xu et al., 2014). It has been seen in studies that an inhibiting condition would lead to the accumulation of VFAs in the system (Dang et al., 2016; Duan et al., 2012). One common way to induce inhibition is by overloading the reactor (M Kayhanian & Hardy, 1994; Schievano et al., 2010). Since the acidogenic bacteria grows significantly faster than the methanogens, VFAs would be produced faster than the methanogens could consume (M Kayhanian & Hardy, 1994). The high concentration of VFAs would decrease the pH of the system (M Kayhanian & Hardy, 1994), which would inhibit the methanogens. The system can be affected by the pH, with the optimal range being 6.8-7.2 (Ward et al., 2008). This inhibition would create a negative feedback loop where VFAs would continue to accumulate from the inhibition of the methanogenesis process, thus causing a further drop in pH (Sun et al., 2016). This scenario was observed in Sun et al., 2016, where total ammonia nitrogen (TAN) concentration as high as 9 g N/L was observed and caused the inhibition of the AD process. This coincided with an accumulation of VFA, with acetic acid concentrations of 25 g/L being observed. This VFA accumulation led to the

subsequent drop to a pH of ~6. A similar acidification was observed when the VFA concentration reached ~17 g/L and the pH reduced down to 5.74 in Qian et al., 2017. This was due to a high percolation rate. Thus, it can be seen that high concentrations of VFA could be detrimental to the AD process.

Although high VFAs is a concern, another value that could be used to evaluate process stability is the VFA to alkalinity ratio (Callaghan et al., 2002; Yeqing Li et al., 2013). This is as the alkalinity could buffer the pH change and keep the system at the desired 6.8-7.2 pH (Duan et al., 2012; Ward et al., 2008; Weiland, 2010). It was suggested that a VFA/Alkalinity ratio >0.8 would result in instability, while a VFA/Alkalinity ratio between 0.4 and 0.8 may induce some instability, whereas a VFA/alkalinity ratio less than 0.4 is considered stable (Callaghan et al., 2002; Yeqing Li et al., 2013; Zickefoose & Hayes, 1976). However, even though Duan et al., 2011 had observed VFA concentrations of 3000-4500 mg/L and TAN concentrations of 3000-4000 mg/L, the VFA/Alkalinity ratio was only 0.19-0.26 (which would be considered stable according to the parameters above, even though the authors of that paper had deemed the process stability as fragile). It was suggested by Duan et al., 2011 that VFA/Alkalinity ratio is not as useful under high solid high ammonia conditions. This is as ammonia could increase the alkalinity of the system. Thus, in situations of high ammonia, the VFA/Alkalinity ratio should be used with discretion (Duan et al., 2012).

The concentrations of VFAs in a system varies greatly. Qian et al., 2017 terminated a reactor after it observed VFA concentration of 17.7 g/L. On the other hand, Jabeen et al., 2015 found during the operation of a plug flow high solid anaerobic digestion of food and risk husk waste that the inhibiting VFA concentration was between 2.4 to 8.3 g/L. It should also be noted that sometimes the VFA accumulation is a result of ammonia inhibition (Sun et al., 2016). The high

ammonia would inhibit the methanogens, which would trigger the negative feedback loop mentioned above leading to varying levels of VFA. Duan et al., 2011 studied the digestion of sewage sludge. Under high TAN conditions, the total VFA concentration was 3-4.5 g/L. However, in Sun et al., 2016, the accumulation of VFA from the digestion of chicken manure and maize silage was upwards of ~60-70 g/L under inhibiting ammonia conditions. This was due to the ammonia inhibition of methanogens, which allowed for VFAs to be accumulated (Sun et al., 2016). Thus, it could be seen that the highest accumulated concentration of VFAs can vary. Table 2.1 below summarizes the highest ranges of VFA observed in anaerobic digestion.

Table 2.1 Concentration of the highest VFAs reported in anaerobic digesters

Reference	Feedstock	Concentration	Comments
(M. Qian et al., 2017)	HSAD of OFMSW and corn straw with percolate recirculation	17691 mg/L	• Complete failure of one reactor condition after 13 days • pH dropped to 5.74
(Schievano et al., 2010)	HSAD of OFMSW	16.6 g/kg in terms of acetic acid	• Process inhibition due to overloading • Limited biogas production due to imbalanced food to inoculum ratio • Inhibition from 2.4-16.6 g/kg as acetic acid • Uninhibited conditions showed <1g/kg as acetic acid VFA concentrations
(Guendouz et al., 2008)	HSAD of MSW	~8 g COD/kg of digestate observed	• Suggested possible reactor overloading leading to high observed VFA

(Sun et al., 2016)	Chicken manure and maize silage	Max concentration of ~60-70 g/L total VFA observed	• High TAN concentration led to accumulation of VFAs (pH dropped below 6)
(Duan et al., 2012)	Sewage sludge	3000-4500 mg/L	• Accumulation of VFAs due to high TAN levels • VFA >10,000 mg/L during initial startup phase at high OLR but recovered • Inhibition was moderate at VFA concentration of 1000–3000 mg/L (FAN: 400-600 mg/L) • Inhibition was significant at VFA concentration of 3000–4500 mg/L (FAN 600-800 mg/L)
(Jabeen et al., 2015)	HSAD of food waste (FW) and rice husk	10445 mg/L	• Acidification at high OLR resulted in pH dropping to 6.54 • VFA jumped from 2413 mg/L to 8344 mg/L when reactor overloaded

Note. Food waste (FW); High Solid Anaerobic Digestion (HSAD); Municipal solid waste (MSW); Organic fraction of municipal solid waste (OFMSW); Total Ammonia Nitrogen (TAN); Volatile Fatty Acid (VFA);

2.4.2 Ammonia

Ammonia is produced from the breakdown of things such as proteins in the substrate (M Kayhanian, 1999). Although ammonia is important to the growth of microbes in anaerobic digestion, the accumulation of ammonia may lead to instability and failure in a reactor (Wang et al., 2013; Yenigün & Demirel, 2013). It is well known that methanogens are often more sensitive

to ammonia than fermentative bacteria (Masoud Kayhanian, 1994; Nakakubo et al., 2008). Hence, an accumulation of ammonia would often lead to the accumulation of other potential inhibitors such as VFAs, thus causing further inhibition (Akindele & Sartaj, 2018; Sun et al., 2016). The inhibition can lead to a reduction in performance (Sun et al., 2016).

Although high ammonia concentration is often regarded as undesirable, the exact concentration is often varying. A review by Yenigün & Demirel, 2013 showed that the inhibition TAN concentration could range from 100% inhibition at 2.5 g/L to only 50% inhibition at 11 g/L. This is as there are many factors that affect ammonia inhibition. These include temperature, pH, as well as the concentration of ammonia (Y. Chen et al., 2008; Rajagopal et al., 2013). In addition to the factors mentioned above, the acclimation of microbes has been suggested to positively affect the ammonia tolerance in an anaerobic digestion system (Nakakubo et al., 2008; Rajagopal et al., 2013). Yan et al., 2019 demonstrated that a stepwise increase of TAN in a reactor with MSW was able to withstand up to 8.5 g NH_4^+-N /L. Similarly, the acclimation of microbes to high ammonia conditions was also demonstrated by Abouelenien et al., 2009. In the study, chicken manure was digested with a seed. After gas production ceased, half the content was replaced with fresh chicken manure for the next batch. High methane production was noticed on the last two batches at 28 and 31 mL/g VS. The authors of that study attributed this production to the acclimation of microbes to the high ammonia conditions. Thus, the addition of acclimated inoculum may play a vital role in determining the success of the reactor.

It is well known that ammonia in the form of free ammonia nitrogen (FAN) can affect AD performance (Duan et al., 2012; Sun et al., 2016). One possible mechanism responsible for this is due to the ability of free ammonia to diffuse into the cells and cause a proton imbalance (Y. Chen et al., 2008; Richard et al., 2019). Similar to ammonia, the inhibitory level of free ammonia

also varies. For example, Peng et al., 2018 noted severe inhibition and a reduction in gas production at FAN 0.3 g/L, whereas Sun et al., 2016 saw slight inhibition at FAN ~0.6 g N/L. As can be seen by equation 1, the free ammonia concentration is influenced by the pH, temperature, and the total ammonia concentration (Hansen et al., 1998).

$$\frac{[NH_3]}{[TNH_3]} = (1 + \frac{10^{-pH}}{10^{-(0.09018+(\frac{2729.92}{T(k)}))}})^{-1} \quad (1)$$

Therefore, thermophilic reactors with a higher temperature are more susceptible to high free ammonia concentrations. An increasing pH would also likely increase the free ammonia concentration (Richard et al., 2019).

As mentioned earlier, the methanogenic archaea are more sensitive to ammonia (Masoud Kayhanian, 1994). However, it has been shown that certain types of methanogens are more sensitive than the other. Yan et al., 2019 noticed a shift in abundance of microbes from *Methanosaeta concilii 2* to *Methanosarcina soligelidi 1* with increasing TAN. It also noted a decrease in alpha diversity when the TAN concentration was increased from 8.5 g NH_4^+-N /L to 9.5 g NH_4^+-N /L (Yan et al., 2019). The methanogenic pathway could be different under high ammonia conditions (Sun et al., 2016; H. Tian et al., 2017). Thus, under high TAN conditions, the microbial biome could be greatly altered, which would significantly affect the methane generation of the system. Understanding the change in microbial community under these conditions would greatly aid in our knowledge of AD. This will be further discussed in the next section.

There are many strategies to remedy the high concentration of ammonia. As mentioned previously, the pH and temperature both greatly influence the free ammonia concentration (Richard et al., 2019). Controlling these two parameters could help alleviate the stress caused by

ammonia. Additionally, allowing time for the microbes to acclimate to high ammonia conditions could also aid in reactor stability (Y. Chen et al., 2008). Other strategies not yet mentioned include changing the mixing ratio of feedstock. For example, Sun et al., 2016 noted varying ammonia concentrations when changing the mixing ratio of chicken manure and maize silage. Similarly, the organic loading rate has also been noted as a cause for inhibition (Yenigün & Demirel, 2013). Lastly, a recent trend in anaerobic digestion is the addition of materials such as granular activated carbon or carbon fiber textiles (Florentino et al., 2019; Sasaki et al., 2007, 2011). The addition of these materials could enhance the AD process under elevated ammonia conditions (Florentino et al., 2019; Sasaki et al., 2007, 2011). Table 2.2 below summarizes some of the TAN and FAN concentrations in anaerobic digesters.

Table 2.2 Reported TAN and FAN concentrations in anaerobic digesters

Reference	Feedstock	Concentration	Comments
(Yan et al., 2019)	MSW	TAN 8.5 g NH_4^+-N /L	• Step wise acclimation of continuously stirred tank reactor (CSTR) at mesophilic conditions • FAN > 800 mg NH3-N /L when TAN concentration was 8.5 g NH_4^+-N /L • Mesophilic conditions
(Peng et al., 2018)	Food Waste	FAN >150 mg/L: process efficiency disturbed FAN > 200 mg/L: process efficiency and stability disturbed FAN > 300 mg/L: inhibition and reduction in gas production	• Long term CSTR of food waste • Observation of steady, quasi steady state, inhibited, inhibited steady, and inhibited states • Mesophilic conditions

(Duan et al., 2012)	Sewage sludge	Moderate inhibition: FAN 0.4-0.6; TAN: 3-4 g/L Significant inhibition: FAN 0.6-0.8; TAN: 3-4 g/L	• Inhibition of ammonia led to VFA accumulation • Mesophilic conditions
(Sun et al., 2016)	Chicken Manure and Maize Silage	Inhibition observed at TAN ~7 g N/L; FAN ~0.6 g N/L (10-20% inhibition in biogas and methane production) Complete inhibition at TAN 9 g N/L	• Changing feedstock ratio led to high ammonia concentration • High VFA also observed leading to pH drop • Mesophilic conditions
(Wang et al., 2013)	Corn Stover	TAN concentrations of 4.3 and 6.0 g/kg resulting in 20-50% reduction in biogas, methane and CO_2 compared to control at 2.5 g/kg	• Urea added as ammonia source • High TAN concentrations reduced reaction kinetics • Significant VFA inhibition observed at 6.0 g/kg condition • Mesophilic conditions
(Masoud Kayhanian, 1994)	MSW	TAN inhibition starts at 1200 mg/dm^3, failure at ~2500 mg/dm^3	• Extreme shift of VFA noted at 1200 mg/dm^3 • Optimal TAN concentration at 600-800 mg/dm^3 • Thermophilic Conditions

Note: Continuously stirred tank reactor (CSTR); Free Ammonia Nitrogen (FAN); Municipal Solid Waste (MSW); Total Ammonia Nitrogen (TAN); Volatile Fatty Acid (VFA)

2.5 Microbiology

As previously mentioned, the anaerobic digestion process contains a wide variety of microbes, each responsible for different tasks. Thus, changes in the microbial communities could result in a change in performance. The microbial community of a digester is vital to its success. Some studies have linked the presence of certain key microbes to higher methane production (Granada

et al., 2018; Han et al., 2017). The understanding and cultivation of these microbes could allow for better performance.

One of the most common ways to inhibit the process is through the accumulation of ammonia or VFAs. Esquivel-Elizondo et al., 2016 noted a decrease in microbial diversity over time and attributed this reduction to the enrichment of certain microbes that could withstand high ammonia conditions. It is generally acknowledged that the methanogens in the AD process are generally more sensitive to ammonia as compared to bacteria (Masoud Kayhanian, 1994). Similarly, within the methanogens, it is believed that hydrogenotrophic are more resilient compared to their acetoclastic counterparts; however, there exists conflicting literature on that point (Y. Chen et al., 2008). For example Sun et al., 2016 conducted an isotope tracer experiment and concluded that the dominant methanogenesis pathway at TAN concentration of ~7-8 g N/L was hydrogenotrophic methanogenesis. Similarly, Tian et al., 2017 noted from an activity test that the hydrogenotrophic methanogens were generally more active when compared to the acetoclastic methanogens at high FAN concentrations. Meanwhile, Esquivel-Elizondo et al., 2016 observed a 25% relative abundance of the acetoclastic methanogen *Methanosaeta* in a semi-continuous reactor at high TAN concentrations and suggested that both the hydrogen utilizing and acetate utilizing methanogens can exist in these conditions. The authors suggested that this might be due to the acclimation of the acetoclastic methanogens at a lower ammonia concentration during an earlier part of their experiment or that the ~2000 mg N/L concentration was not high enough to inhibit the methanogens. Regardless, more research into microbial communities are needed to explicitly understand the effects of inhibitory conditions.

It was suggested that the methanogenesis pathway shifts to the hydrogenotrophic pathway during high ammonia conditions through the syntrophic acetate oxidation (SAO) process (Blomgren et

al., 1990; Sun et al., 2016; Westerholm et al., 2016). In the SAO process, instead of converting acetate to CH_4, the acetate created in the AD process is first converted to H_2, formate, and CO_2. The hydrogenotrophic methanogens would then convert them to CH_4 (Westerholm et al., 2016). Thus, the relationship between syntrophic acetate oxidizing bacteria (SAOB) and hydrogenotrophic methanogens could be a dominant pathway under ammonia inhibition. For example, Fotidis et al., 2014 examined various full-scale reactors and concluded that the dominant pathway at high ammonia and free ammonia concentrations (>2.8 g NH_4^+-N/L; >0.44 g NH_3-N/L) was the SAO-HM (syntrophic acetate oxidation with hydrogenotrophic methanogenesis), whereas at lower concentrations (<1.5 g NH_4^+-N/L), it was facilitated by acetoclastic methanogens such as *Methanosaetaceae* spp.

These shifts in pathways may be linked to the ammonia tolerance of the microbes (Westerholm et al., 2016; Yan et al., 2019). Different microbes have different tolerances and optimal growth conditions. For example, a *Methanosarcina* specie increased in relative abundance while a *Methanosaeta* specie decreased under high ammonia conditions (Yan et al., 2019). This is likely due to the wider range of metabolism and ability for *Methanosarcina* to cluster to reduce inhibition (Boone et al., 1993; Calli et al., 2005a, 2005b; De Vrieze et al., 2012; Yan et al., 2019).

The tolerance of methanogens could affect the relative abundance of bacteria. This was demonstrated by Yan et al., 2019, who suggested the stimulated growth of *Bacillaceae* sp. 20 and *Syntrophaceticus schinkii* 25 was due to a shift in methanogen populations/metabolism. In the experiment, an increased TAN concentration resulted in the shift from acetoclastic methanogen *Methanosaeta concilii* 2 to *Methanosarcina soligelidi* 1. *Methanosaeta concilii* 2 utilizes acetate, whereas *Methanosarcina soligelidi* 1 can utilize a wider variety of substrates such as H_2. The changing pathways then resulted in an increase in abundance of the two bacteria

(*Bacillaceae* sp. 20 and *Syntrophaceticus schinkii* 25) from undetectable levels to 12%. Thus, it can be seen that the microbial population in the AD process is interconnected. A change in one community may result in the enhancement of another.

Another area of interest is the difference between wet and dry AD digesters. As previously mentioned, although both are considered AD, the difference in solid content in the reactor creates new challenges such as mass transfer limitations (Chanakya et al., 1997; Yebo Li et al., 2011; Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). These differences could affect the performance of a digester, especially on the microbial scale. Yi et al., 2014 demonstrated the changes in a microbial community when increasing the total solid content of the food waste fed into the digester. For example, the population of *Choroflexi* was 31% at a TS content of 20%, compared to 65% at 5% TS content. The authors attributed these discrepancies to the increased TS content, leading to a higher organic loading rate (OLR). This higher OLR would result in higher amounts of biodegradable substrates; thus, affecting the microbial community distribution. Similarly, Han et al., 2017 examined microbial samples from 6 full-scale digesters with varying parameters (ex. feedstock, hydraulic retention time, etc.) and noted a correlation between certain microbial families such as *Clostridiaceae* and TS contents. This once again reinforces the idea that changing TS content could affect the process. Additionally, spatial variations could exist within the solid digesters as well. For example, Xing et al., 2020 observed variations in microbial diversity parameters between the upper and lower parts of the digester with leachate/percolate recirculation. They noted a higher Shannon index for upper layers, thus meaning there was a higher diversity in the upper layers. Additionally, they noted a higher proportion of certain phylum (ex. Bacteroidetes and Firmicutes) in the upper layer compared to the lower layer. They also suggested the flushing of products such as VFAs due to percolation may be a reason for the

spatial differences in the microbial community. Thus, not only could there be a variation in microbes due to changing TS content, but recirculation could also affect the community.

Understanding the microbial communities is vital to the success of optimizing the AD process. For example, De Vrieze et al., 2012 suggested a real-time PCR monitoring of the ratio between *Methanosaeta* and *Methanosarcina* as an indicator for reactor stability. They also suggest monitoring the quantity of SAOB to judge the main pathway in the AD reactor. In addition, the presence of certain microbes could be used as indicators for reactor performance. Poirier et al., 2017 conducted an experiment where various support media (i.e. activated carbon, zeolites, etc.) were added to an AD digester under high TAN conditions. It noted a reduction in the family *Porphyromonadaceae* in the higher methane-producing reactors and hypothesized the family member could be associated with inhibiting conditions. Overall, a very limited amount of information is available on the microbial communities in high-solids anaerobic digester with percolate recirculation. Thus, a further understanding of microbes could allow us to better understand reactor performance, allowing it to be monitored or operated more efficiently.

2.6 Summary and research gaps

There is a consensus that excess concentrations of ammonia and VFA is undesirable. As seen from Table 2.1 and 2.2, these values vary greatly depending on the conditions. Therefore, additional investigation into the impacts of these parameters is needed to understand how the various conditions could impact the AD process.

AD can be considered a well-established process. It has been suggested that the process of wet and dry AD are similar and thus the knowledge could be extrapolated between them, however more research into HSAD is needed (Rocamora et al., 2020). Thus, the research gap highlighted

should be further investigated in HSAD. Additionally, the use of percolate recirculation is limited and needs further investigation (Ge et al., 2016; Rocamora et al., 2020; Xing et al., 2020). Although percolate recirculation could be beneficial, an excess amount could be detrimental to the process. For example M. Qian et al., 2017 explored the effects of percolate recirculation rates on the digestion of OFMSW and corn straw. It was observed that at the highest frequency of percolation recirculation rate of 4.8, the VFA concentration drastically increased until the reactor failed. Similarly, the method of percolation could also affect the system. Xing et al., 2020 noted that the performance of the reactor with continuous addition of percolate recirculation differed from the reactor with intermittent percolate recirculation. The intermittent percolation resulted in 78.5 L/kg VS of methane, whereas the continuous system only yielded 56.2 L/kg VS. Thus, more research is needed in the field of HSAD with percolate recirculation. Lastly, it is evident that high solid content and inhibitors can shape the microbial community, which should also be explored further.

Chapter 3 Transitions of microbial communities in the solid and liquid phases during high-solids anaerobic digestion of organic fraction of municipal solid waste

3.1 Introduction

With the world becoming more environmentally conscious, the practice of landfilling is becoming a less attractive option for the disposal of organic fraction of municipal solid waste (OFMSW). One alternative is high-solids anaerobic digestion (HSAD), which can simultaneously degrade waste and generate biogas (Guilford et al., 2019; M. Qian et al., 2017; Rocamora et al., 2020). HSAD systems offer lower digester heating costs and higher tolerance to substrate heterogeneity, as compared to high moisture digestate from conventional wet-type anaerobic digester operated with less than 15% total solids (TS) (Jha et al., 2013; M. Qian et al., 2017; Rapport et al., 2008; Rocamora et al., 2020). HSAD systems also produce low moisture digestate, which would be easier to handle (Jha et al., 2013, 2010). However, HSAD systems usually do not incorporate mixing, which could induce a mass transfer limitation (Fu et al., 2018; Yebo Li et al., 2011; Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). Additionally, HSAD systems often exhibit problems with process disturbances due to localized inhibition and disturbances caused by high ammonia levels and accumulation of volatile fatty acids (Abbassi-Guendouz et al., 2012; X. Chen et al., 2014; Rocamora et al., 2020; Sun et al., 2016; A. H. M. Veeken & Hamelers, 2000).

Several studies demonstrated the positive benefits of liquid digestate (or percolate) recirculation in HSAD systems. The recirculation of percolate could provide homogenization of nutrients within the solid waste (M. Qian et al., 2017; Rocamora et al., 2020). A washing effect caused by recirculation could remove inhibitory compounds from solids to percolate (Michele et al., 2015;

M. Qian et al., 2017; Rocamora et al., 2020). Furthermore, percolate/leachate recirculation could provide additional inoculum in solids (Rocamora et al., 2020; Wilson et al., 2016). Thus, percolate recirculation could provide improvement in methane production and shorter digestion time when compared to reactors with no recirculation (M. Qian et al., 2017; Rocamora et al., 2020; A. H. M. Veeken & Hamelers, 2000). A study reported that introducing leachate/percolate recirculation could increase the amount of methane produced (A. H. M. Veeken & Hamelers, 2000).

HSAD systems have become increasingly popular. For instance, a box or garage-type HSAD reactor with percolate recirculation is one of the most popular batch configurations in Europe (M. Qian et al., 2017; Rocamora et al., 2020). Nonetheless, HSAD operation with percolate recirculation is still considered as emerging, as existing literature provides limited fundamental understanding of process complexity. Notably, the changes in various liquid water quality parameters (e.g., pH, alkalinity, VFAs, ammonia nitrogen, etc.) in percolate during batch operation could correspondingly influence the digester stability (Massaccesi et al., 2013; M. Qian et al., 2017). For instance, a recent study reported that certain percolate recirculation frequency could accelerate hydrolysis and acidogenesis efficiencies. At the same time, it could also cause volatile fatty acids (VFAs) accumulation that could hinder methanogenic activity (M. Qian et al., 2017). These findings suggest that there could be temporal variations in microbial communities during batch operation.

Additionally, the hydrolysis of solid organics would rapidly increase the ammonia nitrogen level in percolate, which would influence percolate microbial communities (Rajagopal et al., 2013; Yan et al., 2019). Although the performance and stability of anaerobic digestion process are highly dependent on active microbial communities, focus on the microbiological diagnosis of

HSAD systems with percolate recirculation found to be very limited in previous studies. To the best of the author's knowledge, no reports are available in the literature on how microbial communities shift from initial inoculum to final digestate and percolate. Such information would be useful in understanding and optimizing HSAD systems.

Consequently, the present study investigated the changes in microbial communities in solid and liquid phases in high-solids anaerobic digestion of OFMSW. Firstly, temporal changes in chemical and microbiological characteristics of percolate were characterized. Secondly, microbial communities in the initial solid inoculum and final digestate samples collected from the top, middle, and bottom sections of the digester tank were characterized. The results of this study will intensify our knowledge on how microbial community shifts and methane generation patterns are associated with dynamic changes in concentrations of volatile fatty acids (VFAs), ammonia nitrogen, alkalinity, and pH values in the system.

3.2 Methodology

3.2.1 OFMSW and inoculums

The biosolids, liquid inoculum OFMSW samples (see Fig. A.1, A.2 and A.3) were collected from the Edmonton Waste Management Centre (EWMC) in Edmonton Alberta, Canada. At the EWMC, the waste is screened through a 3-inch mesh prior to processing at the facility. The OFMSW had initial total solids (TS) and volatile solids (VS) content of 50.70 ± 2.9% and 27.10 ± 4.62%, respectively. The biosolids served as the solid inoculum and had an initial TS and VS content of 24.20 ± 0.162% and 14.00 ± 0.4%, respectively. The biosolids, OFMSW and liquid inoculum samples were all stored at 4°C prior to the start of the experiment. The initial characteristics of the liquid inoculum were as follows: ammonia: 3.63 ± 0.15 g/L, chemical

oxygen demand (COD): 24.69 ± 1.88 g/L, alkalinity: 14.94 ± 2.03 g/L, acetate: 1.61 ± 0.08 g COD/L, propionate: 1.41 ± 0.18 g COD/L, butyrate: 0.75 ± 0.25 g COD/L, pH: 8.50. Before starting the experiment, the liquid inoculum and biosolids were re-acclimated at mesophilic conditions (37°C) in a water bath for two days.

3.2.2 Design and operation of the lab-scale HSAD system

A custom-built lab-scale HSAD system was used in this study. It consisted of a digester tank and a percolate tank (Fig. A.4 and A.5). The digester tank was made of polycarbonate. The working volume of the digester tank was about 16 L, with an inner diameter of ~19 cm and a height of ~55 cm, respectively. The digester tank had one liquid inlet port at the top and one liquid outlet port at the bottom to allow for the recirculation of percolate. The liquid inlet port was connected with a sprinkler to distribute liquid percolate onto the solid waste inside the digester tank. A stainless-steel perforated mesh (~2 mm hole diameter and 1 mm thickness) was placed at 5.8 cm from the bottom of the tank to prevent solid waste leaving the digester tank during recirculation of percolate. The percolate tank was a glass vessel with a working volume of 2 L and was equipped with a mechanical agitator coupled with an electrical motor for the mixing of percolate. The percolate tank also had an inlet and an outlet port for liquid recirculation. The lids of each tank had a gas outlet port that was connected to a wet-tip gas meter (ISES gas meter, ISES-Canada, Vaughan, ON, Canada) with an in-line CO_2 sequestration bottle for direct measurement of methane production. The CO_2 sequestration bottles were filled with 3 M NaOH solution with a thymolphthalein indicator to absorb any acidic gases in the biogas (Ryue et al., 2019). It should however be noted that the gas bags were used for the first 13 days of the experiment before being replaced with the wet-tip gas meter.

For operation, the digester tank was loaded with 2 kg of OFMSW (feedstock) and 2 kg of biosolids (solid inoculum). The OFMSW and biosolids were completely premixed before loading. The percolate tank was filled with 2 L of liquid inoculum. Additional liquid inoculum (~500 mL) was added on day 10, while ~400 mL was added on days 16 and 17 due to low liquid levels in the reactor. An additional 300 mL was added on day 18. The percolate tank was continuously stirred at 300 rpm during the experiment. Both digester and percolate tanks were wrapped with heating tapes, and the temperature was set at 37ºC. The percolate was semi-continuously recirculated between both tanks using a peristaltic pump (Longer Pump BT100-2J, Langer Instruments Corp, Tucson, AZ, USA). The percolation rate was initially set at 4.1 mL/s for 10 minutes every 12 hours. This was later changed to a percolation rate of 2.0 mL/s for 4 hours a day on day 7. It should be noted that by the end of day 25, the percolate level was extremely low and continuous percolation over the four hours was no longer observed. For example, on day 25, there was only enough percolate for 2 hours of continuous percolation. This duration was further reduced as the experiment continued. The methane production was monitored daily. The percolate sample was collected every five days for analysis of water quality parameters and microbial communities.

3.2.3 Microbial community analysis

Four solid samples and five liquid samples were collected for DNA extraction, qPCR, and gene sequencing. The four solid samples included the initial biosolids that were used as the solid inoculum as well as the final digestate taken from the top, middle, and bottom sections of the reactor after the 33-day experiment. The five liquid samples included the initial liquid inoculum and percolate taken on days 5, 10, 15, and 30. The DNA in the samples were extracted using the

PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), and the concentration was determined using a spectrometer (NanoDrop 2000C, Thermo Fisher Scientific, Waltham, MA, USA). The microbial communities in samples were determined using the high-throughput 16S rRNA gene sequencing on an Illumina Miseq. The universal primer set 515F/806R was used to target the V3-V4 region of the 16S rRNA gene. The DNA samples were stored at -70˚C before sending them to the Research and Testing Laboratory (Lubbock, TX, USA) to perform gene sequencing using a 2×300 bp paired-end protocol. The sequencing data were analyzed using the open-source software Quantitative Insights Into Microbial Ecology (QIIME v2), as described previously elsewhere (Barua et al., 2019). Moreover, a quantitative analysis of microbial communities in solids and liquid samples was performed with qPCR. Quantitative PCR (qPCR) was conducted to quantify the microbial cells using QuantiFast SYBR® Green PCR Kit (Qiagen, CA, USA). qPCR mixtures were prepared in 25 uL:1 uL of DNA template, 12.5 uL 2x master mix, 2.5 uL forward and reverse specific primer after a 10 times dilution, and 6.5 uL nuclease-free water. The qPCR reactions were conducted using CFX 96 real-time PCR system with a C1000 Thermal Cycler (Bio-Rad, CA, USA) with the following cycling conditions: initial heat activation cycle at 95˚C for 5 min, 35 cycles at 95˚C for 10 sec and 60˚C for 30 sec, and finally, one cycle at 40˚C for 30 sec. Triplicate reactions were conducted for all samples. The DNA of E. coli was used as the standard.

3.2.4 Analytical methods

The TS and VS concentrations were measured using the standard method from APHA (Clesceri et al., 1999). The total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), and total ammonia nitrogen concentrations were determined using HACH kits (HACH,

Loveland, Colorado, USA). The samples were passed through a 0.20 μm filter to obtain samples for SCOD analysis. The particulate chemical oxygen demand (PCOD) was calculated from the difference between TCOD and SCOD (PCOD=TCOD-SCOD). Free Ammonia Nitrogen (FAN) was calculated using an equation provided elsewhere (Hansen et al., 1998). pH was measured using a bench-top pH meter (AR15 pH meter, Fisher Scientific, Pittsburgh, PA). The concentrations of various volatile fatty acids (VFAs) were measured using an ion chromatograph (Dionex ICS-2100, Dionex, Sunnyvale, CA). Alkalinity was measured using the titration method. 0.1 N HCl was added to samples diluted with deionized water until a pH of 4.5 was reached. The VS removal percentage was calculated with the assumption that no mass change occurred in the solids.

3.3 Results and discussion

3.3.1 Methane production and solids removal

Fig. 3.1 shows methane production during batch operation over 33 days. The daily methane production curve showed three distinct phases (lag phase, exponential phase, and declining phase), which was similar to that typically observed in batch anaerobic digestion process (Rico et al., 2020). The lag phase was between days 0-10. The 5.5 L of methane production on day one could likely be attributed to the readily biodegradable material. The exponential phase followed from days 10 to 22, with a peak of 16 L on day 16. Methane production began to decrease from day 17 until day 33 with some fluctuations. At the end of the experiment, the total cumulative methane production was 170.6 L. The estimated methane yield was 193 L/kg VS. Lee et al., 2019 reported an average methane yield of 186 L/kg VS for a semi-continuous reactor with leachate/percolate recirculation fed with food waste, yard waste and waste activated sludge.

Meanwhile, M. Y. Qian et al., 2016 reported a yield of approximately 270 L/kg VS for a MSW garage type digester with percolate circulation. Thus, our estimated methane yield was within the range of methane yields observed from literature. However, methane yield from source-separated food waste as a sole substrate has been observed to be as high as 477 mL CH_4/g VS (Rico et al., 2020).

The TS and VS contents of the initial mixture of OFMSW and biosolids were 36.2%, 22.1 respectively (results not shown). In the final digestate, TS and VS contents were 19.1%, and 10%, respectively. Thus, ~55% VS removal efficiency was achieved in this study, which was higher than the VS removal efficiency of 38% reported for an OFMSW (food waste + yard waste + waste activated sludge) fed HSAD system with leachate/percolate recirculation (Lee et al., 2019). The high VS removal efficiency could be credited to a faster hydrolysis rate of OFMSW, which could be influenced by operating conditions such as rate of percolate recirculation (M. Qian et al., 2017). However, a portion of particulate organics was transferred to the percolate tank during the recirculation process (discussed later), which could slightly influence the observed VS removal efficiency.

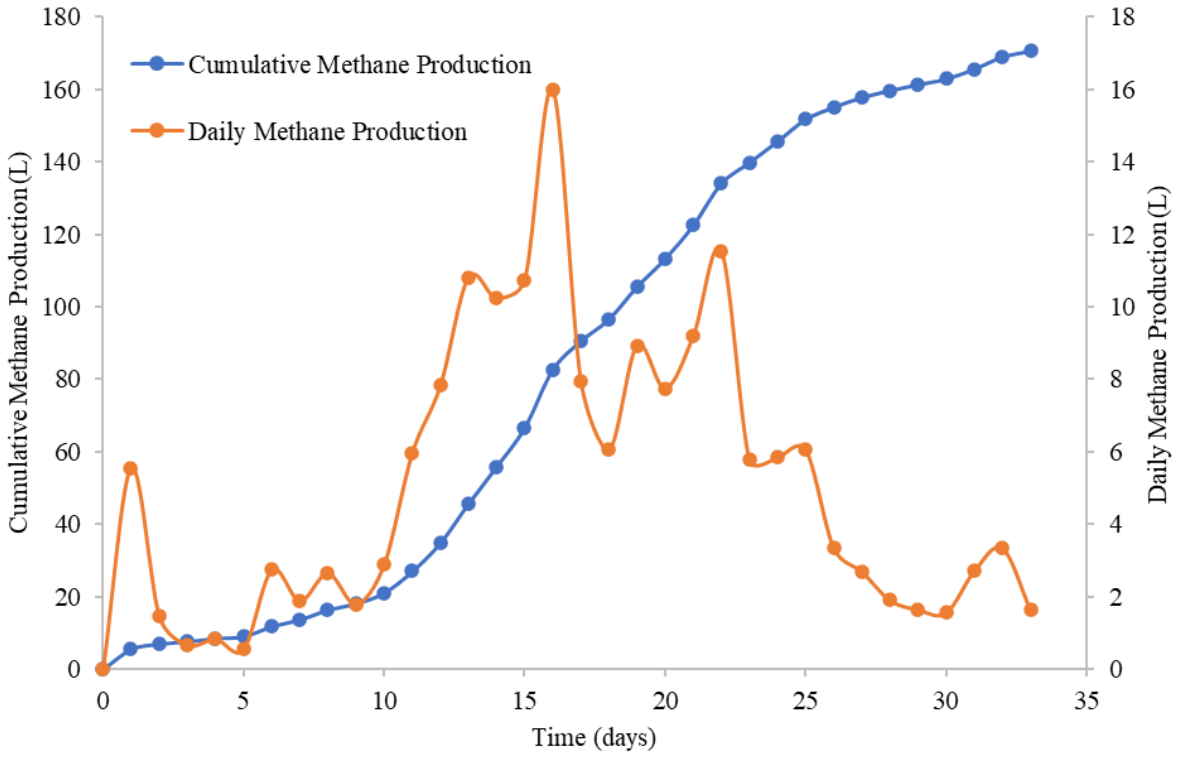

Fig. 3.1 Temporal profile of daily and cumulative methane production

3.3.2 Temporal changes in percolate

3.3.2.1 COD

Fig. 3.2 shows the evolution of percolate COD over time. The initial TCOD concentration was 24,688 ± 1878 mg/L, which increased dramatically to 53,128 ± 3975 mg/L after the first 5 days of operation. This initial increase in TCOD concentration was likely due to the percolation of the liquid through solid waste in the digester tank. The organics released due to hydrolysis were transferred to the percolate tank via recirculation. The TCOD concentration was quite stable between day 5 to day 10, and then began to gradually decrease down to 15,381 ± 1323 mg/L on day 30. Although initial PCOD concentration in the percolate was negligible, PCOD concentrations in the percolate increased over time. Thus, some particulate organics also transferred to the percolate tank through recirculation.

3.3.2.2 VFAs profile

Fig. 3.3 shows the changes in VFAs concentrations in the percolate. After 5 days of operation, the total VFA concentration (the sum of acetate, propionate and butyrate) increased considerably from 3,759 ± 11 to 20,484 ± 85 mg COD/L, which could be attributed to the rapid hydrolysis/fermentation of readily biodegradable fraction of OFMSW. The increase in total VFA concentration continued until day 10. It then sharply decreased between days 15 and 20. This sharp decrease in VFAs also corresponded with a sharp increase in methane production during this operating period (see Fig. 3.1 and 3.3). A stable reduction in total VFA concentrations was observed from days 20-30. A closer analysis of the individual VFAs showed that the acetate concentration increased after the first 5 days of operation, and then sharply decreased between days 10 and 20. The butyrate concentration showed a similar trend. It then returned down to a low concentration (508 ± 108 mg COD/L) on day 20 and was stable for the rest of the operating period. No significant variation in the propionate concentration was observed.

The accumulation of VFAs is considered an indication of either the high rate of hydrolysis or the inhibition of methanogens. High VFA accumulation leads to a significant decrease in digester buffering capacity, which is one of the common reasons for the failure of HSAD systems (Lee et al., 2019; M. Qian et al., 2017). In this study, total VFA concentration reached as high as 21,196 ± 3.8 mg COD/L. Previous studies also reported that VFA concentration could reach a peak value of ~18-24 g/L (Massaccesi et al., 2013; M. Qian et al., 2017). However, the ratio of total VFA to alkalinity is recognized as another parameter to assess digester process stability (Callaghan et al., 2002; Yeqing Li et al., 2013). It was reported that a VFA to alkalinity ratio of <0.4 results in a stable reactor, while a ratio of >0.8 will result in inhibition (Callaghan et al.,

2002; Yeqing Li et al., 2013; Zickefoose & Hayes, 1976). Therefore, pH and alkalinity were measured to evaluate process stability. Between days 5 to 15, the VFA/Alkalinity ratio was substantially higher than 0.8, suggesting a potential of inhibition. However, pH remained higher than neutral throughout the operating period (results not shown), with a slight decrease in pH from 8.5 to 7.25 observed after the first 5 days of operation. As evident from exponential methane production and VFAs utilization profiles between days 15 to 30, there was no sign of significant inhibition. One reason for this could be due to the ammonia released during hydrolysis, which could provide a buffer to maintain the pH. Ammonium could react with carbon dioxide to form ammonium bicarbonate, which could alleviate acidification (Lee et al., 2019; Q. Li et al., 2017; Michele et al., 2015; Qiao et al., 2013). As discussed later, despite the indication of significant hydrolysis (i.e., VFA accumulation), TAN concentrations in the percolate remained almost stable until day 10. This observation suggests that the released ammonia nitrogen was utilized for buffering the system. A previous study also suggested that high ammonia content could assist in maintaining natural pH in percolate despite the very high VFAs levels in OFMSW fed digester (Michele et al., 2015).

3.3.2.3 Ammonia nitrogen

As shown in Fig. 3.4, TAN concentrations fluctuated between 3636 ± 145 and 4735 ± 51 mg N/L over the operating period. However, a gradual increase over the 30 days could be observed. The concentration on day 0 was 3636 ± 145 mg N/L, while the total concentration on day 30 was 4735 ± 51 mg N/L. The relatively constant TAN levels in the first 5 days, combined with a dramatic increase in VFAs resulted in a dramatic decrease in pH and alkalinity. As TAN slowly increased and total VFA began being consumed, the pH and alkalinity slowly increased. As

discussed earlier, ammonia could be utilized to buffer the system. Nonetheless, TAN concentration in this study was considerably higher compared to previous studies (mostly 1.15-2.5 g/L) (M. Qian et al., 2017; Rico et al., 2020). The unionized form of ammonia (i.e., free ammonia) would be more toxic to methanogenic communities. FAN concentration as low as 215 mg/L could result in an inhibition of methane production, while inhibitory concentration reported in the literature ranged from 215 mg/L to 1450 mg/L (Yenigün & Demirel, 2013). As shown in Fig. 3.4, FAN concentrations on days 0, 20, 25, and 30 were >900 mg N/L. However, as discussed earlier, the methane production pattern did not demonstrate any indication of significant inhibition. Previous studies suggested that digesters acclimatized to high ammonia concentration could run sustainably at high free ammonia nitrogen level up to 1633 mg NH_3-N/L (H. Tian et al., 2017). Thus, it is possible that inoculums have already been acclimatized to high ammonia levels. The liquids inoculum was collected from a HSAD facility. Notably, the initial ammonia level in the percolate (liquid inoculum) was high (3635 ± 145 mg N/L). Moreover, as discussed later, methanogens known to have a higher tolerance to high ammonia level flourished in the reactor during the course of operation, which could be another reason the process stability (H. Tian et al., 2017).

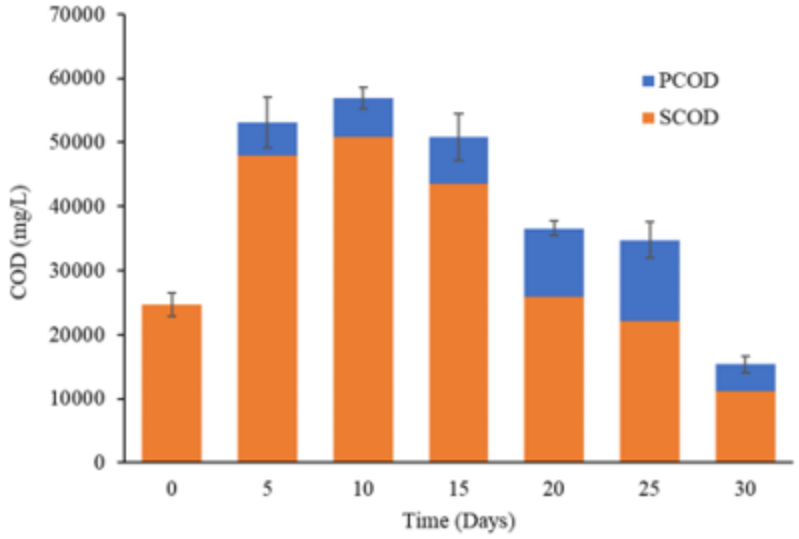

Fig. 3.2 Changes in COD concentrations in the percolate over time

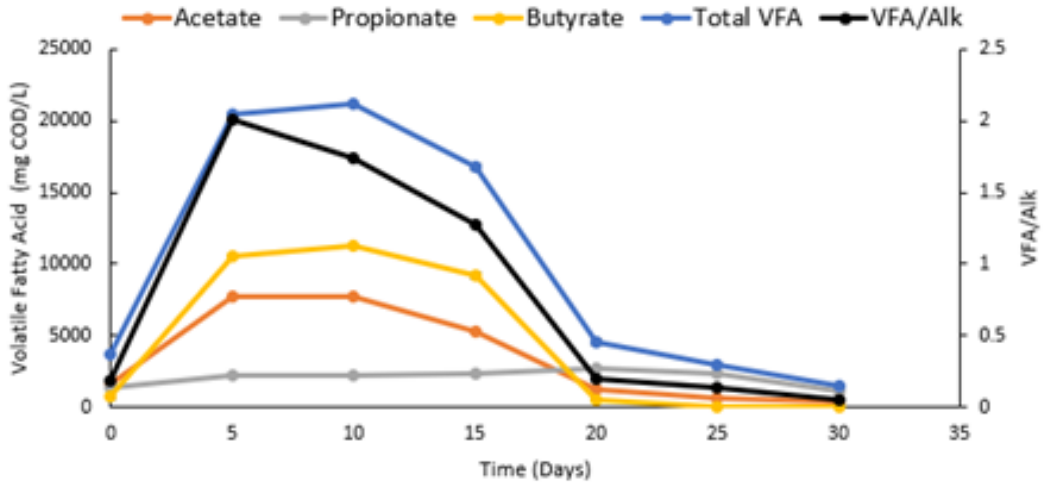

Fig. 3.3 Changes in VFA concentrations and VFA/Alkalinity ratio in the percolate over time

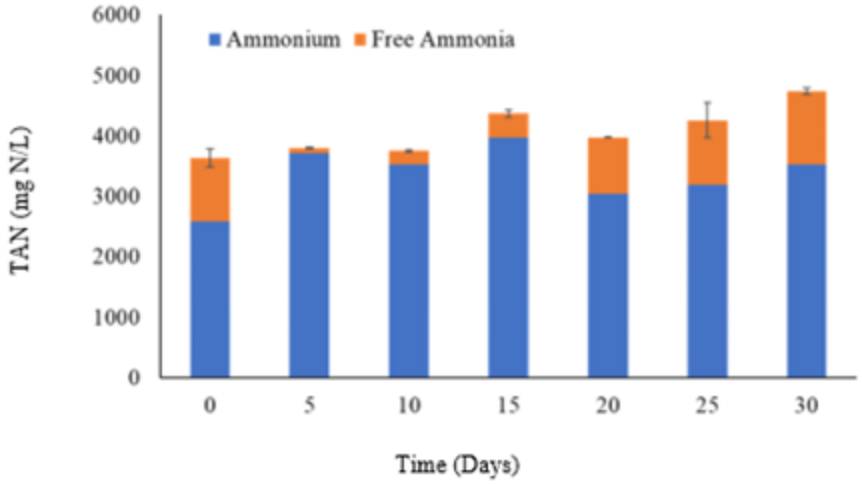

Fig. 3.4 Changes in TAN concentrations in the percolate over time

3.4 Characterization of microbial communities

3.4.1 Microbial diversity and quantity

Several alpha diversity indices, including the observed Operational Taxonomical Unit (OTU), Pielou's evenness, Shannon index, and the phylogenetic distances were calculated to examine the diversity of the microbial communities (see Table 3.1). With the exception of Pielou's evenness, the alpha diversities of the microbial communities decreased in the final digestate samples when compared to the initial biosolids. For instance, the OTUs decreased significantly from 284 in the initial biosolids to 156-200 in the final digestate samples. The phylogenetic distance also decreased from 27.6 initially to 15.9-19.1 in the final digestate.

Comparing the final digestate samples, the digestate from the top and middle of the digester tank showed comparable values in the alpha diversity indices. However, digestate from the bottom of the tank exhibited lower values of OTU, Shannon, and phylogenetic distances. Pielou's evenness was consistent for all three samples. The results indicate that the microbial diversity in the

digestate decreased towards the bottom of the digester tank. This may be the result of substrate gradients developed across the reactor as a result of the percolate inlet being located at the top of the digester tank. A recent study also reported spatial variation of microbial communities in two different layers of a leach-bed reactor (Xing et al., 2020). A decrease in microbial diversity was observed in the liquid percolate over time, with the exception of day 5, which showed a slight increase in microbial diversity. For example, the observed OTUs increased from 158 on day 0 to 179 on day 5, then decreased over the next 25 days to 85 on day 30. A similar trend was also observed for the Pielou's evenness, Shannon's index, and Phylogenetic distance.

Beta diversity was calculated in a principal coordinate analysis (PCoA) plot to examine the similarity of the microbial communities among different samples (Fig. 3.5). The solids (biosolids and digestate) and liquid samples were clearly separated by axis 1 that explained about 39% of the variations. The initial biosolids sample was apparently different from other samples along axis 2 that explained about 29% of the variations. Three final digestate samples taken from the top, middle, and bottom were clustered together, indicating the similarity of their microbial communities. On the other hand, the five liquid samples were gradually scattered along both axes, clearly indicating that the microbial communities in the liquid sample gradually shifted over time.

The qPCR results showed that the quantity of microbes in the solid phase decreased from 1.60×10^{15} copies/g to 7.79×10^{13} – 2.34×10^{14} copies/g (see Table 3.2), which was consistent with the alpha diversity indices. Also, digestates from the top (8.91×10^{13} copies/g) and middle (7.79×10^{13} copies/g) sections had comparable values for microbial cell counts, while the bottom section had a value (2.34×10^{14} copies/g) that deviated from the other two sections. Although the digestate collected from the bottom of the tank had the lowest microbial diversity, the quantity of microbes was highest for this sample. The cell counts for percolate samples also showed a

decrease between day 0 and day 5 from 1.00×10^{14} cells/mL to 7.95×10^{13} cells/mL. It then increased from day 5 to day 30 to a value of 2.26×10^{14} cells/mL. Interestingly, the highest VFA to alkalinity ratio was also observed on day 5 (see Fig. 3.3). Also, methane production also gradually increased after day 5. Thus, these results clearly suggested that dynamic changes in percolate characteristics could considerably influence microbial diversity and community. Also, an opposite pattern was found between microbial diversity and quantity over time.

Table 3.1 Microbial diversity indices

Sample		Observed OTUs	Pielou's evenness	Shannon	Phylogenetic distance
Biosolids		284	0.77	6.3	27.64
Digestate	Top	195	0.83	6.29	18.91
	Middle	200	0.81	6.19	19.13
	Bottom	156	0.82	5.94	15.85
Percolate	Day 0	158	0.81	5.88	16.5
	Day 5	179	0.82	6.17	16.54
	Day 10	140	0.68	4.83	14.61
	Day 15	135	0.62	4.4	14.2
	Day 30	85	0.63	4.05	9.57

Table 3.2 Results of quantitative analysis of microbial communities with qPCR

Sample		Microbial Abundance
Digester tank	Initial (copies/g)	1.60×10^{15}
	Top (copies/g)	8.91×10^{13}
	Middle (copies/g)	7.79×10^{13}
	Bottom (copies/g)	2.34×10^{14}
Percolate tank	Day 0 (copies/ml)	1.00×10^{14}
	Day 5 (copies/ml)	7.95×10^{13}
	Day 10 (copies/ml)	1.11×10^{14}
	Day 15 (copies/ml)	2.39×10^{14}
	Day 30 (copies/ml)	2.26×10^{14}

3.4.2 Archaeal communities

Fig. 3.6 shows the relative abundance of methanogens at the genus level, which also indicates the low microbial diversity in the percolate. The initial percolate was dominated by only three main archaeal genera, which were *Methanocelleus* (67.6%), candidate genus *vadin CA11* (6.7%), and *Methanosarcina* (25.7%). *Methanosarcina* completely disappeared from the percolate after day 5. The percolate samples taken from days 5-15 showed a very similar archaeal community, which were dominated by only *Methanoculleus* (65.3-70.5%) and candidate genus *vadin CA11* (29.5-34.7%). On day 30, only the candidate genus *vadin CA11* remained in the percolate sample.

Methanoculleus is known as a hydrogenotrophic methanogen (L. Li et al., 2016; H. Tian et al., 2018; Ziganshina et al., 2014). *Methanoculleus* sp. has been previously found in HSAD systems and seems to thrive in high ammonia conditions (Bayrakdar et al., 2017; Buhlmann et al., 2019; J. Li et al., 2014; Suksong, Mamimin, et al., 2019). High stress situations (high ammonia and VFAs) has also been reported to be correlated with the dominance of *Methanoculleus sp.* (Dang et al., 2017; Franke-Whittle et al., 2014; Goux et al., 2015; Lerm et al., 2012; L. Li et al., 2016). This might explain the disappearance of *Methanoculleus* on day 30, as the total VFA concentration started to decrease after 10 days of operation. The candidate genus *vadin CA11* belongs in the family *Methanomassiliicoccaceae* (Bravo et al., 2019). Their exact role is unclear, but it has been suggested to metabolize methanol (Buhlmann et al., 2019; Gagen et al., 2017; Kalyani et al., 2017). Similar to *Methanoculleus*, candidate genus *vadin CA11* also appeared to flourish at high TAN concentrations (Buhlmann et al., 2019).

The digestate samples showed a more diverse methanogenic community, as compared to the percolate (Fig. 3.6). This is also highlighted by the higher diversity indices, as shown in Table 3.1. Comparing the initial biosolids to the final digestate samples, genera *Methanocorpusculum*

and *Methanosaeta* declined in digestate samples, while *Methanoculleus* and *Methanosarcina* appeared. A previous study also reported that a *Methanosarcina* specie relatively increased while a *Methanosaeta* specie declined with increasing TAN concentrations (Yan et al., 2019).

In the final digestate samples, *Methanoculleus*, *Methanolinea*, and methanogen in the order of *Methanobacteriales* were present in all three sections. The relative abundance of *Methanobacteriacles* (12.5-22.9%) was similar in all three sections. However, the proportion of *Methanolinea* increased (11.9% vs. 46.4% vs. 43.8%) from top to bottom, while the proportions of *Methanoculleus* decreased (45.5% vs. 35.7% vs. 22.9%). The genera *Methanolinea, Methanoculleus,* methanogen in the order of *Methanobacteriales*, and *Methanocorpusculum* are known as hydrogenotrophic methanogens (Joshi et al., 2018; Karakashev et al., 2005; Kim et al., 2013; Luo & Angelidaki, 2012; H. Tian et al., 2018; Z. Tian et al., 2015; Ziganshin et al., 2013). On the other hand, *Methanosaeta* and *Methanosarcina* are known as acetoclastic methanogens. Depending on the environmental conditions, *Methanosarcina* could also facilitate hydrogenotrophic methanogenesis (Boone et al., 1993; Qu et al., 2009; Yan et al., 2019). *Methanosaeta* completely disappeared in the final digestate, which could be attributed to their exposure to high ammonia levels from the percolate recirculation. Several studies previously reported that *Methanosarcina* would be more tolerant of ammonia inhibition, as compared to *Methanosaeta* (Calli et al., 2005a, 2005b; Yan et al., 2019). Interestingly, *Methanosarcina* also disappeared from the percolate, while they remained in the final digestate. At high ammonia level, *Methanosarcina* species can form clusters to lower the effects of ammonia inhibition (Calli et al., 2005a, 2005b; De Vrieze et al., 2012), which could explain the relatively higher abundance of *Methanosarcina* species in the top section of digester tank where percolate was fed. Nonetheless, known hydrogenotrophic methanogens, which have a higher tolerance to ammonia

inhibition (Sun et al., 2016; H. Tian et al., 2017), dominated the microbial communities in this study. Exposure to high ammonia levels in percolate and its recirculation within the digester tank could explain their higher abundance in both percolate and digester tank.

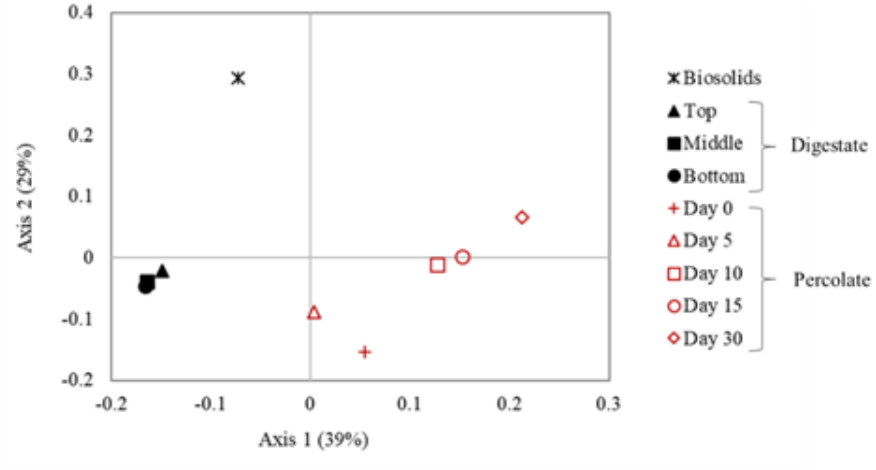

Fig. 3.5 Principal coordinate analysis (PCoA) of microbial communities in the solid and liquid samples based on weighted UniFrac distance matrix

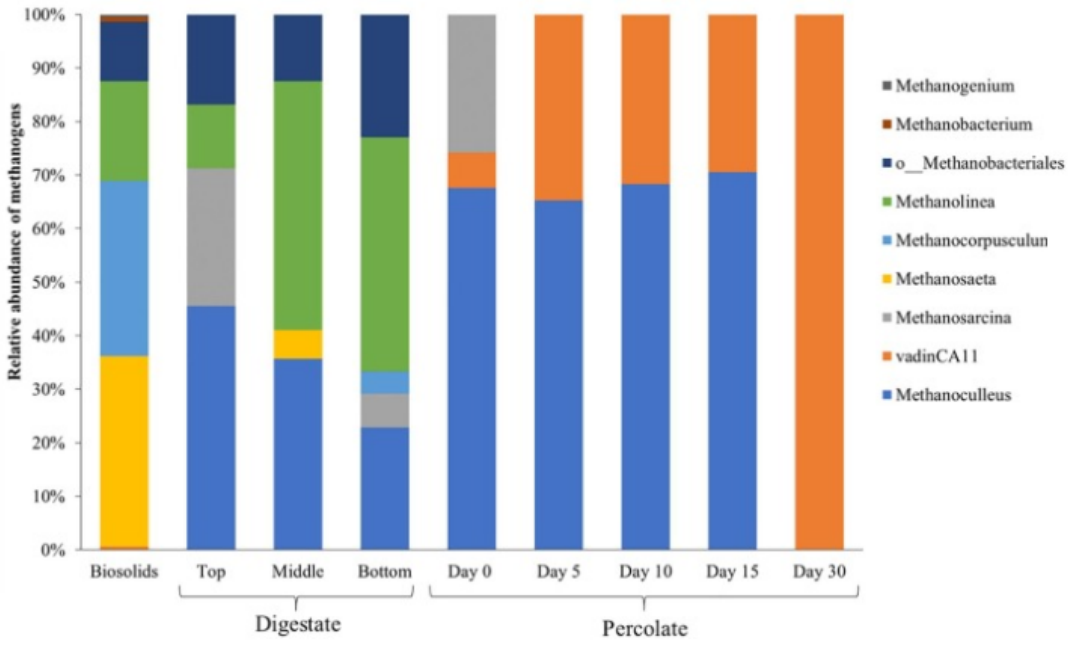

Fig. 3.6 Relative abundance of methanogens at genus level

3.4.3 Bacterial communities

Fig. 3.7 shows the relative abundance of bacterial communities at the phylum level. There was a substantial shift in the bacterial community structures between the initial biosolids and final digestate samples, however the final bacterial communities across the top, middle, and bottom sections were nearly identical. In the digestate samples, the relative abundance of *Firmicutes* increased (8.7% vs. 53.7-55.6%) while proportions of both *Proteobacteria* (29.9% vs. 5.6-7.8%) and *Bacteroidetes* (26.8% vs. 15.9-20.7%) decreased considerably. In the percolate samples, the relative abundance of bacteria also changed over time. From day 0 to day 30, the relative abundances of *Firmicutes* (53.0%-22.4%) decreased gradually, while and *Bacteroidetes* increased from day 0 to 5 (13.1 – 21.8%), then (21.8-6.6%) decreased gradually. However, the proportions of *Proteobacteria* gradually increased (26.4-68.8%) over time.

Fig. 3.8 shows the detailed results of the bacterial communities at the family level. To further the analysis of the bacterial community, the bacterial families that accounted for more than 5% of their population in at least one of the samples were investigated more closely (see Fig. 3.9). The bacterial community at the family level reveals that the initial biosolids sample was dominated by *Pseudomonadaceae* (21.5%), *Anaerolinaceae* (9.6%), and *Cloacamonaceae* (9.2%). The final digestate samples were all dominated by *Anaerolinaceae* (7.0–11.6%), *Porphyromonadaceae* (7.2–9.7%), *Halanaerobiaceae* (3.4–9.0%), *Caldicoprobacteraceae* (3.3–7.2%), and *Tissierellaceae* (2.7–5.6%), but to slightly different degrees. Members of the *Pseudomonadaceae* family were reported to be able to break down cellulose and other substrates (Palleroni, 1981; Weiß et al., 2013). It showed a drastic decrease in relative abundance between the biosolids and final digestate samples (21.5% vs. 1.6-2.5%). Members of *Anaerolineaceae* are suggested to be able to ferment carbohydrates to acetate and H_2 (Jiang et al., 2019; Sekiguchi et al., 2003;

Yamada et al., 2006; Yi et al., 2014; Zamanzadeh et al., 2016). The dominance of *Anaerolinaceae* in all the digestate samples was expected due to the presence of carbohydrates in food waste in the OFMSW (Yi et al., 2014; Zamanzadeh et al., 2016). *Porphyromonadaceae* appears to be associated with high ammonia conditions and some members of the family are suggested to be important bacteria in degrading carbohydrates and proteins (Gao et al., 2019; Hahnke et al., 2015; Ozbayram, Akyol, et al., 2018; Ozbayram, Kleinsteuber, et al., 2018; Sakamoto, 2014). *Porphyromonadaceae* increased significantly from 3.9% initially, to an average of 8.2% at the end of the experiment. Members of *Cloacamonaceae* are suggested to be responsible in the conversion of propionate into H_2, CO_2, and acetate (Dennehy et al., 2017; Esquivel-Elizondo et al., 2016; Hagen et al., 2014; Pelletier et al., 2008). It was dominant in the initial biosolids sample but accounted for <1% of relative abundance in the final digestate solids. Members of the *Halanaerobiaceae* family is said to be able to ferment carbohydrates (Ince et al., 2020; Oren, 2014). Members of *Caldicoprobacteraceae* is said to be able to degrade various organic substrates (Bouanane-Darenfed et al., 2014; Suksong et al., 2020). Based on previous studies, species within this family increased in digesters when the ammonia level increased (Lv et al., 2019; Müller et al., 2016). Nevertheless, it had a higher relative abundance in the middle section of the final solids than the other two sections.

Unlike the solids, *Anaerolinaceae* was not detected in any of the percolate samples. The most abundant bacteria were *Pseudomonadaceae* (13.5–30.7%), followed by *Porphyromonadaceae* (3.5–10%), *Tissierellaceae* (1.9–9.2%), and *Lachnospiraceae* (0.4–5.1%). Interestingly, *Pseudomonadaceae* decreased from 24.5% at day 0 to 13.5–14% on day 5-10, and then increased to 25.4–30.7% during day 15–30. Both *Tissierellaceae* and *Porphyromonadaceae* increased in relative abundances from 4.8-5.0% at day 0 to 9.2-10% on day 5. It then decreased from day 5 to

day 30. As mentioned before, members of *Pseudomonadaceae* are suggested to be able to break down cellulose, while members of *Porphyromonadaceae* are suggested to be able to degrade carbohydrates. Members of *Tissierellaceae* are suggested to be able to degrades complex substrates (Granada et al., 2018; Navarro-Díaz et al., 2016; Niu et al., 2009; Pagliano et al., 2019). Members of *Lachnospiraceae* are suggested to be able to degrade xylose and other sugars (Cotta & Forster, 2006; Han et al., 2017; Suksong, Kongjan, et al., 2019; Zheng et al., 2018). Members within these family (*Lachnospiraceae* and *Tissierellaceae*) also appear to be related to ammonia (Esquivel-Elizondo et al., 2016; Gao et al., 2019; Han et al., 2017; Müller et al., 2016). Thus, similar to the archaea population, bacterial species that seems to be associated with ammonia (e.g., members within *Porphyromonadaceae, Tissierellaceae, Lachnospiraceae*, and *Caldicoprobacteraceae*) were present.

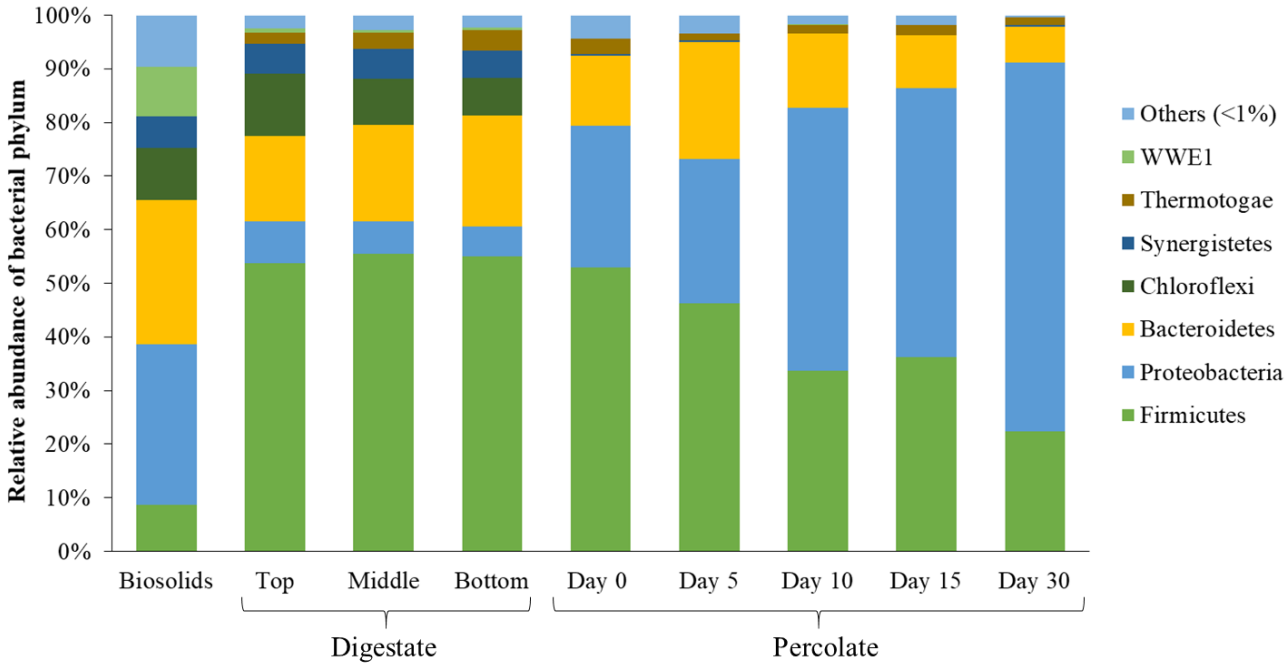

Fig. 3.7 Relative abundance of bacterial community at phylum level

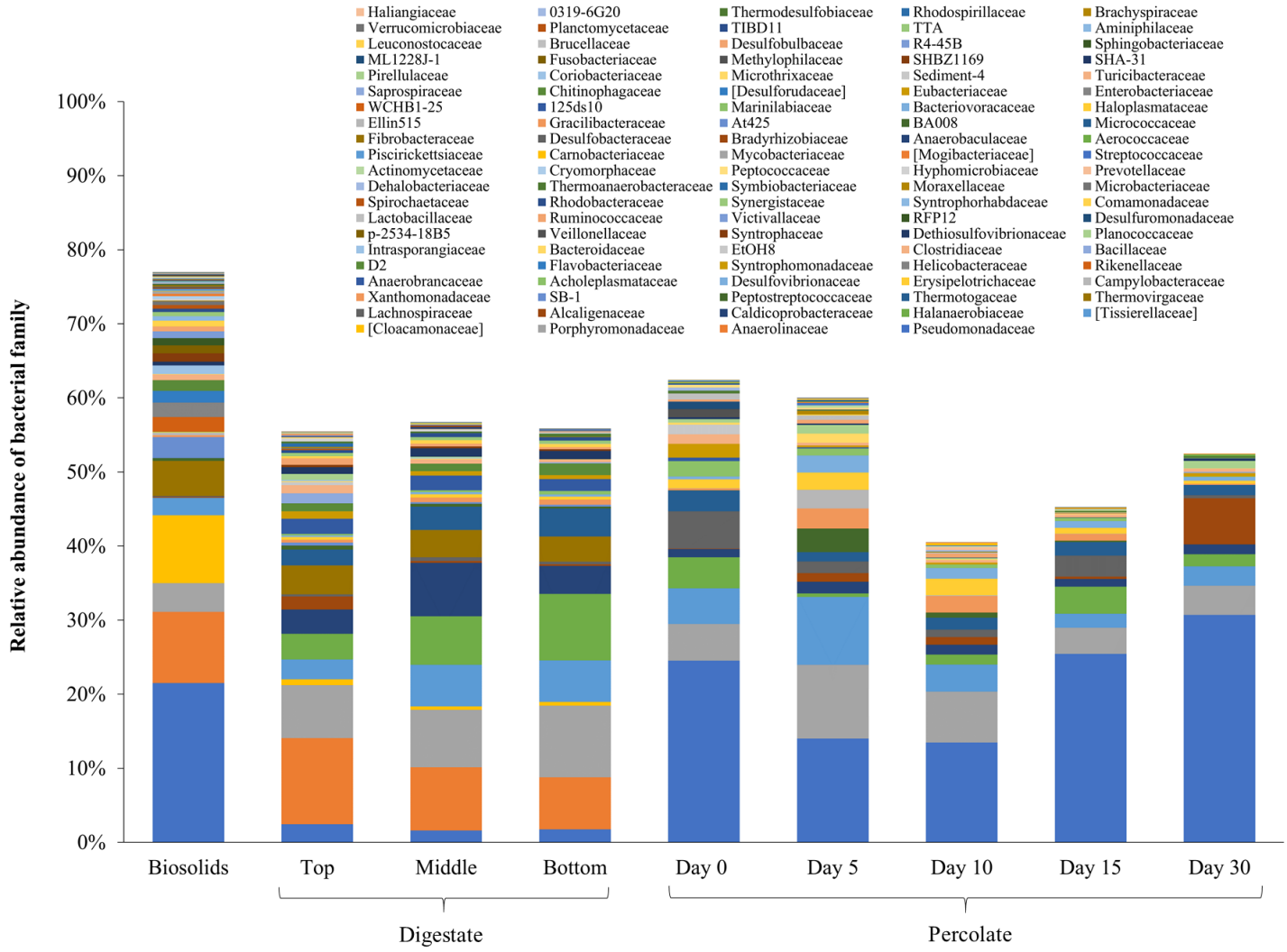

Fig. 3.8 Relative abundance of bacterial community at family level

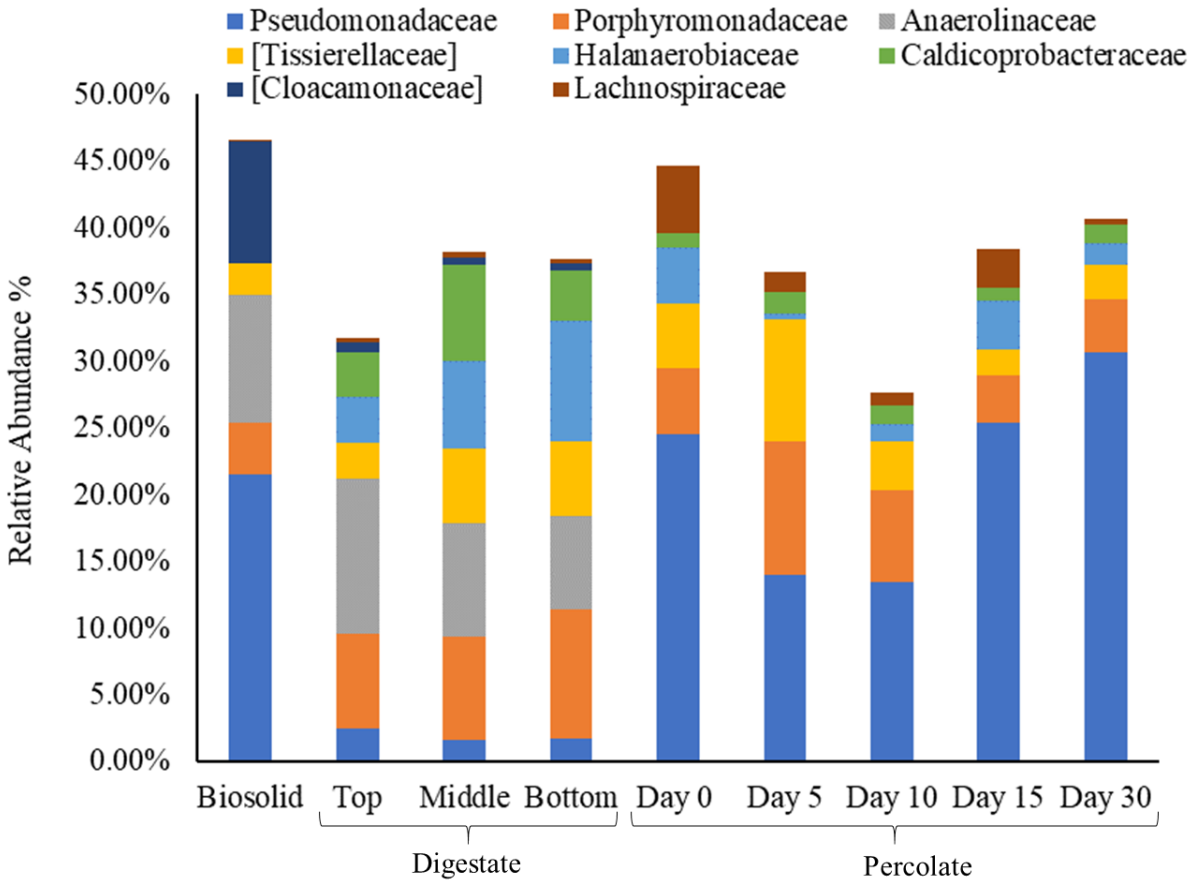

Fig. 3.9 Relative abundance of bacterial community at family level of bacteria that had greater than 5% relative abundance

Chapter 4 Conclusions and recommendations

4.1 Conclusions

The results of this study showed how microbial communities changed in a HSAD with percolate recirculation during batch operation. The initial rapid hydrolysis/fermentation of OFMSW could rapidly increase VFAs/Alkalinity ratios to a level usually known to cause process instability; however, ammonia nitrogen released due to hydrolysis could provide self-buffering capacity. Nonetheless, the microbial communities shifted towards known hydrogenotrophic methanogens and ammonia-tolerant bacteria/methanogens along with a considerable decrease in microbial diversity. These changes were explained by the dynamic changes in VFAs/Alkalinity, TAN, and pH. Also, it was evident that the percolate feeding location could induce a spatial heterogeneity in the microbiome in the digester tank.

4.2 Limitations and recommendation

Although chapter 3 demonstrated interesting results, further research is still needed to further bridge the gap between wet and dry anaerobic digestion. Future investigations into this topic should explore the microbial community to gain better insight into the exact mechanism. As well, there were several limitations to the experiment which should be addressed.

- Calibration should be improved for future experiments. It was recognized over time that the calibration of the gas meter used during the experiment should have been done more frequently. The calibration of the gas meter in this experiment was done once at the beginning and once at the end. This lack of calibration may lead to some inaccuracies in

gas measurement. However, a similar experiment conducted later which had calibration completed every 2-3 days showed similar gas production. Similarly, better calibration for the VFA samples could aid in future experiments. It was noted that some samples had higher VFA concentrations than the calibration standards (This meant some of the sample concentrations had to be extrapolated). This would lead to inaccurate VFA concentration readings for some samples. However, the trend of the VFA (the high concentration initially followed by the decline) is still considered valuable information. Nevertheless, future VFA samples should be diluted further to ensure it is within the calibration standard.

- Samples for microbial analysis was obtained for the top middle and bottom sections. The extraction process required <0.3 g of digestate per section. Duplicate analysis should be conducted for each section if localized inhibition/mass transfer limitation exists.
- Although the heating tape was set to 37°C, the solid digestate was unable to efficiently transfer the heat. Thus, the digestate in the middle was a lower temperature compared to the digestate near the walls (31-35°C). Additionally, the heating tape provided for the percolate tank did not provide heating from the bottom (only the sides), thus during low levels of percolate, lower temperature was observed (33-37°C). Nevertheless, these temperatures are still within the mesophilic temperature range. However, it is recommended for future experiments to place the percolate tank in a water bath for more consistent heating and to increase the temperature provided to the solid digester tank to ensure higher temperatures in the centre.
- A clogging issue was noted which resulted in the significant reduction of percolate volume over time. This was likely caused by one of two reasons. The first being the

clogging of the pipes due to transfer of solids into the liquid digester tank. The second could be the consolidation/compaction of the solid material as percolation and the digestion process occurred. This consolidation of material would lead to a reduction in pore space and reduce the ability of percolate to freely flow. The addition of bulking material is recommended to maintain the pore space in future experiments.

- It should be noted that an elemental analysis of the waste was not conducted for the experiments. The elemental analysis would allow for the calculation of a theoretical methane yield, and thus, allow us to further evaluate the reliability of the results. Further research should incorporate an elemental analysis as a check.
- One recommendation from this experiment is to conduct analysis on the final digestate to determine the presence of localized inhibition. A variation in parameters such as TS, VS and or COD could potentially indicate the presence of localized inhibition. This would require an alternation in process parameter or design in order to reduce the effects of localized inhibition. Conducting tests on different sections (i.e. taking samples from top middle and bottom vs. taking samples from the middle) could also provide data which is more representative of the final digestate if localized inhibition is present.
- The location of introduction for percolate in the digester could affect the presence of localized inhibition. Therefore, future studies should focus on the location of percolate recirculation as well as different reactor shapes/sizes (i.e. cylindrical vs square digesters) and their effects on AD performance. Additionally, future studies could also focus on repeated batches. In this experiment, the analysis was conducted for one batch. Future research should be done to monitor consecutive batches (i.e. taking half the digestate and mixing it with new feedstock).

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Appendix A

Fig. A.1 Initial samples received from EWMC of the biosolid

Fig. A.2 Initial samples received from EWMC of the percolate

Fig. A.3 Initial samples received from EWMC of the waste

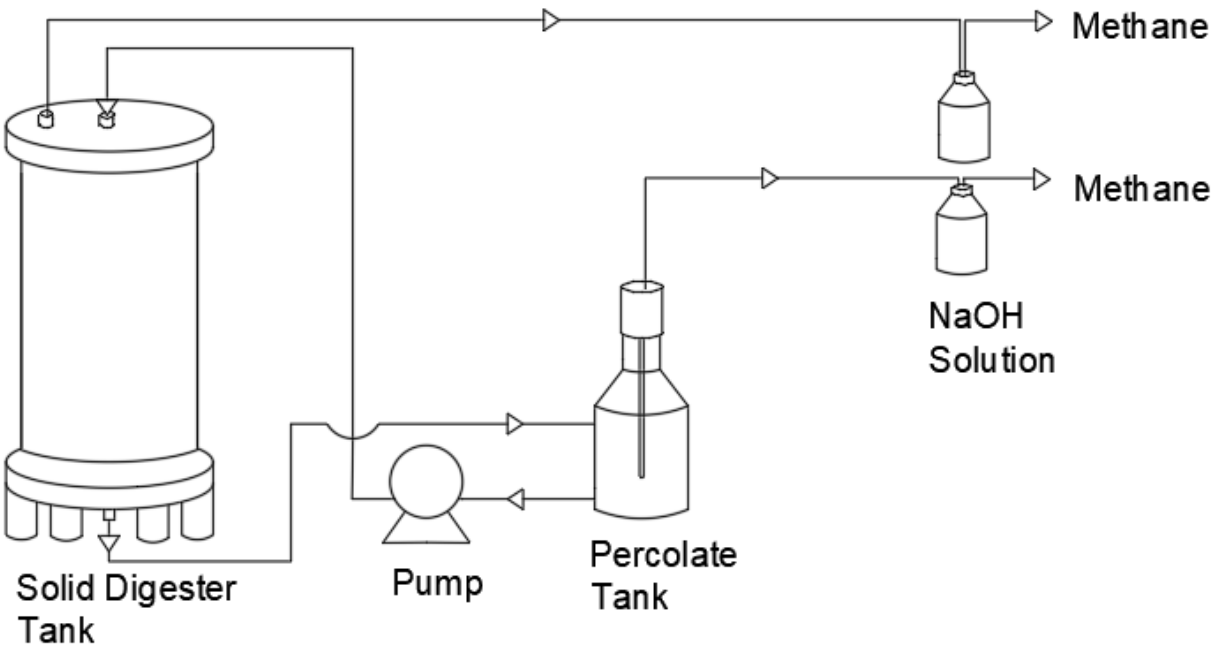

Fig. A.4 Diagram of reactor setup with percolate tank and digester tank

Fig. A.5 Photo of solid digester with digestate