EFFECT OF OZONE PRETREATMENT ON METHANE PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF SINGLE-STAGE ANAEROBIC DIGESTERS

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EFFECT OF OZONE PRETREATMENT ON METHANE PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF SINGLE-STAGE ANAEROBIC DIGESTERS

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ABSTRACT

EFFECT OF OZONE PRETREATMENT ON METHANE PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF SINGLE-STAGE ANAEROBIC DIGESTERS

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Increasing amount of sludge produced by wastewater treatment plants poses many problems in environmental, public health and economic aspects. Anaerobic digestion (AD), the most common sludge stabilization method, is used to reveal energy potential of sludge as methane-rich biogas. Various pretreatment methods are applied to enhance AD efficiency by facilitating the rate limiting hydrolysis step. Ozone pretreatment is a remarkable method for improving methane production, sludge reduction and pathogen removal. Therefore, this study investigated the effect of ozone pretreatment with varying doses of 0.03, 0.06 and 0.09 g O_3/g TSS on methane production and microbial communities of mesophilic single-stage anaerobic digesters operated as semi-batch. Anaerobic digesters were monitored in terms of pH, temperature, chemical oxygen demand, total nitrogen, solids content, total volatile fatty acids, methane production and microbial community structure. Changes in methane and microbial community structure were determined by gas chromatography and fluorescence *in situ* hybridization (FISH) methods, respectively. Pretreatment with 0.06 g O_3/g TSS resulted in 47% methane increase

by achieving the highest methane content of 78%. FISH analyses revealed that *Methanosaeta* spp. were the most dominant methanogens in this pretreated digester.

Keywords: Single-stage anaerobic digester, Ozone pretreatment, Methane production, *Methanosaeta* spp.

ÖZ

OZON ÖN ARITIMININ TEK AŞAMALI ANAEROBİK ÇÜRÜTÜCÜLERDEKİ METAN ÜRETİMİ VE MİKROBİYAL KONSORSİYUM ÜZERİNE ETKİSİ

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Atıksu arıtma tesislerinde üretilen çamur miktarının giderek artması çevresel, halk sağlığı ve ekonomik açıdan birçok sorunu beraberinde getirmektedir. En yaygın çamur stabilizasyon yöntemi olan anaerobik çürütme (AÇ), çamurun enerji potansiyelini metan bakımından zengin biyogaz olarak ortaya çıkarmak için kullanılmaktadır. Çeşitli ön arıtım yöntemleri hız sınırlayıcı hidroliz adımını kolaylaştırarak AÇ verimliliğini artırmak için uygulanmaktadır. Ozon ön arıtımı, metan üretiminin iyileştirilmesi, çamur azaltımı ve patojen giderimi için uygulanan dikkate değer bir yöntemdir. Bu nedenle, bu çalışmada 0.03, 0.06 ve 0.09 g O_3/g TSS dozlarındaki ozon ön arıtımının yarı kesikli işletilen mezofilik tek aşamalı anaerobik çürütücülerdeki metan üretimi ve mikrobiyal konsorsiyum üzerindeki etkisi araştırılmıştır. Anaerobik çürütücüler pH, sıcaklık, kimyasal oksijen ihtiyacı, toplam nitrojen, katı madde içeriği, toplam uçucu yağ asitleri, metan üretimi ve mikrobiyal konsorsiyum açısından izlenmiştir. Metan ve mikrobiyal konsorsiyumdaki değişiklikler sırasıyla gaz kromatografisi ve floresan *in situ* hibridizasyon (FISH) yöntemleri ile belirlenmiştir. 0.06 g O_3/g TSS ile yapılan ön arıtım, 78%'lik en yüksek metan içeriğine ulaşarak metan üretiminde %47'lik bir artış sağlamıştır.

Yapılan FISH analizleri, bu ön arıtımda çalıştırılan anaerobik çürütücüde *Methanosaeta* türlerinin en baskın metanojenler olduğunu ortaya çıkarmıştır.

Anahtar Kelimeler: Tek aşamalı anaerobik çürütücü, Ozon ön arıtımı, Metan üretimi, *Methanosaeta* türleri

To my family

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Love you all… but not you coronavirus! :)

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ABBREVIATIONS

AD: Anaerobic Digestion

ASBR: Anaerobic Sequencing Batch Reactor

COD: Chemical Oxygen Demand

CSTR: Continuously Stirred Tank Reactor

DAPI: 4′,6-Diamidino-2-Phenylindole Dihydrochloride

DGGE: Denaturing Gradient Gel Electrophoresis

EGSB: Expanded Granular Sludge Bed

FA: Formamide

FISH: Fluorescence *in situ* Hybridization

FITC: Fluorescein Isothiocyanate

GC: Gas Chromatograph

GHG: Greenhouse Gas

HRT: Hydraulic Retention Time

MAR-FISH: Microautoradiography Combined with FISH

NGS: Next Generation Sequencing

OLR: Organic Loading Rate

PBS:EtOH: Phosphate-Buffered Saline and Ethanol

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

qPCR: Quantitative Polymerase Chain Reaction

Raman-FISH: Raman Microspectroscopy Combined with FISH

RAS: Return Activated Sludge

SIP: Stable Isotope Probing

SRB: Sulfate Reducing Bacteria

SRT: Solids Retention Time

SSCP: Single-Strand Conformation Polymorphism

TE: Tris-EDTA

TN: Total Nitrogen

T-RFLP: Terminal-Restriction Fragment Length Polymorphism

TS: Total Solids

TSS: Total Suspended Solids

UASB: Upflow Anaerobic Sludge Blanket

VFA: Volatile Fatty Acids

VS: Volatile Solids

VSS: Volatile Suspended Solids

WAS: Waste Activated Sludge

WWTP: Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

In today's world, environmental problems and energy requirements have increased as consequences of rapid population growth and industrialization. In parallel with high water consumption trend, larger amounts of waste and wastewater have been generated day by day. Resulting wastewater and sewage sludge imposes a burden on wastewater treatment plants (WWTPs) in environmental and economic aspects. Although biological process seems to be a primary issue in the wastewater treatment, management of residual sludge is one of the main problems to be solved in terms of technical and ecological applications (Yüksekdağ et al., 2020). Along with the environmental investments in wastewater treatment, sustainable sludge treatment and disposal have gained importance with the technological developments and the legal regulations made during the European Union harmonization process (*Evsel/Kentsel Arıtma Çamurlarının Yönetimi Projesi*, 2015).

Daily average production of sewage sludge per household varies between 40-60 g for both municipal and industrial facilities (Salan, 2014). In Turkey, it is estimated that 1600 tons of activated sludge are produced daily by assuming 60 g dry matter/day per capita (*Evsel/Kentsel Arıtma Çamurlarının Yönetimi Projesi*, 2015). Since sludge management is a complicated and costly process, not only sludge reduction and stabilization but also energy recovery from the sludge have drawn a great interest (Şahinkaya, 2011). Therefore, scientific studies on innovative and more sustainable treatment processes for a long-term sludge management have been increasing (Rulkens, 2004).

Because of hazardous composition, sludge should be stabilized to eliminate organics and pathogens before disposal so that putrefaction does not occur (Metcalf & Eddy, 2003). Anaerobic digestion (AD) is the most common and well-practiced sludge stabilization method (Tyagi & Lo, 2016). As compared to other stabilization methods such as lime addition and composting, AD is one step ahead because it provides both reduction of sludge volumes and the production of a renewable energy source: biogas (Zupančič & Grilc, 2012). Typical biogas is composed of mainly 55-65% methane, 35-45% carbon dioxide and trace gases (Adebayo et al., 2015). Biogas obtained from anaerobic digesters can be stored by transforming into electricity and heat energy in combined heat and power plants to be used directly at WWTPs or to be given to the municipal electricity grid (Dilek, 2015). Moreover, biogas can be treated to get biomethane for using it as a biofuel just like natural gas after removing other gases (U.S. Energy Information Administration, 2020).

AD is a biological process in which organic portion of sludge is converted to biogas by microorganisms under anaerobic conditions and at certain temperatures (Anukam et al., 2019). Recently, some pretreatment methods on AD have emerged in order to enhance biogas or methane production and to reduce the amount of sludge (Ariunbaatar et al., 2014). Ozone pretreatment is one of the most outstanding methods particularly for already existing anaerobic digesters due to being feasible, cheap in low doses, easily applicable and adoptable to system (Bougrier et al., 2007). Use of ozone in appropriate doses prior to anaerobic digesters increases biogas and methane production without any chemical residue, pathogens and change in salt concentration (Ariunbaatar et al., 2014). However, it should be determined which ozone doses to be applied for an efficient operation providing higher methane production.

Challenging part of improving AD comes from its microbiology. AD includes specific microorganisms with different physiological properties, growth conditions and metabolic activities (Amani et al., 2010). Performance of AD highly depends on

complex interactions and dynamics among microbial communities (Camacho & Ruggeri, 2018). Until recently, roles and requirements of microbial communities are not well-understood due to inadequate knowledge about microbiology of AD (Gerardi, 2003). Thus, molecular techniques have been used to understand of microbial composition in AD (Shin et al., 2019). As one of these techniques, fluorescence *in situ* hybridization (FISH) method enables to quantify microbial communities and their specific members (Dinova et al., 2018). In this way, AD process can be improved by determining and favoring microorganisms effective in methane production.

In the literature, correlation between microbial communities and methane production is not well-established. Moreover, there is no information about how ozone pretreatment affects methane production with respect to microbial shifts. Optimization of ozone doses to obtain a meaningful increase in methane production is another object of interest. Therefore, this study aimed at evaluating changes in methane production and microbial community structure in ozone pretreated anaerobic digesters. For that purpose, single-stage mesophilic anaerobic digesters pretreated with varying ozone doses of 0.03, 0.06 and 0.09 g O_3/g TSS were monitored in terms of sludge characteristics, methane production and microbial community structure in this study.

CHAPTER 2

LITERATURE REVIEW

2.1. Sludge problem and its treatment

Sludge accumulates as a semi-solid residue in all sorts of wastewater treatment. The amount of wastewater and sludge generated by WWTPs is increasing day by day due to excessive water consumption. Although the quantity of produced sludge in a WWTP is about 1% of the quantity of treated wastewater, treatment and disposal of sludge takes much longer than of wastewater (Turovskiy & Mathai, 2006). For that reason, sludge treatment is more expensive than wastewater treatment, therefore, proper sludge management in a cost-effective way is essential.

In many conventional municipal WWTPs as shown in Figure 2.1, two types of sludge are generated as primary and secondary sludge. Primary sludge is composed of floating and settled solids collected at primary sedimentation tank while secondary or waste activated sludge (WAS) is composed of suspended solids and microbial cells collected at secondary sedimentation tank after biological treatment of wastewater (Mondala et al., 2009). Traditionally, primary and secondary sludge are combined and called as mixed or raw sludge for further sludge treatment (Stehouwer, 2010).

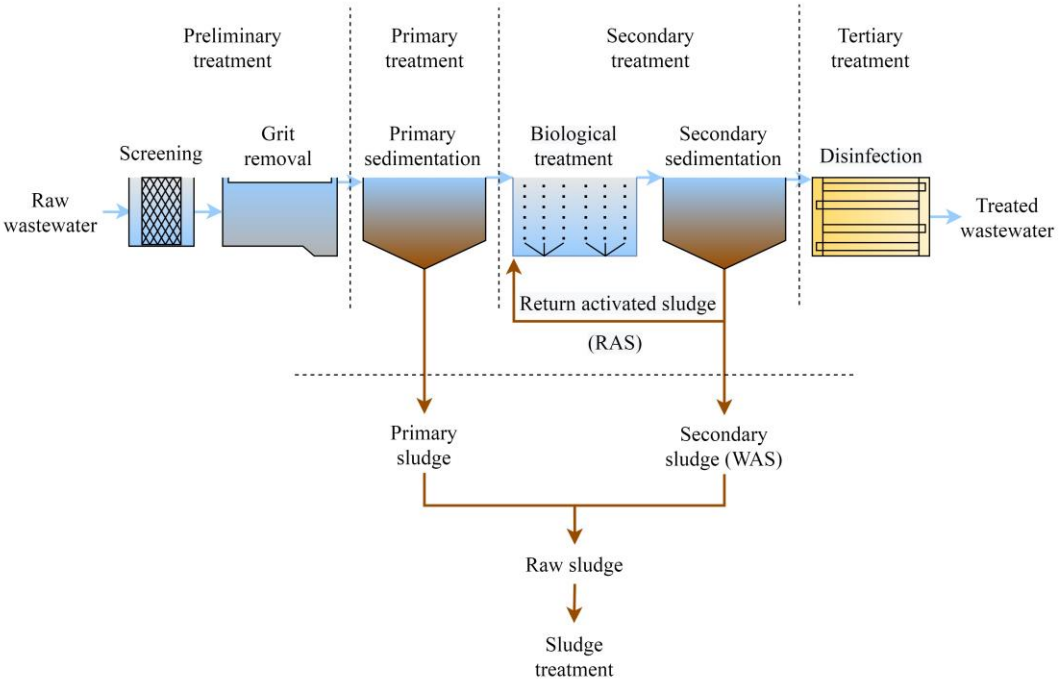

Figure 2.1. Schematic diagram of typical wastewater treatment processes

Even though sludge composition varies for each WWTP, raw sludge is mainly composed of organics, inorganics, nutrients, heavy metals and pathogens (Bharathiraja et al., 2014). Because of unstable, degradable and pathogenic nature, raw sludge has potential risks for both environment and public health (Stehouwer, 2010). Therefore, several sludge treatment processes have been developed for stabilizing sludge, reducing sludge volume and pathogens. Typical sludge treatment processes involve thickening, stabilization, conditioning, dewatering and disposal as shown in Figure 2.2 (Englande et al., 2015).

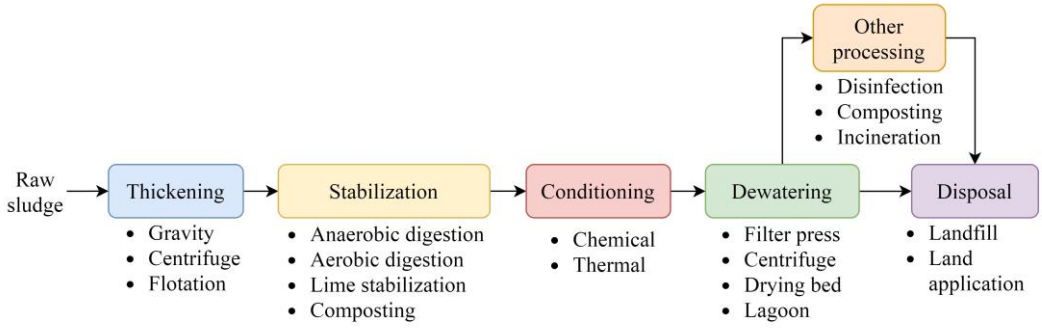

Figure 2.2. Typical sludge treatment processes adapted from Englande et al. (2015)

First step of sludge treatment is usually thickening, where sludge volume is reduced by concentrating solids in sludge with the removal of aqueous portion (Sanin et al., 2011). Secondly, sludge is stabilized to reduce biodegradable organics and pathogens causing odor and putrefaction problems (Metcalf & Eddy, 2003). Stabilized sludge can be conditioned with chemicals or heat for enhancing dewatering characteristics of sludge (Olivier et al., 2018). Dewatering is done for sludge volume reduction by removing moisture in order to make handling and disposal easier and cheaper. Other processes such as disinfection, composting and incineration can be applied when required before disposal. Lastly, treated sludge is either disposed in landfills or applied to lands for agricultural use if applicable (Baily, 2009).

Among sludge treatment processes, stabilization is the most important step since its operation specifies and affects following treatment and disposal options. Sludge stabilization is based on the principle of biological or chemical degradation of volatile compounds and disinfection. Common stabilization options are anaerobic or aerobic digestion, lime stabilization and composting (Water Environment Federation, 2007). The selection of which stabilization method used depends on the amount of sludge treated, regulations, cost, available technologies and its integration with other units. Overview of sludge stabilization methods is given in Table 2.1.

Table 2.1. Overview of sludge stabilization methods (Petta, 2008)

Method	Description	Advantages	Disadvantages
Anaerobic digestion (AD)	Biodegradation of organic matter without oxygen at specific temperatures in an enclosed reactor	- Biogas and energy recovery - Less sludge production with higher sludge stability - Lower operational cost - Beneficial by-products can be used as fertilizer - Greenhouse gases reduction - Odor reduction - Good pathogen reduction - Smaller area requirement	- Higher capital cost - Longer retention times - Harder operation and maintenance - Sensitive process - Less effluent quality
Aerobic digestion	Biodegradation of organic matter with continuous oxygen supply in an aerated tank	- Simpler operation and less maintenance - Lower capital cost - Better effluent quality - Higher stability and fertilizer value of sludge - No odor problem	- Higher energy requirement - Higher operational cost - Poor dewatering characteristics of sludge - No useful by-products
Lime stabilization	Addition of lime to obtain high pH for elimination of pathogens	- Easier operation - Lower operational cost - Good pathogen control - Good emergent stabilization	- Higher sludge production - Odor problem
Composting	Mixing of sludge with carbon rich bulking agents in piles or windrows for biological conversion	- Easier operation - Lower operational cost - Agricultural use of sludge - Good volatile solids reduction - Good pathogen inactivation	- Larger area requirement - Need of bulking agents - Weather dependent - Odor problem

2.2. Anaerobic digestion (AD)

Because of the operational and financial problems encountered in aerobic sludge processes, interest on anaerobic processes providing energy output has increased. AD, which have been widely used for decades, has begun to be considered as a well-accepted and preferred waste-to-energy method for sludge stabilization (Meegoda et al., 2018).

From the environmental point of view, AD offers many benefits as (Pullen, 2015):

- Producing biogas, a renewable energy source that can be used as electricity, heat or fuel,
- Ensuring well-stabilized sludge with great dewatering capacity that can be used as a fertilizer,
- Reducing pathogens threatening public health,
- Controlling odor which is an aesthetic concern,
- Handling high organic loading rates,
- Lowering sludge volumes to be disposed and greenhouse gas (GHG) emissions from landfills,
- Economic and sustainable process in long term considering energy output,
- Suitable for pre- and post-treatment methods.

The biological growth rate and additional nutrient requirement in AD are less than in aerobic processes. Only 5-15% of organic carbon is converted into biomass in anaerobic processes (Libhaber & Jaramillo, 2012). Thus, sludge disposal after AD is easier and lower cost than aerobic process. On the other hand, AD has some limitations. Due to low growth rate, AD needs longer retention times (Libhaber & Jaramillo, 2012). It is a sensitive process because of the delicate balance among microorganisms involved. AD is costly to build and technically skilled labor is required in its operation and maintenance. Also, there is an explosion risk due to methane content of biogas produced (Vesilind, 2003).

AD process takes place in a closed oxygen-free tank called as anaerobic digester. Anaerobic digesters provide two main valuable products as biogas and digestate. Schematic diagram of an anaerobic digester is shown in Figure 2.3. Biogas production is the key property of AD. The quality and quantity of biogas produced depends on several factors such as feedstock composition, digester configuration and operating conditions. Biogas is composed of mostly methane, carbon dioxide and other trace gases as given in Table 2.2 (Adebayo et al., 2015).

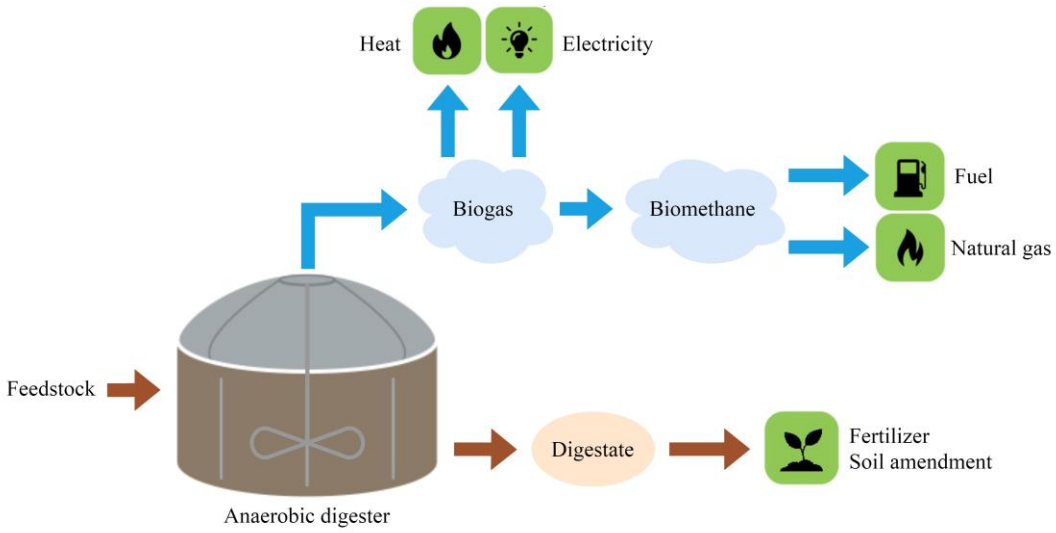

Figure 2.3. Schematic diagram of anaerobic digester

Table 2.2. Typical biogas composition (Adebayo et al., 2015)

Content	**Range (%)**
Methane (CH_4)	55-65
Carbon dioxide (CO_2)	35-45
Nitrogen (N_2)	0-10
Hydrogen (H_2)	0-1
Hydrogen sulfide (H_2S)	0-3
Ammonia (NH_3)	0-3
Carbon monoxide (CO)	0-3
Oxygen (O_2)	0-2

The most valuable component of biogas is methane. The calorific value of biogas depends on its methane content, but it is stated in the literature as in the range of 16.8-23.0 MJ/m^3 (Masłoń, 2020). Energy release after the combustion or oxidation of methane turns biogas into a clean renewable fuel (Bhatia, 2014). Biogas has wide applications all around the world. After biogas is captured and stored, it can be utilized for generating heat and electricity or upgraded into a biomethane for using as natural gas and vehicle fuel (Tanigawa, 2017). Operational parameters and types of digesters are important determining factors for biogas and methane produced in anaerobic digesters.

Throughout years, AD has been made great progress in process knowledge, design and control. The privilege of energy recovery ensures that AD maintains its importance. Today, many WWTPs prefer AD to meet their electricity and energy needs from biogas produced (Metcalf & Eddy, 2003). Excess portion of biogas produced is stored for future uses or given to electric network. Therefore, lots of researches and efforts have been made to improve AD for more biogas production.

2.2.1. Process description

AD is a process which organic matter in a feedstock is converted to methane-rich biogas by microorganisms in the absence of O_2 (Tang & Sillanpää, 2018). Various microorganisms and biochemical reactions are involved in that conversion of complex organics to biogas. According to the specific microorganisms responsible for the process, AD follows four consecutive steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Figure 2.4 (P. Wang et al., 2018).

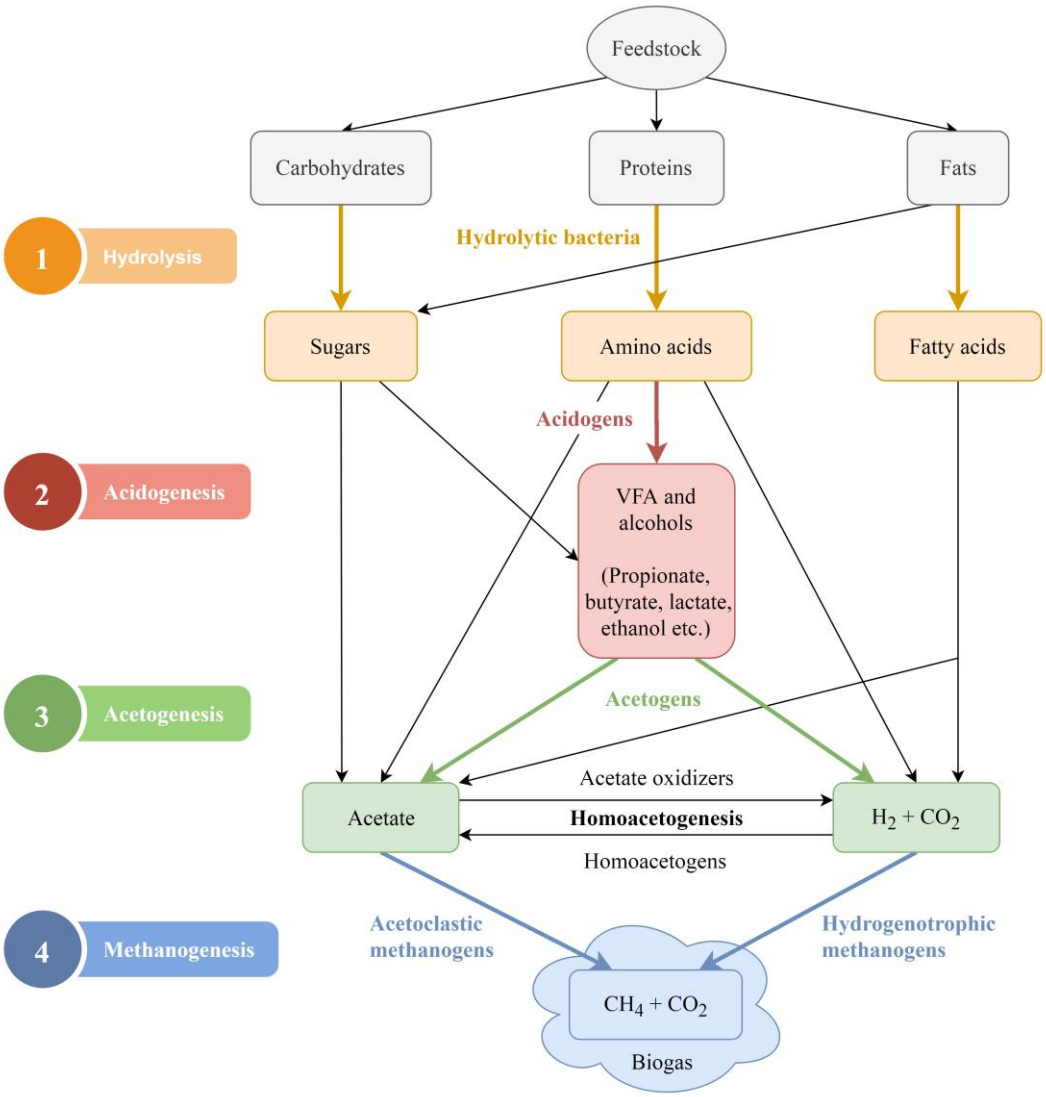

Figure 2.4. AD process adapted from Wang et al. (2018)

Hydrolysis: Hydrolysis is the first step of AD in which insoluble complex organic polymers (e.g. carbohydrate, protein, and fat) are converted into simpler monomers (e.g. sugar, amino acid and fatty acid) by strict anaerobic hydrolytic microorganisms with the help of extracellular enzymes (Chen & Neibling, 2014). Hydrolysis is an important step because it allows microorganisms to use non-utilizable large molecules as a substrate. The rate of degradation during hydrolysis depends on the nature of the substrate (Adekunle & Okolie, 2015). Some resistant compounds like

cellulose cannot be easily broken down so, they are hydrolyzed slowly (Schnürer & Jarvis, 2009). Thus, hydrolysis is the rate-limiting step affecting overall AD process (Anukam et al., 2019).

Acidogenesis: Acidogenesis or fermentation is the second step of AD. In acidogenesis, hydrolyzed compounds are converted into volatile fatty acids (VFA) such as acetic, propionic and butyric acid by acidogens (Merlin Christy et al., 2014). Other end products of this step are alcohols, hydrogen (H_2), carbon dioxide (CO_2) (Merlin Christy et al., 2014). Additionally, trace amounts of ammonia (NH_3) and hydrogen sulfide (H_2S) which are the reasons of odor problem are formed in this step. This conversion leads to pH drop that favors the action of acidogens and acetogens since they prefer slightly acidic conditions (Náthia-Neves et al., 2018). VFA production in this step is a precursor for methane (CH_4) production. The composition of VFA varies depending on the factors such as operational parameters, microbial communities and sludge composition (Lukitawesa et al., 2020).

Acetogenesis: The third step of AD is acetogenesis where VFA and alcohols produced in acidogenesis are converted into acetate (CH_3COO^-), H_2 and CO_2 by acetogens (Chen & Neibling, 2014). H_2 released during the process show toxic effects on acetogens so, a symbiosis relationship between acetogenesis and the next step methanogenesis is crucial for using that H_2 (Anukam et al., 2019). It is hard to differentiate acidogenesis and acetogenesis because their biochemical reactions are typified by the production of acetate and H_2, which are the substrates for methanogens (Bajpai, 2017). The achievement of the acetogenesis determines the efficiency of biogas production since 70% of CH_4 is generated through the reduction of acetate (Nguyen et al., 2019). Another pathway of acetogenesis is homoacetogenesis which is the conversion of H_2 and CO_2 into acetate by homoacetogens. Reverse of this conversion is accomplished by acetate oxidizers (Patel et al., 2017).

Methanogenesis: The last and most important step is methanogenesis. Methanogens which are strict anaerobes convert acetate and H_2 into biogas containing CH_4 and CO_2 in this step (Anukam et al., 2019). VFA accumulation in acidogenesis at high organic loads may restrict methanogenesis due to sensitivity of methanogens to pH drops. Depending on methanogens and substrates, methanogenesis can follow two pathways as acetoclastic and hydrogenotrophic (Gerardi, 2003). Acetoclastic methanogens use acetate as a substrate to produce CH_4 whereas hydrogenotrophic ones use H_2 and CO_2 (Náthia-Neves et al., 2018). Generally, 28% of CH_4 produced in AD comes from H_2 and CO_2 while 72% from acetate (Metcalf & Eddy, 2003). In collaboration with all these microorganisms, AD process is carried out.

2.2.2. Operational parameters

The operation of anaerobic digesters is monitored by some parameters which are the indicators of process efficiency. Even one of the parameters is outside of the desired range, process performance will be negatively affected (Kundu et al., 2017). Main operational parameters need to be considered are presented in the following sections.

2.2.2.1. Solids and hydraulic retention times (SRT and HRT)

Solid retention time (SRT) and hydraulic retention time (HRT) are important parameters to determine organic matter and volatile solids amount to be fed into anaerobic digesters (Alepu et al., 2016). SRT is the average time that sludge or biomass remains in anaerobic digester while HRT is the average time that wastewater remains (Gerardi, 2003). SRT equals to HRT for anaerobic digesters with no recycle (Metcalf & Eddy, 2003). After this point, SRT term will be used to refer retention time. SRT is calculated by dividing reactor volume into flowrate. It is desired that SRT to be short because shorter SRT lowers capital cost and reactor size by increasing AD efficiency (X. S. Shi et al., 2017). If it is too short, washout of methanogens and pH drops may occur (Alepu et al., 2016). The optimal SRT value changes with temperature, sludge composition and reactor type (Manser, 2015).

Most of mesophilic anaerobic digesters are operated with 15-30 days SRT (Alepu et al., 2016). Minimum 10 days SRT is required at 35°C operating temperature (Filipe & Grady, 1998).

2.2.2.2. pH

pH value of anaerobic digester is critical for the stability due to sensitivity of the microorganisms towards pH changes. As a result of accumulation of VFA produced in acidogenesis, pH of anaerobic digester fluctuates during the process. Despite acidogens favor acidic pH like 5.0, methanogens prefer alkaline pH close to neutral. Majority of anaerobic microorganisms involving methanogens thrive 6.5-7.5 pH (Nayono, 2010). The optimal range of pH is considered as 6.8-7.2, but the system can tolerate 6.5-8.0 pH (Cioabla et al., 2012). However, it is observed that methane production rate declines if pH is below 6.3 and above 7.8 (Nayono, 2010). In such situation, alkalinity addition can be used to buffer pH variations when it is necessary (Manser, 2015).

2.2.2.3. Temperature

Anaerobic digesters should be maintained at constant operating temperatures that determine degradation rate of particularly hydrolysis and methanogenesis (Nayono, 2010). As temperature increases, the degradation accelerates so, more effective operation happens with a shorter SRT and smaller reactor size (Cloete & Muyima, 1997). Temperature also influences other factors like sludge settling characteristics and gas transfer (Nayono, 2010). There exist three temperature regimes for anaerobic digesters as psychrophilic (<20°C), mesophilic (30-40°C) and thermophilic (50-60°C) (Connaughton et al., 2006). Psychrophilic digesters are unusual because of low microbial activity and large space requirement (Connaughton et al., 2006). Traditionally, mesophilic digesters are widely spread since thermophilic digesters have some drawbacks such as more energy need, poor stability and reliability. Yet, thermophilic digesters provide better quality of digested sludge, shorter retention

times, higher pathogen removal, degradation and methane production (Labatut et al., 2014). Most commonly, anaerobic digesters in WWTPs are operated as mesophilic with a typical temperature range of 35-37°C. In case of changing temperature in the digester more than 2-3°C per day, methane production may decrease and foaming problem may show up (Schnaars, 2012).

2.2.2.4. Carbon to nitrogen (C/N) ratio

Carbon to nitrogen (C/N) ratio is a measure of nutrient balance for anaerobic microorganisms to perform an efficient AD process. It shows a relationship between C and N amount of feedstock. C/N ratio is affected by pH, temperature and the total concentrations of C and N. The optimum C/N ratio should lie between 25:1 and 30:1 (Korres et al., 2013). The fact that C/N ratio is higher than its optimal range causes N deficiency which reduces degradation and methane production. When the ratio is too low, excess N accumulates as NH_3 that raises pH above 8.5. This creates a toxic environment to methanogens and again inhibits methane production (Sarangi et al., 2018). Thus, total nitrogen (TN) is an important factor that should be monitored for denitrification in AD.

2.2.2.5. Solids content

For specifying digester sludge characteristics, sludge is quantified in terms of total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS). TS, dry matter in sludge independently of organic and inorganic, represents total sludge amount produced in WWTPs. In digester sludge, most of TS is in the form of TSS (Andreoli et al., 2007). VS, a measure of organic matter in sludge, are used for indirect determination of active biomass which is difficult to measure directly (Arnaiz et al., 2006). Especially reduction in VS represents anaerobic digester efficiency. In anaerobic digesters, TS are reduced by 30-60% whereas VS are reduced by 35-50% (Pennsylvania Department of Environmental Protection, 2016). VS/TS ratio is an indicator of the organic portion of sludge and

level of digestion. Typical range of VS/TS ratio for digestate is 0.60-0.65 (Andreoli et al., 2007). Additionally, TSS and VSS are important parameters to observe solubilization when pretreatment methods are applied and also to control scum or foaming problem (Estokova & Balintova, 2018).

2.2.2.6. Volatile fatty acids (VFA)

Monitoring VFA concentrations in anaerobic digesters can be tricky because VFA also influence pH. If pH is close to neutral, VFA do not indicate any toxicity on methanogens at concentrations below 10000 mg/L (Spinosa & Vesilind, 2001). Since there are many VFA found in anaerobic digesters, identifying a certain level of every individual VFA is not feasible (Franke-Whittle et al., 2014). Each anaerobic digester has its own normal VFA levels which are determined by feed sludge composition and other operating parameters (Franke-Whittle et al., 2014). Although the relevance of individual VFA with AD performance is still under research, concentration of total VFA in anaerobic digesters should be between 8 and 300 mM (Spinosa & Vesilind, 2001).

2.2.2.7. Organic loading rate (OLR)

Anaerobic digesters are fed based on organic loading rate (OLR) which is the daily amount of organic matter in chemical oxygen demand (COD) or VS basis to be fed into the anaerobic digester per unit of time (Labatut & Pronto, 2018). OLR is calculated by dividing VS of feed sludge into SRT (Orhorhoro et al., 2018). Optimum OLR value is stated as in the range of 1.6-4.8 kg TS/m^3.d in the literature, but it is highly related with feed sludge composition, temperature and SRT value of each system (Metcalf & Eddy, 2003). If there is a change in organic composition of feed sludge at the same SRT, OLR will change too (Nayono, 2010). High OLRs cause a reduction in methane production and pH because of VFA accumulation (Orhorhoro et al., 2018).

Other factors rather than operational parameters may impair anaerobic digester performance. Some inhibitory or toxic compounds such as O_2, H_2S, NH_3 and heavy metals may exist in anaerobic digesters and cause process instability (Nayono, 2010). Release of H_2S may lead to odor and corrosion problems. Moreover, insufficient mixing, improper feeding and rapid temperature changes may lead to foaming problem (Schnaars, 2012; Coyne et al., 2017).

2.2.3. Digesters

Anaerobic digesters can be designed and operated with many different process configurations depending on feedstock, feeding, multi-staging and operating temperature.

2.2.3.1. Feedstock

Instead of sludge, anaerobic digesters can treat different feedstocks such as manure, food waste, solid waste and agricultural waste. If their nutrient contents (e.g. C/N ratio) are not well-balanced for a successful AD, multiple feedstocks can be mixed in certain proportions for adjusting nutrient requirements, referred as co-digestion (Chow et al., 2020). Besides, solid content of the feedstock defines wet or dry anaerobic digesters. Dry digestion is applied for feedstocks having TS concentrations greater than 15%, whereas wet digestion for smaller than 15% (Van et al., 2020). Many anaerobic digesters in municipal WWTPs are wet digesters as undigested sewage sludge has usually low solid content (Walling et al., 2019).

2.2.3.2. Feeding

Based on the way the feedstock is fed, anaerobic digesters can be operated as batch, continuous and semi-batch as shown in Figure 2.5. In a batch digester, all feedstocks are added to the digester once at the beginning and then sealed without any inflow or outflow until the digestion is completed. On the contrary, feedstocks are

constantly added and digestate is removed simultaneously throughout the digestion in a continuous digester. Semi-batch or semi-continuous digesters work intermittently as feedstocks are added and digestate is removed periodically. Homogeneous composition throughout the reactor is provided by mixing. Today, high-rate digesters offering uniform feeding, heating and mixing are the most common digester type. Examples of this form of anaerobic digesters include completely stirred tank reactor (CSTR), upflow anaerobic sludge blanket (UASB), anaerobic sequencing batch reactor (ASBR) and expanded granular sludge bed (EGSB) etc. (Rao, 2005; Ingham et al., 2008; Náthia-Neves et al., 2018).

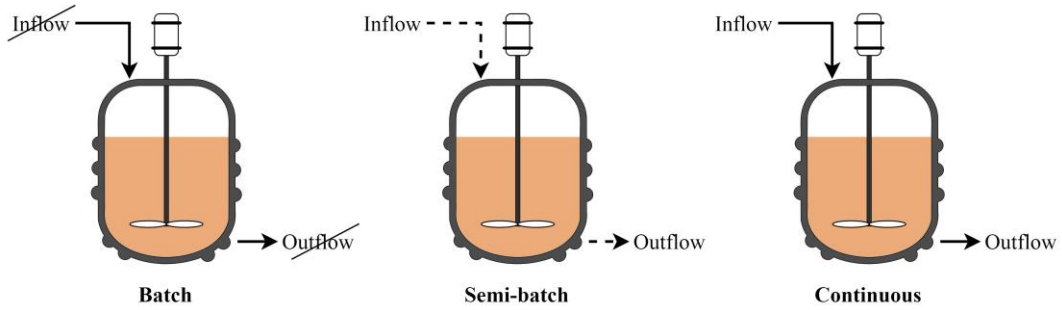

Figure 2.5. Schematic diagram of anaerobic digester types by feeding

2.2.3.3. Multi-staging

Anaerobic digesters can be operated usually as single- or two-stage and unusually three-stage as shown in Figure 2.6 (EPA, 2006). In conventional single-stage anaerobic digesters, AD process happens in a single reactor where acidogens and methanogens are kept together. However, acidogens and methanogens are different from each other in terms of physiology, growth kinetics, nutritional requirements and sensitivity to environmental conditions. This makes the stability and control of AD difficult. Therefore, two-stage anaerobic digesters can be performed by separating acidogens and methanogens physically in two reactors with different environmental conditions (Azbar & Speece, 2001).

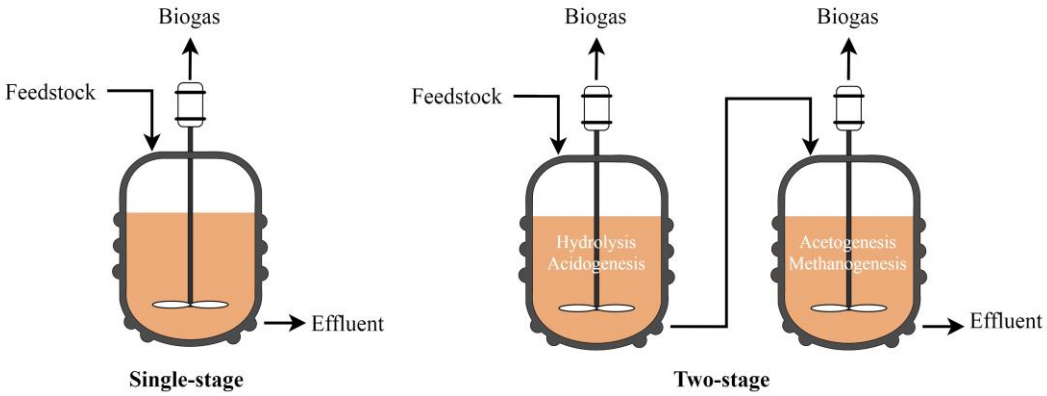

Figure 2.6. Schematic diagram of anaerobic digester types by staging

2.3. Pretreatment methods

The performance of AD process depends on the extent of how hydrolysis step has been accomplished. Many limitations of AD such as longer retention times, larger reactor size and insufficient degradation are associated with the rate-limiting hydrolysis step (Yi et al., 2013). The structural property of sludge makes hydrolysis even more problematic. Organics in sludge are adsorbed by extracellular polymeric substances within microbial cells that are protected by cell membranes and walls. In hydrolysis, cell walls are disrupted and organics are released for acidogens. Yet, microbial cell walls and complex extracellular polymeric substances are very resistant to degradation (Appels et al., 2008). Thus, hydrolysis pose an obstacle for biogas and methane yield obtained from AD.

The efficiency of AD can be increased by facilitating hydrolysis. For this reason, several pretreatment methods have been developed and still under investigation. The main purpose of pretreatment is to make substrates in sludge easily available for microorganisms (Atelge et al., 2020). In other words, pretreatment promotes hydrolysis externally with cell disruption causing the release of more accessible intracellular substances for subsequent microorganisms (Zhen et al., 2017).

Pretreatment of sludge prior to AD provides (Singh & Kalia, 2017):

- Improvement of the process,
- Acceleration of biodegradation,
- Increase in biogas and methane production,
- Minimization of sludge having better sludge characteristics,
- Additional pathogen removal and stability,
- Reduction in foam and bulking problem.

As shown in Figure 2.7, pretreatment methods are comprised of physical, chemical and biological processes or combinations of those (Kamusoko et al., 2019).

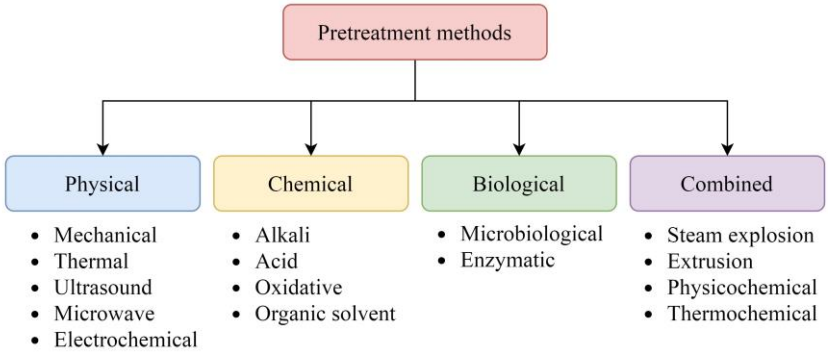

Figure 2.7. Pretreatment methods adapted from Kamusoko et al. (2019)

Physical pretreatment: In physical pretreatment methods, physical forces are applied to increase surface area of sludge by reducing particle size and altering sludge structure. As a result, sludge becomes eligible to microbial and enzymatic attacks (Karuppiah & Azariah, 2019). Sludge composition is not affected and no toxic compounds are formed in physical pretreatment (Abraham et al., 2020). Physical pretreatment alone is not very effective because of high energy requirement. It is usually combined with other pretreatment methods (Nair & Sivakumar, 2020). Mechanical, thermal and ultrasound pretreatments are prominent physical pretreatment methods.

Chemical pretreatment: Chemical pretreatment methods are used to disrupt microbial cells and breakdown complex organics by means of alkalis, acids, oxidants or organic solvents (Pilli et al., 2020). The impact of chemical pretreatment is related to sludge characteristics. It is not suitable for easily biodegradable compounds rich in carbohydrates because faster degradation of carbohydrates causes VFA accumulation and the failure of methanogenesis (Fernandes et al., 2009). Due to the advantage of pH and alkalinity adjustment, alkali pretreatment is preferable but oxidative and acid pretreatments are also applied to increase biogas production (Ariunbaatar et al., 2014). However, types of reagents and their dose adjustments are critical because excessive doses may exhibit toxic effects on anaerobic microorganisms (Pilli et al., 2020). Most often, alkali and acid pretreatments were combined with thermal pretreatment (Tyagi & Lo, 2011).

Biological pretreatment: Biological pretreatment methods are based on a principle of degradation and solubilization of organics with microorganisms or enzymes (Atelge et al., 2020). Microorganisms that produce enzymes endogenously can be used for improving hydrolysis as well as enzyme additions (Karuppiah & Azariah, 2019). Although biological pretreatment is a safe, ecofriendly and energy-saving method, expensiveness and preservation of enzymes obstruct its application (Karuppiah & Azariah, 2019; Zhao et al., 2019).

Combined pretreatment: In order to eliminate disadvantages of each pretreatment, two or more pretreatment methods can be applied together as sequentially or simultaneously. Despite combined pretreatments have proven to be more effective and economic in biogas and methane yield, they are complex and need more research (Atelge et al., 2020).

2.3.1. Ozone pretreatment

Of oxidative chemical pretreatment methods, ozone pretreatment is the most widely used. Ozone (O_3) is not only a strong oxidant but also a disinfectant (Bougrier et al., 2007). Unlike other chemical pretreatments, pretreatment with ozone neither leaves any chemical residues behind nor increases salt concentration. Ozone pretreatment decreases pathogens, micro-pollutants, odor and foaming problems (Qasim & Zhu, 2017; Banu et al., 2018). Moreover, it improves sludge characteristics such as settleability, dewaterability and viscosity (Battimelli et al., 2003). It is relatively faster process that generates less sludge compared to other chemical methods (Trzcinski, 2018).

Ozone pretreatment aims further hydrolysis by transforming hardly biodegradable compounds into more easily biodegradable ones (Tyagi & Lo, 2011). It solubilizes and partially oxidizes refractory organics available in sludge without allowing complete oxidation (Carballa et al., 2007). Mechanism behind can be described as the disintegration of sludge flocs first, then cell lysis and finally oxidation of released soluble organics from the cells (Tyagi & Lo, 2011). Ozone functions in two ways; directly reacting with particulate and dissolved compounds (Equation 2.1) and indirectly forming hydroxyl radicals (Equation 2.2) to oxidize those (Jafarinejad, 2017). As a result of assisted hydrolysis and enhanced biodegradability, AD improves by yielding more biogas and methane.

$$O_3 + R \rightarrow RO + O_2 \qquad \text{(Equation 2.1)}$$

$$2O_3 + 2H_2O \rightarrow 2HO\cdot + 2HO_2\cdot + O_2 \qquad \text{(Equation 2.2)}$$

Ozone dose to be applied is an important factor for an efficient pretreatment. Higher ozone doses lead to complete oxidation or destruction of essential anaerobic microorganisms (Zhen et al., 2017). As a consequence, COD mineralization occur and CO_2 portion of biogas rises while CH_4 portion declines as an indicator of poor biogas quality (Trzcinski, 2018). Also, use of high ozone doses is not desirable

because of the pretreatment cost. Lower ozone doses may become both effective and economic. In the literature, the optimum ozone dose for pretreatment is suggested to be between 0.05 and 0.5 g O_3/g TS, but it depends on pretreatment conditions and sludge characteristics (Zhen et al., 2017). Considering the balance between acquired efficiency and cost, ozone doses between 0.03 and 0.05 g O_3/g TSS are recommended (Tyagi & Lo, 2011).

Previous researches available for ozone pretreatment have been generally focused on either biodegradation and solubilization or biogas and methane production for different feedstocks. Various ozone doses have been proposed for gaining the highest biogas and methane yield in AD treating sludge. A summary of the studies investigating biogas/methane production from sludge in ozone pretreated mesophilic anaerobic digesters are tabularized in Table 2.3. Still, there is not sufficient knowledge for optimizing ozone doses to be applied in pretreatment prior to mesophilic semi-batch anaerobic digesters as biogas/methane production, microbial changes and costs are considered.

Table 2.3. Literature survey on ozone pretreatment

Sludge	Feeding	Ozone doses	Biogas production	Methane production	Reference
PS+WAS	Batch	0.05 g O_3/g COD 0.1 g O_3/g COD 0.2 g O_3/g COD	–	+50% +80% +30%	(Weemaes et al., 2000)
WAS	Batch	0.02 g O_3/g TSS 0.05 g O_3/g TSS 0.1 g O_3/g TSS 0.2 g O_3/g TSS 0.5 g O_3/g TSS	–	+26% +75% +114% +130% +135%	(Yeom et al., 2002)
WAS	Semi-batch	0.015 g O_3/g TS (SRT: 14 d) 0.015 g O_3/g TS (SRT: 28 d) 0.05 g O_3/g TS (SRT: 14 d) 0.05 g O_3/g TS (SRT: 28 d)	–	+17% +5% +109% +82%	(Goel, Takutomi, et al., 2003)
Thickened WAS	Batch	0.1 g O_3/g TS 0.16 g O_3/g TS	+8% +25%	+11% +23%	(Bougrier et al., 2006)
WAS	Batch	0.015 g O_3/g TS 0.025 g O_3/g TS 0.04 g O_3/g TS 0.06 g O_3/g TS 0.09 g O_3/g TS 0.12 g O_3/g TS 0.15 g O_3/g TS 0.18 g O_3/g TS	+30% +14% +24% +58% +72% +104% +144% +91%	–	(Bougrier et al., 2007)
WAS	Batch	0.1 g O_3/g TS	–	+25%	(Erden & Filibeli, 2011)
WAS	Semi-batch	0.05 g O_3/g TS 0.07 g O_3/g TS	-6% +17%	–	(Braguglia et al., 2012)
WAS PS+WAS	Batch	0.043 g O_3/g TSS (WAS) 0.063 g O_3/g TSS (WAS) 0.08 g O_3/g TSS (WAS) 0.1 g O_3/g TSS (WAS) All doses (PS+WAS)	+5% +21% NA but decreased NA but decreased NA but increased	–	(Silvestre et al., 2015)
WAS PS+WAS	Semi-batch	4.8 mg O_3/g TS (PS+WAS) 9.5 mg O_3/g TS (PS+WAS) 73.2 mg O_3/g TS (PS+WAS) 3.5 mg O_3/g TS (WAS) 7.7 mg O_3/g TS (WAS) 53.6 mg O_3/g TS (WAS)	–	+6% -14% -21% +30% +16% +5%	(Chiavola et al., 2019)

PS, primary sludge; WAS, waste activated sludge; PS+WAS, mixed or raw sludge; NA, not available;
+, percent increase for specified ozone dose; -, percent decrease for specified ozone dose

2.4. Microbiology of anaerobic digesters

Over the last few years, the most striking and advancing field of AD has been its microbiology. In AD process, microbial communities are constituted by microorganisms that interact each other to grow in the same habitat. Since full knowledge of microbial communities and dynamics is required for controlling and improving AD much better, many efforts have been made to understand microbial communities in anaerobic digesters (Narihiro & Sekiguchi, 2007). Understanding of key microorganisms and the determination of microbial profile involved in anaerobic digesters have always been one of the prerequisites for increasing biogas and methane production.

As previously shown in Figure 2.4 and described in section 2.2.1., AD includes hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each step is carried out by different anaerobic microorganisms as hydrolytic bacteria, acidogens, acetogens and methanogens. Anaerobic digester performance is related to microbial activities and synergetic relationship between those microbial communities. Presence and abundance of the communities highly depend on many factors such as operating conditions, feedstock type and substrate characteristics (Nguyen et al., 2019). AD is performed by several microbial communities in the domains of *Bacteria* and A*rchaea*. Mainly, there are five important microbial communities in anaerobic digesters with respect to the substrates utilized. These communities are composed of acidogens, acetogens, sulfate reducers, denitrifiers and methanogens as shown in Figure 2.8 (Andreoli et al., 2007).

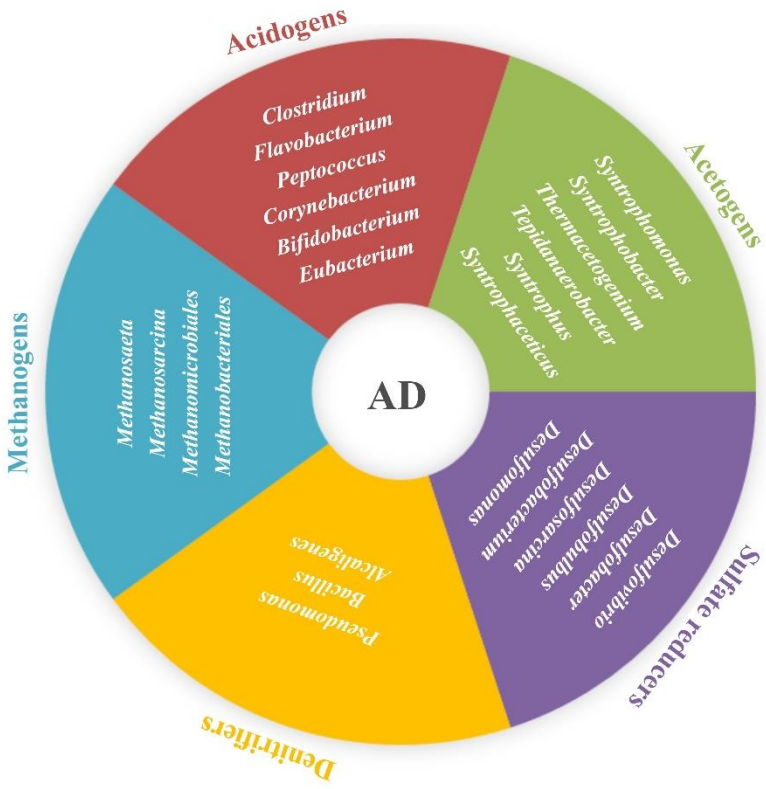

Figure 2.8. Schematic diagram of microbial communities and their representative members in anaerobic digesters

Acidogens: Acidogens use hydrolyzed compounds as substrates to produce intermediate products such as VFA and alcohols. Acidogens are fast-growing microorganisms that involve both facultative and obligate fermentative anaerobic bacteria (Anukam et al., 2019). They cannot survive at extreme temperatures and prefer pH of 5.0-6.0 (Wainaina et al., 2019). Acidogens are found in the phyla of *Firmicutes, Bacteroidetes, Proteobacteria, Acidobacteria* and *Actinobacteria.* The examples of acidogens in anaerobic digesters are *Clostridium, Flavobacterium, Peptococcus, Corynebacterium, Bifidobacterium, Bacillus, Pseudomonas* and *Eubacterium* etc. (Nguyen et al., 2019). Hydrolytic bacteria and acidogens have very similar microbial characteristics even sometimes they are used interchangeably in some sources.

Acetogens: Acetogens use intermediates produced by acidogens to generate acetate, H_2 and CO_2. This intermediate conversion is vital for biogas production because the intermediates cannot be used directly by methanogens. Therefore, there is a syntrophic relationship between acetogens and methanogens because of H_2 transfer (Schön, 2010). Unlike acidogens, acetogens are relatively slow-growing obligate anaerobic bacteria and they are sensitive to changing environmental conditions (Anukam et al., 2019). The generation time for acetogens is usually longer than 3 days (Gerardi, 2003). Acetogens can be found in many different phyla, but mostly in *Firmicutes* and *Proteobacteria.* Many acetogens in anaerobic digesters belong to the genera of *Syntrophomonas*, and *Syntrophobacter* (Ali Shah et al., 2014). *Thermacetogenium, Tepidanaerobacter, Acetobacterium, Clostridium, Syntrophus, Syntrophaceticus, Pelotomaculum* and *Smithllela* can be given as examples of acetogens (Venkiteshwaran et al., 2016; Westerholm & Schnürer, 2019). Among them, *Acetobacterium woodii* and *Clostridium aceticum* are homoacetogens (Patel et al., 2017).

Sulfate reducers: Sulfate reducers, i.e. sulfate reducing bacteria (SRB), are one of the important communities in anaerobic digesters as they adversely affect methanogenesis and so, methane production. Sulfate reducers use sulfate as a substrate to produce H_2S which is an inhibitory compound for methanogens (Liu et al., 2018). Aqueous H_2S shows inhibition in two ways: One originates from the competition between sulfate reducers and methanogens for using the same substrates like acetate and H_2, while the other is through H_2S toxicity to methanogens. Moreover, gaseous H_2S at high levels may cause odor and corrosion problems in the digesters. If excess sulfate is present in the system, sulfate reducers are favored over other anaerobes, especially methanogens, and the digester performance decreases (Madden et al., 2014). Sulfate reducers in mesophilic anaerobic digesters are found in the phylum of *Proteobacteria* with the genera of *Desulfovibrio, Desulfobacter, Desulfobulbus, Desulfosarcina, Desulfobacterium, Desulfcoccus* and *Desulfomonas* etc (Liu et al., 2018).

Denitrifiers: Denitrifiers, i.e. denitrifying bacteria, play a fundamental role in anaerobic digesters since denitrification process may take place in AD (Andreoli et al., 2007). Denitrifiers are facultative anaerobes that use nitrate (NO_3^-) to produce N_2 (Gerardi, 2006). When denitrifiers predominate in anaerobic digesters, they primarily utilize organic carbon used by methanogens (Bernet et al., 2001). Thus, there is a competition between denitrifiers and methanogens for acetate as sulfate reducers. Coexistence of both leads to suppression of methanogens by nitrogen oxides and decrease in methane content of biogas (Bless, 2018). According to Clarens et al. (1998), denitrification products inhibited some *Methanosarcina* spp. and methane production stopped due to *Pseudomonas* spp. Many denitrifiers in anaerobic digesters are found in the genera of *Pseudomonas, Bacillus, Alcaligenes* (Gerardi, 2006).

Methanogens: Methanogens use acetate or H_2 and CO_2, which are produced by acetogens, in order to produce methane. Methanogens are slow-growing obligate anaerobic archaea and they are extremely sensitive to environmental changes especially to O_2 (Anukam et al., 2019). Most of methanogens prefer pH ranges of 6.0-8.0 and none can grow at pH values below 5.6 (Garcia et al., 2000). The generation time for methanogens at 35°C is 3 days (Gerardi, 2003). So far, over 65 methanogenic species have been discovered in phylum of *Euryarchaeota* and grouped in five orders: *Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanococcales* and *Methanopyrales* (Nguyen et al., 2019). They are classified according to their structure, substrate usage and growth temperature (Gerardi, 2003). Methanogens are divided into two groups as acetoclastic and hydrogenotrophic methanogens depending on utilizing acetate or H_2 and CO_2, respectively (Náthia-Neves et al., 2018). Although present acetoclastic methanogens are less than hydrogenotrophic ones, a vast majority of CH_4 is produced by acetoclastic methanogens (Schön, 2010). Compared to acetoclastic methanogens, hydrogenotrophic ones are more tolerant but relatively lower in

anaerobic digesters (Laiq Ur Rehman et al., 2019). Hydrogenotrophic methanogens involve the orders of *Methanomicrobiales, Methanobacteriales* and *Methanococcales,* whereas acetoclastic methanogens involve only the order of *Methanosarcinales* (Ziganshin et al., 2016).

The existing literature on methanogens in anaerobic digesters is extensive and well-studied. Despite methanogens generally represent 2-5% of the total community, they have high activity relative to their abundance (Westerholm & Schnürer, 2019). Methanogens commonly detected in sludge treating anaerobic digesters belong to the orders of *Methanosarcinales, Methanomicrobiales* and *Methanobacteriales* (Westerholm & Schnürer, 2019). Among them, the most abundant methanogens are found in the family of *Methanosaetaceae* or *Methanosarcinaceae* which belong to acetotrophic *Methanosarcinales* order (Nguyen et al., 2019). Unlike *Methanosaeta* spp., *Methanosarcina* spp. are more tolerant to inhibitors and they can use hydrogenotrophic pathway additionally (Castellano-Hinojosa et al., 2018). Majority of the studies stated that *Methanosaeta* spp. dominated in anaerobic digesters (Gao et al., 2016; Q. Zhang et al., 2019). In other studies, the most dominant methanogens were reported as either *Methanosarcina* spp. alone or together with *Methanosaeta* spp. (Ziganshin et al., 2016; Khan et al., 2018).

2.4.1. Microbial analyses

Due to complex interactions among microorganisms involved in AD, isolation and identification of individual microorganisms have always been a problem. There is a great interest on the microbiome in different anaerobic digesters in the past century (Lim et al., 2020). In the light of the researches done, several culture dependent and independent molecular approaches have been proposed to identify microbial communities and their diversity in anaerobic digesters. Current knowledge of microbial communities obtained from culture dependent methods is restricted and incomplete since only 1% of the microorganisms are culturable and most of them have not been cultivated or isolated. Low growth rates and unknown growth

requirements of anaerobic microorganisms make culturing difficult. Additionally, the microorganisms having syntrophic relationships like in microbial communities cannot be grown as a monoculture (Sikora et al., 2016). Nowadays, culture independent molecular methods that are less time-consuming and modern have been often used for monitoring microbial communities in AD. These molecular methods have been improved to understand how feedstock, operational parameters and digester configurations can be affected by microbial changes and how this changes interfere AD stability and efficiency (Vanwonterghem et al., 2014).

There are many molecular microbial analyses to comprehend diversity, dynamics, quantification and function of microbial communities. Figure 2.9 summarizes widely used methods for analyzing microbial communities in anaerobic digesters (Cabezas et al., 2015).

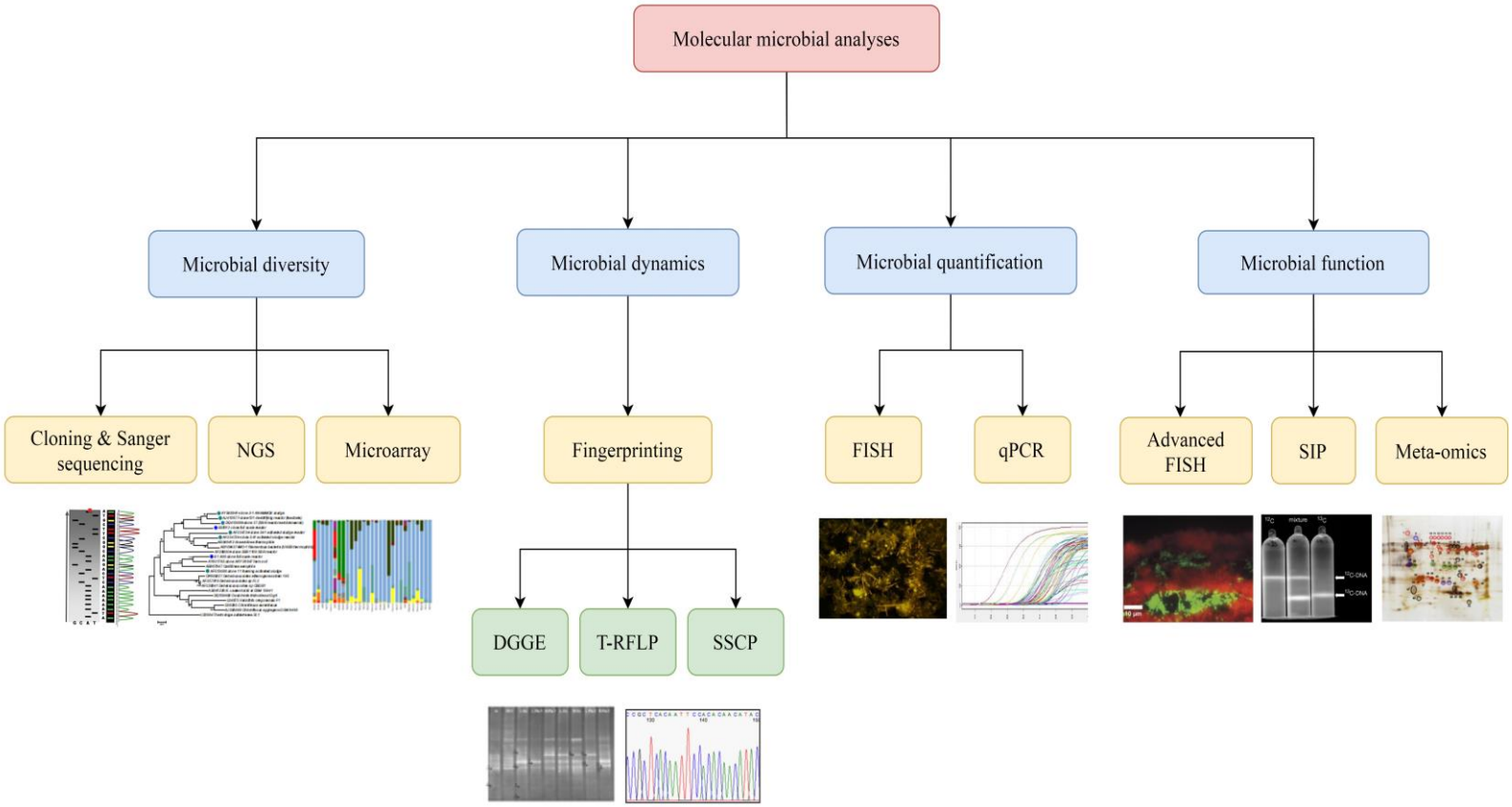

Figure 2.9. Overview of molecular microbial analyses used for microbial communities in AD adapted from Cabezas et al. (2015). NGS, next generation sequencing; DGGE, denaturing gradient gel electrophoresis; T-RFLP, terminal-restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; FISH, fluorescence in situ hybridization, qPCR, quantitative polymerase chain reaction; SIP, stable isotope probing

Microbial diversity: Most of the information on microbial diversity in anaerobic digesters are determined through polymerase chain reaction (PCR) followed by the analysis of marker genes, most widely 16S rRNA (Cabezas et al., 2015; Lim et al., 2020). Bacterial and archaeal taxonomy and phylogeny can be found by 16S rRNA gene sequencing and compared with databases. However, this traditional method does not provide detailed analysis when studying with multiple samples and it is slow and costly (Lim et al., 2020). Also, cloning in a plasmid vector and Sanger sequencing has started to be used in the last decade. In order to eliminate the drawbacks of these methods, a novel next generation sequencing (NGS) method which is cheap, rapid and high throughput has been developed in the last few years. A few researches are available that use NGS for microbial communities in anaerobic digesters treating industrial wastewaters and solid wastes, but not for municipal sludge (Werner et al., 2011; Sundberg et al., 2013). Another method is semi-quantitative DNA microarrays that can identify bacterial and archaeal species or detecting functional genes (Cabezas et al., 2015). Microarrays supply fast and relatively economic application once the array has been engineered, but a microarray designed for all communities in anaerobic digesters has not been available yet except ANAEROCHIP for the detection of methanogens in thermophilic digesters (Cabezas et al., 2015; Lim et al., 2020).

Microbial dynamics: Examination of microbial dynamics may be suitable for observing fluctuations in community structure under varying operational conditions. Microbial dynamics are monitored by fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP) and single-strand conformation polymorphism (SSCP) (Čater et al., 2013; Cabezas et al., 2015). The main advantage of these methods is to get fingerprinting patterns not only from all or active communities having 16S rDNA and 16S rRNA respectively, but also methanogens having *mcr*A gene. *mcr*A is a marker gene which encodes a catalyzing enzyme responsible for methane production and it is present in all methanogens. Thus, *mcr*A gene expression can be correlated

by methanogenic activity (Afzal et al., 2019). Among fingerprinting methods, PCR followed by DGGE is more popular and frequently used than T-RFLP and SSCP since it is straightforward and relatively rapid (Cabezas et al., 2015). Nonetheless, it is limited to dominant community members due to low resolving power, specificity of the bands on the gel and reliability (Cabezas et al., 2015; Lim et al., 2020). Until now, DGGE has many practices for sludge from UASB reactors and CSTRs operating at different conditions and configurations (Kundu et al., 2013; Ziembińska-Buczyńska et al., 2014). Even it is rare, some authors performed T-RFLP and SSCP for community changes (Leclerc et al., 2004; C. Zhang et al., 2014).

Microbial quantification: Quantifying populational changes of microorganisms alone or microbial communities in anaerobic digesters is essential for relating them with AD efficiency. The methods of quantitative polymerase chain reaction (qPCR) and fluorescence in situ hybridization (FISH) can be used to decide how many microorganisms from the different groups exist in the digesters (Cabezas et al., 2015). qPCR is based on PCR with real time monitoring targeted amplicons via SYBR Green dye or TaqMan technology (Cabezas et al., 2015). Like PCR, it can be employed with the help of 16S rRNA or *mcr*A genes (Čater et al., 2013; Cabezas et al., 2015). Precise and reliable qPCR can be applied many samples at once rapidly. On the other hand, it is vulnerable to bias resulting from nucleic acids extraction (Cabezas et al., 2015). Present literature on qPCR applications for anaerobic digesters cover methanogenic analyses mostly (Traversi et al., 2012; Kim, Lim, et al., 2013; Khan et al., 2018). Alternatively, quantification can be fulfilled by FISH method (Lim et al., 2020).

Microbial function: The most compelling part of microbial analyses is to discover the roles and activity of microbial communities in AD since interspecies relations and metabolic pathways are still mysterious. Although identification of microorganisms gives clue about their metabolic potential, it is not possible to know their function completely considering that one microorganism can appear more than

one metabolic pathway (Cabezas et al., 2015). That's why, combination of aforementioned methods or more innovative approaches have been built up (Cabezas et al., 2015; Lim et al., 2020). Advanced versions of FISH such as microautoradiography combined with FISH (MAR-FISH) and Raman microspectroscopy combined with FISH (Raman-FISH) has begun to be used with progressing spectroscopy and high resolution imaging (Rastogi & Sani, 2011). Stable isotope probing (SIP) enables to determine active microorganisms incorporating heavy ^{13}C-labeled substrate within their system (Cabezas et al., 2015; Lim et al., 2020). A new approach of meta-omics comprise meta-genomics, meta-transcriptomics, meta-proteomics and meta-bolomics (Cabezas et al., 2015). Unlike others, this approach have a great advantage of characterizing microbial composition, diversity, metabolism and gene expressions comprehensively (Lim et al., 2020). In spite of high costs, limited references and open to improvement, it is a promising technology. Numerous studies on meta-omic practices in AD have already started to arise and it will eventually ascend in the upcoming years (Zakrzewski et al., 2012; Kirkegaard et al., 2017). In the end, every molecular approach has pros and cons, none of them is the best for accessing to the genetic and functional diversity of complex microbial communities (Rastogi & Sani, 2011). The choice of which one to be used alters for each circumstance and expectation.

2.4.1.1. FISH method

FISH is a prevailing non-PCR-based method to identify phylogeny and enumerate specific microbial communities in biofilms and activated sludge from WWTPs (Nielsen et al., 2009). It is used for *in situ* monitoring of microbial abundancy and shifts in anaerobic digesters. FISH method is based on the principle of observing microorganisms using fluorescently labeled oligonucleotide probes that target their genetic material without damaging the cells. Probes are designed as the short sequences of single strand DNA or RNA complementary to genes in microorganisms of interest. These probes contain nearly 15-25 nucleotides and labeled with fluorescent dyes at 5' end (Lim et al., 2020). The probes typically target 16S rRNAs

since they are present in all living microorganisms and there is an extensive knowledge and databases like probeBase (Greuter et al., 2016). After binding and hybridizing the targeted 16S rRNA gene sequences, the probes emitting fluorescent signals are visualized under fluorescence microscope. Besides, it can be possible to understand metabolic state of the cells from signal intensity associated with cell activity and growth rates (Domańska et al., 2014). The images taken from microscopy are analyzed in a software program in order to determine the relative quantity of microorganisms in microbial communities (Nielsen et al., 2009). Also, flow cytometry can be used for a high resolution automated analysis (Rastogi & Sani, 2011). FISH mechanism is illustrated in Figure 2.10 (BioVisible, 2006).

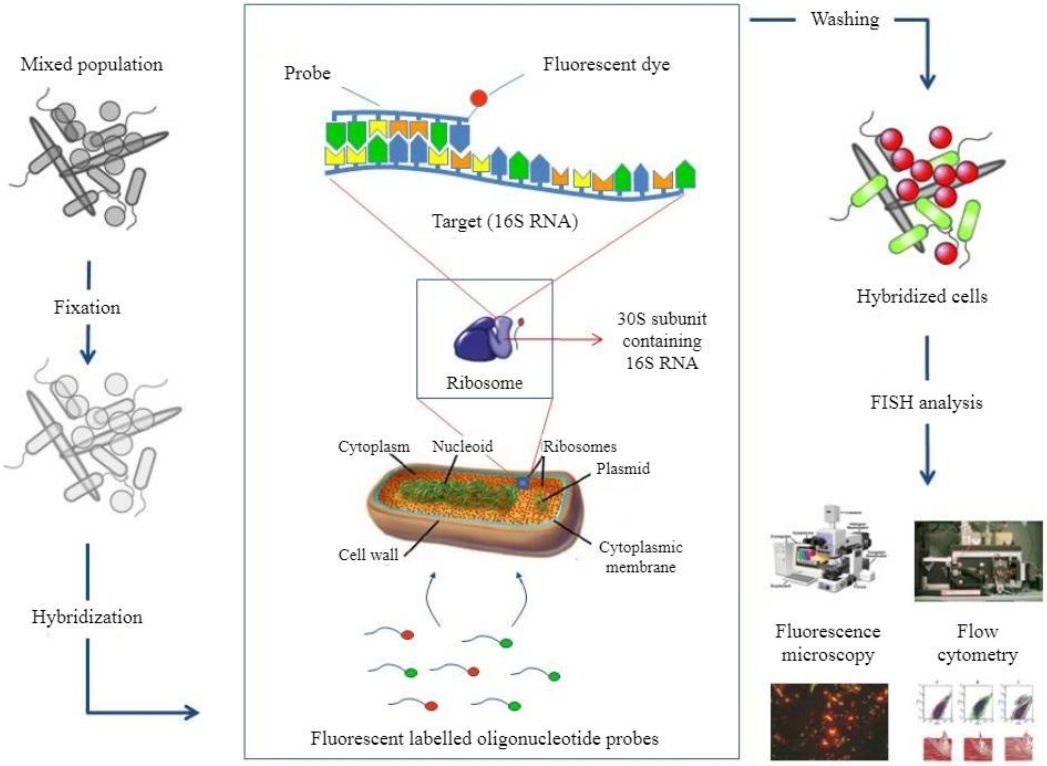

Figure 2.10. Mechanism of FISH method (BioVisible, 2006)

Regardless of the microorganisms and samples to be examined, FISH method involves the main steps of (Nielsen et al., 2009):

- Optimization of probes,
- Fixation of samples,
- Permeabilization of microbial cell walls,
- Hybridization with probes,
- Washing of non-binding probes,
- Visualization with microscopy,
- Quantification with a software or flow cytometry.

In addition to being relatively rapid, simple and reliable, FISH method avoids biases related with PCR and nucleic acid extraction (Su et al., 2012; Cabezas et al., 2015). On the other hand, it is limited to already existing probes of only known microorganisms and it does not give precise results when used in heterogeneous samples (Čater et al., 2013; Lim et al., 2020). Detection limit of FISH method may be insufficient in some cases and special fixation protocols for gram positive cells may be needed (Cabezas et al., 2015). Moreover, microscopic analyses can be time-consuming and subjective due to the operator judgements (Ali Shah et al., 2014).

Most of the essential probes available today are designed as pursuant to early studies on FISH method. Raskin et al. (1994) was the first researcher that designed and used methanogenic probes on anaerobic reactors by FISH method. This study laid the foundations of *Methanosarcina* and *Methanosaeta* spp. abundancy in anaerobic sewage sludge digesters. After that, coexistence of sulfate reducers and methanogens in anaerobic aggregates with their populational structures were studied by Santegoeds et al. (1999). Zheng and Raskin (2000) investigated *Methanosaeta* spp. at genus level in terms of different acetate concentrations and sludge types for thermophilic and mesophilic anaerobic bioreactors. Crocetti et al. (2006) analyzed 16S rRNA gene sequences from 3000 methanogens and other *Euryarchaeota*

phylogenetically and checked previously published probes for target group accuracy. In a research by Díaz et al. (2006), *Methanosaeta concilii, Methanosarcina mazei* and *Methanospirillum* spp. were identified in methanogenic granules of anaerobic sludge bed reactor treating brewery wastewater. According to Reyes et al. (2015), low levels of denitrifiers, high levels of sulfate reducers such as *Desulfobacteriaceae* and *Desulfovibrionales* were found in the anaerobic digester in addition to high level of methanogens. Most recently, Khan et al. (2018) showed that acetoclastic methanogens were more abundant than the hydrogenotrophic ones and *Methanosaetaceae* was the most abundant acetoclastic methanogens in three full-scale mesophilic anaerobic digesters. There are many other studies used advanced FISH methods on microbial communities in AD in order to overcome the limitations of standard FISH method, but they are not within the scope of this study.

To conclude, present researches of FISH method in anaerobic digesters are oriented mostly to methanogens and descended to the genus level because of well-established probes (Lu & Hu, 2017). Yet, advances in probe technology and other molecular approaches have started to allow the discovery of representative members in microbial communities and their probes. In spite of those advancements, there is no comprehensive study in the literature exploring all microbial communities rather than methanogens at genus level in anaerobic digesters. Likewise, influence of ozone pretreatment on these microbial communities is still unknown. Therefore, not only biogas and methane production, but also microbial communities (i.e. acidogens, acetogens, sulfate reducers, denitrifiers and methanogens) in anaerobic digesters and their responses to the selected ozone doses were examined in this study by FISH method.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sludge sampling

For the lab-scale single-stage anaerobic digesters operated in this study, sludge samples were collected from Ankara Central WWTP. Ankara Central WWTP has a daily capacity of treating 765,000 m^3 wastewater and producing 60,000 m^3 biogas with 60-70% methane content. 80-85% of the electricity demand of the plant is met from biogas produced in the anaerobic digesters. Mesophilic anaerobic digesters in the plant has 11,250 m^3 capacity and operated at 35.5°C with 14 days SRT (Ankara Water and Sewage Administration, 2019). Flow diagram of the plant and the locations where sludge samples collected are presented in Figure 3.1.

Seed sludge was collected once from the anaerobic digesters and used as inoculum at start-up. During the operation, feed sludge was collected on a weekly basis from the thickened raw sludge line which was the influent of anaerobic digesters. After sludge samples were transported safely into the laboratory, characteristics of seed and feed sludges were determined. Seed sludge was immediately used at start-up to prevent the inhibition of anaerobic microorganisms. Feed sludge was stored for a week at +4°C to minimize degradation until its use in ozone pretreatment and daily feeding.

a)

b)

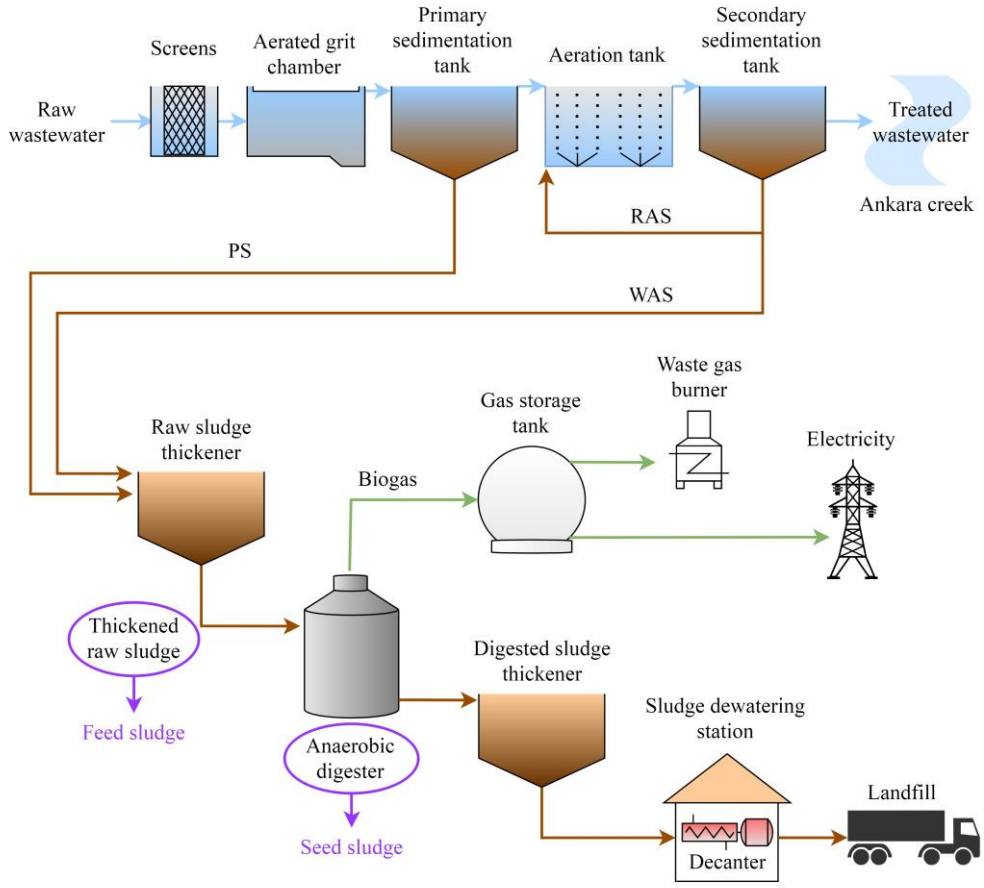

Figure 3.1. Pictures (a) and schematic diagram of Ankara Central WWTP (b)

3.2. Start-up and operation of anaerobic digesters

For this study, single-stage anaerobic digesters were installed and operated under semi-batch and mesophilic conditions. For the setup of the anaerobic digesters, a lab-scale reactor system was ordered from Germany with the distributorship of Çalışkan Laboratuvar Ürünleri Tic. Ltd. Şti. The system was made of Schott branded borosilicate glass reactors and 304 stainless steel pipes. It included 5 L reactors, 2 L graduated cylinders, pipes, valves, rubber O-rings, hose adapters and clamps. Additionally, magnetic stirrers were adapted to the system for uniform mixing. For the start-up, the components of the system were connected and graduated cylinders were filled with saturated sodium chloride (NaCl) solution. The system was sealed for making sure it was air-tight. The system was installed in a hot room operating at 35.5°C and purged with N_2 gas to maintain anaerobic conditions before the operation. Pictures of the installed system and operating conditions are given in Figure 3.2 and Table 3.1.

Figure 3.2. Pictures of single-stage anaerobic digesters operated in the study

Table 3.1. Operating conditions of anaerobic digesters used in the study

Parameter	Digester			
	C	O1	O2	O3
pH	6.5-8.0	6.5-8.0	6.5-8.0	6.5-8.0
Temperature (°C)	35.5	35.5	35.5	35.5
Total volume (L)	5	5	5	5
Effective volume (L)	3	3	3	3
Feeding and sampling (mL/d)	200	200	200	200
SRT (d)	15	15	15	15
Ozone dose (g O_3/g TSS)	**0**	**0.03**	**0.06**	**0.09**

C, control digester without ozone pretreatment; O1, O2, O3; digesters with ozone pretreatment with varying ozone doses of 0.03, 0.06 and 0.09 g O_3/g TSS

At the beginning of the operation, 3 L of seed sludge was poured into the digesters and purged with nitrogen gas (N_2). Every day during the operation, biogas and sludge samples were taken from the digesters for characterization and microbial analyses. After sampling, anaerobic digesters were fed on a daily basis. Until reaching steady-state, 200 mL of feed sludge was purged with N_2 and fed to the digesters. After steady-state conditions had been reached, the same amount of feed sludge was ozonated at various ozone doses except for control then, purged and fed into the digesters similarly. The operation of the anaerobic digesters are summarized in Figure 3.3.

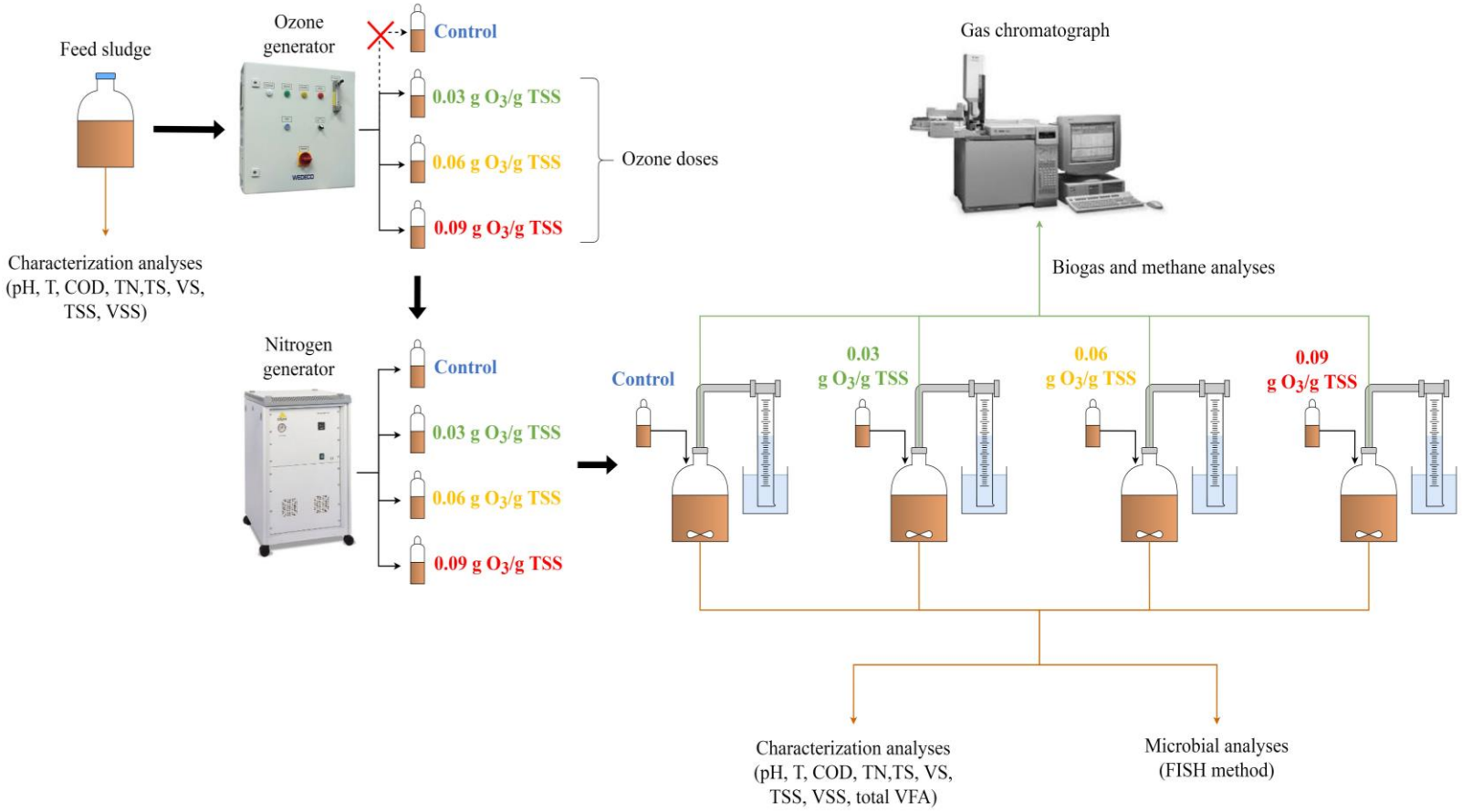

Figure 3.3. Schematic diagram of the anaerobic digesters and their operation

3.3. Ozone pretreatment

Three ozone doses of 0.03, 0.06 and 0.09 g O_3/g TSS were used for pretreating feed sludge. Ozone gas was supplied by WEDECO OCS Modular 4 HC ozone generator (WEDECO, Germany) as shown in Figure 3.4. The generator had a maximum ozone production capacity of 4 g/h and a maximum flowrate of 140 L/h. Ozone concentration and flowrate were adjusted by potentiometer of the generator. Ozone generator was calibrated before ozonation. Ozone calibration curve was provided in Appendix A.

Figure 3.4. Ozone generator used in the study

Ozone calculations for the generator were made on a weekly basis since required amounts of ozone depended on TSS concentration of feed sludge collected weekly. Required amounts of ozone for desired doses were calculated from Equation 3.1 by using TSS concentration of feed sludge, which was determined through characterization analyses. Ozone concentrations were calculated by keeping the constant flowrate at 50 L/h from Equation 3.2.

$$m_{O3}=V_{feed} \times TSS_{feed} \times D_{O3} \quad \text{(Equation 3.1)}$$

$$C_{O3}=\frac{m_{O3}}{Q_{O3} \times t} \quad \text{(Equation 3.2)}$$

m_{O3} : Amount of ozone, mg

V_{feed} : Volume of feed sludge, mL

TSS_{feed} : TSS concentration of feed sludge, g/L

D_{O3} : Ozone dose, g O_3/ g TSS

C_{O3} : Ozone concentration, mg/L

Q_{O3} : Ozone flowrate, L/h

t : Ozonation time, h

Details of ozone calculations are given in Table 3.2. Potentiometer setting in the calculations was obtained from the performance curve of the generator which was shown in Figure 3.5. As a result of these calculations, ozone concentration and potentiometer setting were found as 37.66 mg/L and 4.7 for the first week of ozone pretreatment (7^{th} week), while 54.91 mg/L and 6.8 for the second week (8^{th} week), respectively.

Table 3.2. Ozone calculations

Week	V_{feed} (mL)	TSS_{feed} (g/L)	TSS amount in feed sludge (mg)	D_{O3} (g O_3/g TSS)	m_{O3} (mg)	t (min)	Ozone mass flow (mg/h)	Q_{O3} (L/h)	C_{O3} (mg/L)	Potentiometer setting*
1	200	26.15	5230	**0.03**	156.90	5	1882.80	50	37.66	4.7
						10	941.40		18.83	-
						15	627.60		12.55	-
				0.06	313.80	5	3765.60	50	75.31	-
						10	1882.80		37.66	4.7
						15	1255.20		25.10	-
				0.09	470.70	5	5648.40	50	112.97	-
						10	2824.20		56.48	-
						15	1882.80		37.66	4.7
2	200	38.13	7626	**0.03**	228.78	5	2745.36	50	54.91	6.8
						10	1372.68		27.45	-
						15	915.12		18.30	-
				0.06	457.56	5	5490.72	50	109.81	-
						10	2745.36		54.91	6.8
						15	1830.24		36.60	-
				0.09	686.34	5	8236.08	50	164.72	-
						10	4118.04		82.36	-
						15	2745.36		54.91	6.8

V_{feed}, volume of feed sludge; TSS_{feed}, TSS concentration of feed sludge; D_{O3}, ozone dose; m_{O3}, amount of ozone; t, ozonation time; Q_{O3}, ozone flowrate; C_{O3}, ozone concentration;
*Potentiometer setting was selected for corresponding ozone flowrate and concentration from the performance curve

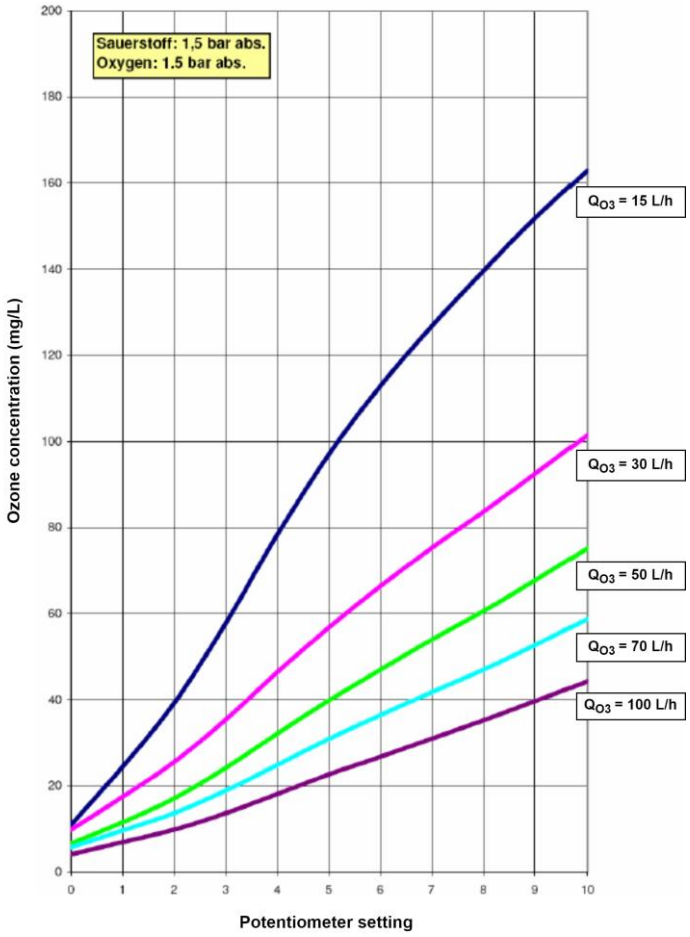

Figure 3.5. Performance curve of the ozone generator

After ozone calculations, a gas washing bottle containing 200 mL of feed sludge was connected to the ozone generator and the junction points were covered with parafilm to prevent any leakage. Three bottles were ozonated for 5, 10 and 15 minutes at predetermined settings for 0.03, 0.06 and 0.09 g O_3/g TSS, respectively. Control bottle was not ozonated. All four bottles were purged with N_2 and then, feed sludge in the bottles were fed to the digesters.

N_2 purging: Before given to the digesters, seed and feed sludges were purged with N_2 in order to eliminate O_2 and preserve anaerobic conditions. Considering sludge volumes, 3 L of seed sludge was purged for 2.5 h at start-up and 200 mL feed sludge

was purged for 10 min before each feeding. N_2 gas for purging was supplied by Peak Scientific NM30LA-MS nitrogen generator (Peak Scientific, United Kingdom) as shown in Figure 3.6.

Figure 3.6. Nitrogen generator used in the study

3.4. Characterization analyses

200 mL of sludge samples was taken from the anaerobic digesters daily by a vacuum pump and they were analyzed in terms of pH, temperature, COD, TN, solids content and total VFA. Additionally, 500 µL biogas samples were withdrawn and analyzed for methane content. Characterization analyses were carried out within the same day immediately after sampling to avoid changes caused by ambient temperature and degradation.

3.4.1. pH and temperature

pH and temperature of sludge samples were measured by HACH sensION 2 waterproof pH/ISE/mV meter (HACH, USA) according to Standard Method 4500H (APHA-AWWA-WEF, 2017). The pH meter was calibrated by pH 4, pH 7 and pH

10 buffer solutions before analyses. pH and temperature measurements were performed as duplicates.

3.4.2. COD

For COD analyses, sludge samples were diluted by 1:50 with ultrapure water (ddH_2O). COD concentrations of sludge samples were determined by HACH LCK 514 COD cuvette tests with a range of 100-2000 mg/L O_2 (HACH, USA) according to its manual. The cuvette tests were evaluated by HACH DR 2800 spectrophotometer (HACH, USA). COD analyses were performed as duplicates and the results were expressed as g/L. COD removal efficiencies were calculated on a daily basis from COD concentration of feed sludge and effluent COD concentrations. All daily COD removals were averaged and expressed as percentage.

3.4.3. TN

For TN analyses, sludge samples were diluted by 1:50 or 1:100 with ddH_2O. TN concentrations of sludge samples were determined by HACH LCK 338 Laton TN cuvette tests with a range of 20-100 mg/L (HACH, USA) according to its manual. The cuvette tests were evaluated by HACH DR 2800 spectrophotometer (HACH, USA). TN analyses were performed as duplicates and the results were expressed as g/L.

3.4.4. Solids content

TS, VS, TSS and VSS concentrations of sludge samples were determined by gravimetric method according to Standard Methods 2540B, 2540D, 2540E and 2540G (APHA-AWWA-WEF, 2017). When it was difficult to filter sludge samples for TSS and VSS analyses due to semisolid structure, the samples were diluted by 1:10 in order to filter easily. All solids determination analyses were performed as duplicates and the results were expressed as g/L. Solids removal efficiencies were

calculated on a daily basis from solids concentration of feed sludge and effluent solids concentrations. All daily solids removals were averaged and expressed as percentage. VS/TS and VSS/TSS ratios were calculated and averaged.

3.4.5. Total VFA

Total VFA concentrations of sludge samples were determined by simple titration method proposed by Anderson and Yang (1992). After initial pH of the samples were determined, 50 mL of the samples were titrated with 0.1 N sulfuric acid (H_2SO_4) until reaching two pH end points; firstly to pH 5.1 and secondly to pH 3.5. Total VFA concentrations were calculated by using following equations as stated in the procedure. Equations were solved in Excel program with the readings obtained from the titration experiments. Total VFA analyses were performed as duplicates and the results were expressed as mM.

$$A1 = \frac{[HCO_3^-]([H]_2-[H]_1)}{[H]_2+K_1} + \frac{[VA]([H]_2-[H]_1)}{[H]_2+K_2} \qquad \text{(Equation 3.3)}$$

$$A2 = \frac{[HCO_3^-]([H]_3-[H]_1)}{[H]_3+K_1} + \frac{[VA]([H]_3-[H]_1)}{[H]_3+K_2} \qquad \text{(Equation 3.4)}$$

A1 : Molar equivalent of H_2SO_4 consumed to reach the first end point (pH 5.1)

A2 : Molar equivalent of H_2SO_4 consumed to reach the second end point (pH 3.5)

$[HCO_3^-]$: Bicarbonate (HCO_3) concentration

[VA] : VFA ion concentration

$[H]_1$: Hydrogen ion concentration of the sample, $10^{-\text{initial pH}}$

$[H]_2$: Hydrogen ion concentration at the first end point, $10^{-5.1}$

$[H]_3$: Hydrogen ion concentration at the second end point, $10^{-3.5}$

K_1 : HCO_3 dissociation constant, 6.6×10^{-7}

K_2 : VFA dissociation constant, 2.4×10^{-5}

3.4.6. Biogas and methane production

Water displacement method was used for the determination of biogas production. Graduated cylinders connected to the digesters were filled with saturated NaCl solution at start-up as mentioned before. NaCl solution was used to minimize the solubility of individual gases like CO_2 in biogas (Walker et al., 2009). Initial liquid levels in graduated cylinders were recorded before sludge sampling. The valves between the digesters and the cylinders were opened so that produced biogas was transferred to the cylinders. Final liquid levels were recorded after the level had dropped. Biogas productions were measured from the differences between final and initial liquid levels.

For the determination of methane content, biogas samples were withdrawn from the digesters by sterilized 500 µL Hamilton SampleLock syringe (Hamilton Company, USA) for the injection into gas chromatograph (GC). Biogas compositions were analyzed by Agilent Technologies 6890N Network GC (Agilent Technologies, USA) as shown in Figure 3.7. GC was equipped with a HP-Plot Q capillary column and a thermal conductivity detector using carrier gases of helium (He) and N_2. Biogas analyses were done with VOLKTCD.M method by GC software. In this method, the temperature was 45°C for the first min and gradually increased to 65°C with a rate of 10°C/min. Methane analyses were performed as duplicates.

GC was calibrated with two standard gas mixtures and pure methane before analyses. The first mixture contained 25% CH_4, 55% CO_2 and 20% N_2. The second one contained 65% CH_4, 25% CO_2 and 10% N_2. GC calibration curve was given in Appendix A. As a result of biogas analyses, methane contents of biogas samples were obtained as percentage from GC software. Methane productions were calculated by multiplying biogas productions and the methane contents. Biogas and methane productions were expressed as mL. Yields were calculated from the productions obtained and VS concentration of feed sludge added into the digesters. Biogas and methane yields were expressed as mL biogas/g VS and mL methane/g VS, respectively.

Figure 3.7. GC used in the study

3.5. Microbial analyses by FISH method

The rest of the sludge samples from characterization analyses were used for microbial community analyses through FISH method. Sludge samples were fixed and stored weekly for FISH analyses considering relatively slow microbial adaptation. The FISH method used in the study was conducted by following modifications for activated sludge in biological wastewater treatment proposed by Nielsen et al. (2009). FISH procedure used in the study is summarized in Figure 3.8. All experiments were conducted as triplicates.

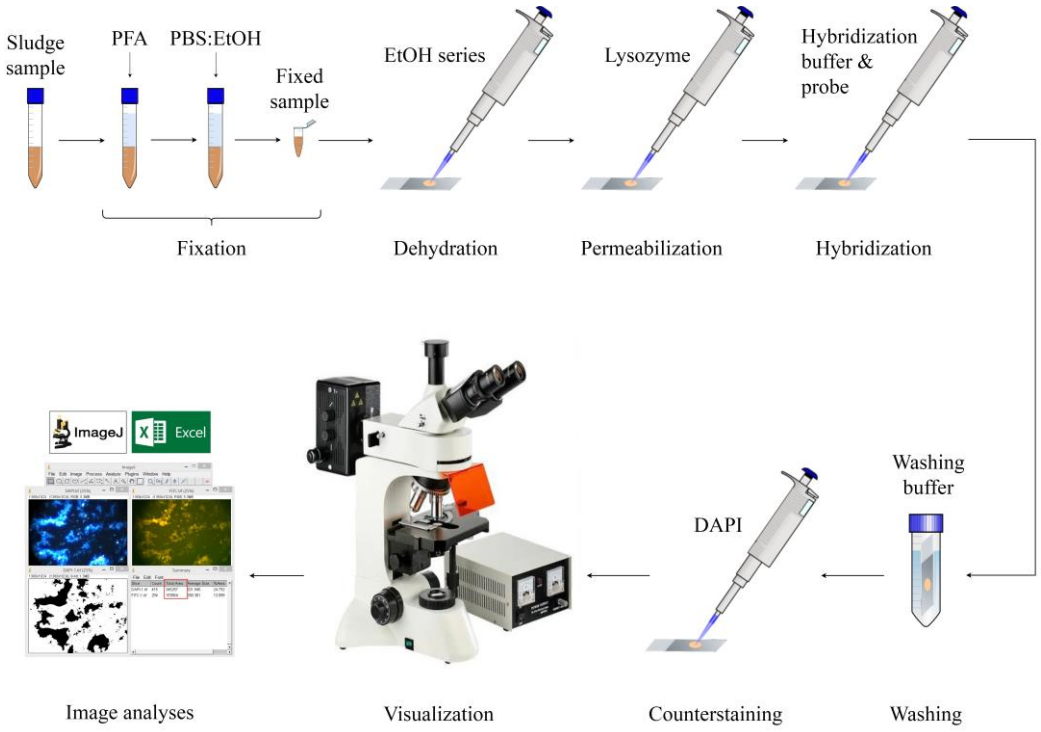

Figure 3.8. Schematic diagram of FISH procedure followed in the study

3.5.1. Probes used and their optimizations

The representative microorganisms of microbial communities in the digesters and their specific probes were selected according to the literature. Before FISH analyses of sludge samples, optimization experiments were done with the probes and corresponding pure culture of microorganisms in order to determine optimal stringency conditions providing the highest hybridization efficiency (Huber et al., 2018). Lyophilized pure cultures for optimization analyses were supplied by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany). Pure cultures were revived and fixed in the anaerobic chamber at Gülhane Military Medical Academy Microbiology Laboratory (GATA, Turkey) by following the procedure of the supplier company.

16S rRNA-targeted oligonucleotide probes for target microorganisms were selected from probeBase (Greuter et al., 2016). The probes labeled with fluorescein isothiocyanate (FITC) at 5' end were supplied by Alpha DNA (Alpha DNA, Canada). Lyophilized probes were re-suspended in Tris-EDTA (TE) buffer. Stock and working solutions were prepared by diluting the probes according to the procedure of the manufacturer. The properties of the probes used in the study and target microorganisms are given in Table 3.3. Probes of EUB338, EUB338 II and EUB338 III were used to make EUBmix for detecting total *Bacteria*. NONEUB probe was used as negative control.

For the optimization, hybridization experiments were carried out using target and non-target microorganisms for each FITC-labeled probe. Pure cultures of the probes were used as positive control and *Escherichia coli* (DH5α) was used as negative control in optimization experiments. The probes were hybridized with the controls at different stringency conditions by changing formamide (FA) and NaCl concentrations according to Table 3.4 (Nielsen et al., 2009). c (Table 3.4). For instance, FA concentration for MX825 probe was stated in probeBase as 50% (Greuter et al., 2016). Therefore, 45, 50 and 55% FA concentrations were used in hybridization buffers. Corresponding NaCl concentrations of 0.040, 0.028 and 0.020 M (i.e. 300, 180 and 100 µL) were used in washing buffers (Table 3.4). Similarly, each probe was hybridized with its pure culture and *E. coli* (DH5α) at 46°C for 2 h. Same FISH procedure from sections 3.5.3 to 3.5.6 were performed for optimization experiments as triplicates.

Table 3.3. Overview of the probes used in the study

Microbial community	Probe	Target microorganism	DSM no	Sequence of probe (5'→3')	[FA] (%)	References
Acidogens	HoAc1402	*Acidobacteria*	DSM 22465	CTTTCGTGATGTGACGGG	10	(Greuter et al., 2016)
	SS_HOL1400	*Acidobacteria*	DSM 22743	TTCGTGATGTGACGGGC	20	(Greuter et al., 2016)
	Clost I	*Clostridium* spp.	DSM 10612	TTCTTCCTAATCTCTACGCA	20	(Greuter et al., 2016)
	Actino221	*Actinobacteria*	DSM 20639	CGCAGGTCCATCCCAGAC	30	(Greuter et al., 2016)
	CFB563	*Flavobacterium*	DSM 18451	GGACCCTTTAAACCCAAT	20	(Greuter et al., 2016)
Acetogens	DSBAC355	*Syntrophobacterales*	DSM 10017	GCGCAAAATTCCTCACTG	35	(Greuter et al., 2016)
	GTAG992	*Thermacetagenium*	DSM 26808	CCAGGTCCGCAGAGATGTCA	45	(Narihiro et al., 2012)
	SYN835	*Syntrophobacter*	DSM 2805	GCAGGAATGAGTACCCGC	35	(Díaz et al., 2006)
	GTE1002	*Tepidanaerobacter*	DSM 21804	TCCGTTTCCGGTCTCTACCA	55	(Narihiro et al., 2012)
Sulfate reducers	DSV687	*Desulfovibrio* spp.	DSM 2480	TACGGATTTCACTCCT	15	(Greuter et al., 2016)
	DSB129	*Desulfobacter* spp.	DSM 17510	CAGGCTTGAAGGCAGATT	15	(Greuter et al., 2016)
	DBB660	*Desulfobulbus* spp.	DSM 10215	ATTCCACTTTCCCCTCTG	60	(Greuter et al., 2016)
	DSC193	*Desulfosarcina variabilis*	DSM 2060	AGGCCACCCTTGATCCAA	35	(Greuter et al., 2016)
Denitrifiers	Pae997	*Pseuodomonas* spp.	DSM 1110	TCTGGAAAGTTCTCAGCA	0	(Czerwionka et al., 2014)
	Bmy843	*Bacillus* spp.	DSM 4337	CTTCAGCACTCAGGTTCG	35	(Del'Duca et al., 2015)
	DEN124	Acetate-denitrifying cluster	DSM 14793	CGACATGGGCGCGTTCCGAT	40	(Greuter et al., 2016)
Methanogens	MS1414	*Methanosarcina* spp.	DSM 2256	CTCACCCATACCTCACTCGGG	50	(Raskin et al., 1994)
	MG1200	*Methanomicrobiales*	DSM 1539	CGGATAATTCGGGGCATGCTG	20	(Raskin et al., 1994)
	MX825	*Methanosaeta* spp.	DSM 17206	TCGCACCGTGGCCGACACCTAGC	50	(Raskin et al., 1994)
	MB1174	*Methanobacteriales*	DSM 2257	TACCGTCGTCCACTCCTTCCTC	45	(Raskin et al., 1994)
Archaea	ARC915	*Archaea*	-	GTGCTCCCCCGCCAATTCCT	35	(Sekiguchi et al., 1999)
Bacteria	EUB338*	Most *Bacteria*	-	GCTGCCTCCCGTAGGAGT	0-50	(Greuter et al., 2016)
	EUB338 II*	*Planctomycetales*	-	GCAGCCACCCGTAGGTGT	0-50	(Greuter et al., 2016)
	EUB338 III*	*Verrucomicrobiales*	-	GCTGCCACCCGTAGGTGT	0-50	(Greuter et al., 2016)
	NONEUB	Negative control	-	ACTCCTACGGGAGGCAGC	0	(Greuter et al., 2016)

*EUB338, EUB338 II and EUB338 III probes were mixed and called as EUBmix for targeting total bacteria; DSM no, accession numbers of the pure cultures obtained from the company; [FA], suggested formamide concentrations in the literature

3.5.2. Fixation of sludge samples

Sludge samples were fixed for inactivation of microbial cells and enzymatic activities. For the fixation, 6 mL of fresh sludge samples were transferred to 15 mL falcon tubes and centrifuged at 10000 rpm for 5 min. After the supernatant was removed, 3 mL of cold 4% paraformaldehyde (PFA) was added onto the pellet and mixed through vortexing. The tubes were kept on ice for 4 h and PFA was separated from the pellet after centrifuging at 10000 rpm for 5 min. 3 mL of cold 1:1 phosphate-buffered saline and ethanol solution (PBS:EtOH) was added onto the pellet and mixed. Fixed samples were transferred into 1.5 mL eppendorf tubes as triplicates and stored at -20°C until use.

3.5.3. Dehydration and permeabilization

Fixed samples were dehydrated and permeabilized to prepare sample for hybridization. After melting fixed samples at room temperature, they were centrifuged to prevent clogging of the pipettes. 10 µL of the sample was applied on a microscope slide. The sample was spread out with pipette tip and air-dried. 50, 80 and 96% ethanol were applied for 3 min each to dehydrate microbial cells and the slide was air-dried. 10 µL of cold lysozyme was applied and cover glass was placed on the sample to permeabilize cell walls. The slides were incubated at 37°C for 30 min in humid environment. Cover glass was removed by washing with ddH_2O and the slide was air-dried.

3.5.4. Hybridization and washing

In order to hybridize the samples with probes, 2 mL of hybridization buffer was prepared depending on optimum FA concentrations of each probe according to Table 3.4. 8 µL of hybridization buffer and 1 µL of probe working solution were applied and a cover glass was placed on the sample. The slides were incubated at 46°C for 2 h in humid and dark environment.

Table 3.4. Composition of hybridization and washing buffers (Nielsen et al., 2009)

	Hybridization buffer					Washing buffer			
[FA] (%)	**FA (µL)**	**ddH_2O (µL)**	**5 M NaCl (µL)**	**1 M Tris/HCl (µL)**	**10% SDS (µL)**	**1 M Tris/HCl (µL)**	**10% SDS (µL)**	**5 M NaCl (µL)**	**0.5 M EDTA (µL)**
0	0	1600	360	40	2	1000	50	9000	0
5	100	1500	360	40	2	1000	50	6300	0
10	200	1400	360	40	2	1000	50	4500	0
15	300	1300	360	40	2	1000	50	3180	0
20	400	1200	360	40	2	1000	50	2150	500
25	500	1100	360	40	2	1000	50	1490	500
30	600	1000	360	40	2	1000	50	1020	500
35	700	900	360	40	2	1000	50	700	500
40	800	800	360	40	2	1000	50	460	500
45	900	700	360	40	2	1000	50	300	500
50	1000	600	360	40	2	1000	50	180	500
55	1100	500	360	40	2	1000	50	100	500
60	1200	400	360	40	2	-	-	-	-
65	1300	300	360	40	2	-	-	-	-
70	1400	200	360	40	2	-	-	-	-

[FA], formamide concentration; ddH_2O, ultrapure water; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; EDTA, Ethylenediaminetetraacetic acid

During hybridization, 50 mL of washing buffer was prepared for each FA concentration to remove non-specific hybridizations according to Table 3.4. Washing buffer was preheated in a 48°C water bath. Cover glass placed in hybridization step was removed by rinsing the slide with washing buffer. The slides were transferred 50 mL falcon tubes containing preheated washing buffer and incubated at 48°C water bath for 15 min. The slides were removed, dipped in cold ddH_2O and air-dried. After hybridization, the samples were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (1 µg/mL) at 4°C for 10 min in the dark. The slides were rinsed with ddH_2O and air-dried. Slides were stored at -20°C in the dark without substantial loss of signal intensity until visualization.

3.5.5. Visualization

Stained slides at -20°C were brought to room temperature in the dark room for microscopic visualization. Slides were examined by BEL Photonics FLUO3 fluorescence microscope (BEL Engineering, Italy) equipped with a HBO 100W mercury lamp as shown in Figure 3.9. The microscope had objective lenses of 4x/0.1, 10x/0.25, 40x/0.65 and 100x/1.25 with the filters of ultraviolet (UV), violet (V), green (G) and blue (B). All microorganisms stained with DAPI were visualized as blue in color through UV filter with 330-400 nm spectrum. Target microorganisms hybridized with FITC-labeled probes were visualized as green to yellow in color through B filter with 420-485 nm spectrum. Triplicate slides were viewed at 100x magnification after immersion oil was applied. For each slide, 10 representative areas for microorganisms were selected and images were captured accordingly. Images from same areas were taken under both UV and B filters with the help of CCD camera and software of the microscope.

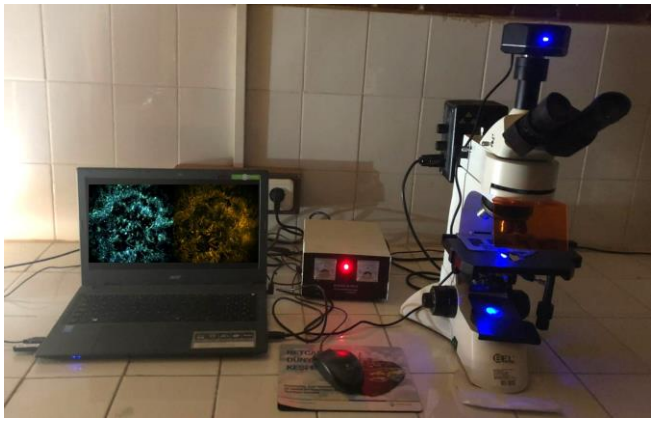

Figure 3.9. Fluorescent microscope used in the study

3.5.6. Image analyses

Images taken from the microscopy were analyzed by ImageJ software (Schneider et al., 2012). The principle behind image analyses is the classification of microorganisms depending on their fluorescence signal intensity. Microorganisms giving higher intensity than a certain threshold are considered as target (Zhou et al., 2007). ImageJ allows adjusting that threshold value and measuring signal intensity differences in images.

As shown in Figure 3.10, ImageJ analyses in the study were performed according to the procedure by Bankhead (2014) with the following commands:

- *File → Open:* The image was opened.
- *Image → Duplicate:* The image was copied.
- *Image → Type → 8-bit:* The image was converted to 8-bit color.
- *Image → Adjust → Threshold:* Threshold value of 8-bit image was adjusted to cover microorganisms with reference to the original image.
- *Analyze → Analyze Particles:* Pixel analysis of the image was done according to count, total area and size etc.

For each image, threshold values were adjusted manually since fluorescence intensity varies for each experiment. Pixel areas of DAPI and FITC images taken under UV and B filters were measured at determined threshold values. Blue fluorescence represented DAPI stained all microorganisms and green fluorescence represented FITC-labeled probes (Li et al., 2007).

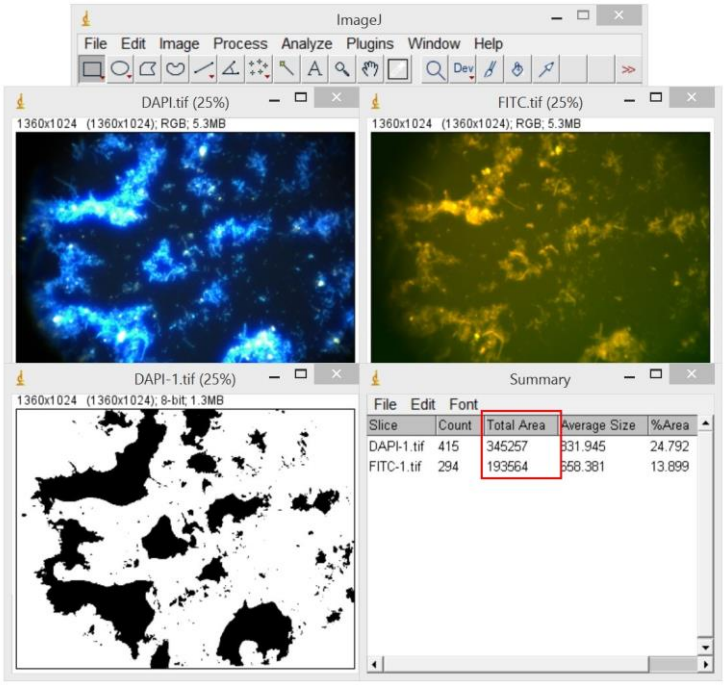

Figure 3.10. Screenshot of image analyses performed by ImageJ

After analyzing the images, total area data shown in summary window (Figure 3.10) were extracted into Excel program. Target microorganisms within the microbial communities and domains were quantified by using Equation 3.5 (Icgen & Yilmaz, 2014).

$$\text{Population of target microorganism (\%)} = \frac{\text{Pixel area}_{\text{FITC}}}{\text{Pixel area}_{\text{DAPI}}} \qquad \text{(Equation 3.5)}$$

In the calculations, pixel areas obtained from the hybridizations with EUBmix and ARC915 probes were applied to get an idea about the distribution of microbial communities by domains of *Bacteria* and *Archaea*, respectively. Pixel areas obtained from the hybridization with NONEUB probe were subtracted to exclude non-specific hybridization, background interference and autofluorescence (Icgen et al., 2007). Relative abundancies of microbial communities were calculated by assuming total microbial population including *Bacteria* and *Archaea* as 100%. Similarly, relative abundancies of target microorganisms were calculated by assuming harboring

microbial community population as 100%. All FISH experiments and analyses were performed as triplicates. Mean and standard deviations of obtained data from different images were determined. Same FISH procedure was also followed to optimize the probes.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Characterization of seed and feed sludges

Seed and feed sludges used for the operation of anaerobic digesters were characterized in terms of pH, temperature, COD, TN and solids content. Seed sludge was determined once for the start-up and feed sludge, collected on a weekly basis, was characterized for each week. Characteristics of seed and feed sludges are given in Table 4.1. OLRs for the digester feeding were calculated for each feed sludge taken. Feed sludge was not ozonated up to five weeks since the digesters did not reach steady-state condition. After steady-state condition was reached at the end of the 6^{th} week (day 42), feed sludges collected at 7^{th} and 8^{th} weeks were exposed to ozone pretreatment. Therefore, ozone requirements for desired doses were calculated according to TSS concentrations of feed sludges at those weeks as mentioned in section 3.3 (Table 3.2). For feed sludges of 1^{st} and 2^{nd} week, characterization values were found higher especially in COD and solids content. This was due to the high loading of wastewater coming to Ankara Central WWTP in that period as consulted with the operator. VS/TS and VSS/TSS ratios of feed sludges were in the ranges of 0.64-0.77 and 0.53-0.79, respectively. Organic content of feed sludges used in this study were high since VS/TS ratios were greater than 0.50 (X. Wang et al., 2016). Additionally, digesters were not operated at constant OLR in order to stimulate the real case. In this study, OLRs varied between 1.45 and 3.81 g VS/L.d. Typically, mesophilic digesters with 15 days SRT have OLR values ranging from 0.95 to 3.8 g VS/L.d (Metcalf & Eddy, 2003). Therefore, no problem related to high or low loading conditions was encountered.

Table 4.1. Characteristics of seed and feed sludges used in the study

	Seed sludge	Feed sludge					Ozone pretreatment		
Parameter	**1st week**	**1st week**	**2nd week**	**3rd week**	**4th week**	**5th week**	**6th week**	**7th week**	**8th week**
pH	7.25±0.03	6.26±0.04	6.54±0.11	6.40±0.18	6.52±0.23	6.75±0.08	6.33±0.31	6.71±0.17	6.64±0.06
Temperature (°C)	31.20±0.10	14.50±0.10	13.60±0.20	14.70±0.30	15.40±0.20	16.00±0.20	15.20±0.40	15.70±0.00	18.70±0.10
COD (g/L)	11.10±0.15	32.90±0.07	30.15±0.02	23.52±0.13	27.83±0.15	26.79±0.12	28.4±0.01	29.18±0.09	22.72±0.04
TN (g/L)	2.48±0.04	3.20±0.03	3.13±0.01	2.90±0.04	3.04±0.04	2.58±0.02	2.29±0.01	2.82±0.05	2.37±0.02
TS (g/L)	25.36±0.07	85.89±0.16	74.66±0.08	51.23±0.14	47.22±0.07	45.34±0.19	32.55±0.06	28.64±0.11	39.38±0.13
VS (g/L)	11.96±0.06	57.16±0.12	49.73±0.07	34.27±0.11	31.65±0.09	28.86±0.26	25.05±0.05	21.71±0.14	26.98±0.17
TSS (g/L)	17.60±0.12	68.80±0.08	54.21±0.13	46.39±0.09	40.18±0.13	39.82±0.15	27.38±0.12	26.15±0.03	38.13±0.09
VSS (g/L)	10.40±0.14	36.40±0.11	28.92±0.04	29.68±0.12	26.07±0.18	27.07±0.14	21.64±0.08	20.25±0.09	25.30±0.11
VS/TS	0.47±0.01	0.67±0.02	0.67±0.02	0.67±0.02	0.67±0.02	0.64±0.02	0.77±0.03	0.76±0.03	0.69±0.02
VSS/TSS	0.59±0.02	0.53±0.02	0.53±0.02	0.64±0.02	0.65±0.02	0.68±0.02	0.79±0.03	0.77±0.03	0.66±0.02
OLR (g VS/L.d)	-	3.81±0.12	3.32±0.07	2.28±0.11	2.11±0.09	1.92±0.26	1.67±0.05	1.45±0.14	1.80±0.17

COD, chemical oxygen demand; TN, total nitrogen; TS, total solids, VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; VS/TS, VS to TS ratio; VSS/TSS, VSS to TSS ratio; OLR, organic loading rate; ±, standard deviation

4.2. Characterization of anaerobic digesters

Sludge samples taken from the anaerobic digesters were characterized in terms of pH, temperature, COD, TN, solids content, total VFA, biogas and methane production. Until reaching steady-state conditions, pH, temperature, biogas and methane productions were determined on a daily basis while COD, TN, solids content and total VFA were determined on a weekly basis. Ozone pretreatment was not applied to the digesters at unsteady-state conditions. After 42 days of unsteady-state operation, steady-state conditions were assumed when daily variation of data was within ±10% fluctuation. When steady-state conditions were reached, ozone pretreatment was started to be applied into feed sludge. At steady-state conditions, anaerobic digesters were pretreated with 0.03, 0.06 and 0.09 g O_3/g TSS for 15 days until biogas production did not significantly change. Control digester continued to be operated without ozone pretreatment. All operational parameters were determined on a daily basis at steady-state conditions. Only mean values of replicate experimental results were illustrated in the graphs. Detailed results including standard deviations were provided in Appendix B. Additionally, average COD, TS, VS, TSS and VSS removal efficiencies were calculated to determine the degree of biodegradation and solubilization. Unsteady- and steady-state results were given individually for each parameter and discussed in following sections:

4.2.1. pH

pH values of anaerobic digesters were monitored on a daily basis throughout the operation. No pH control was required since feed sludges were acted as daily buffers due to lower pH values. pH values of unstable digesters varied between 6.97 and 8.17 (Figure 4.1). For the first three weeks, pH values were fluctuated sharply as expected due to the adaptation of the digesters. For the last two weeks, pH values did not change significantly as daily variations were not more than ±0.2. With ozone pretreatment, pH values of stable digesters varied between 7.55 and 7.97 (Figure 4.2). pH of control digester was almost stable while ozone pretreated digesters

showed fluctuations occasionally. Particularly, pH changes were more drastic than others for the digester pretreated with the highest ozone dose of 0.09 g O_3/g TSS. In this study, increase in ozone doses resulted in pH fluctuations. Ki et al. (2003) recorded that increasing ozone doses resulted in gradual decrease in pH. On the other hand, Chiavola et al. (2019) observed a slight increase in pH at increasing ozone doses for both mixed and WAS sludge. Kosowski et al. (2020) showed that ozonation did not significantly affect pH of sludge. Therefore, pH may need to be controlled when higher ozone doses required. pH values in this study did not exceed the optimal pH range of 6.5-8.0 for digesters (Cioabla et al., 2012).

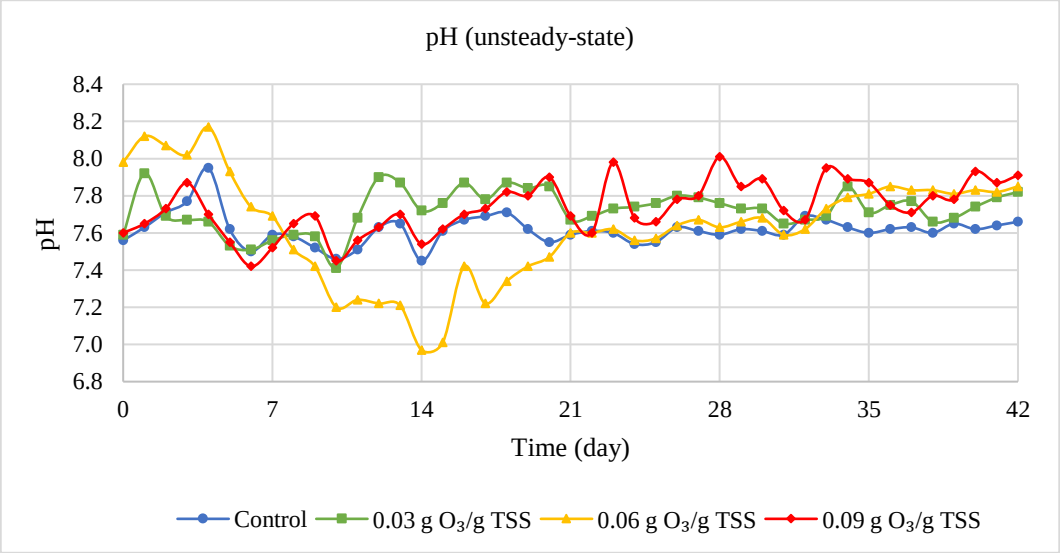

Figure 4.1. pH changes during unsteady-state operation without ozone pretreatment

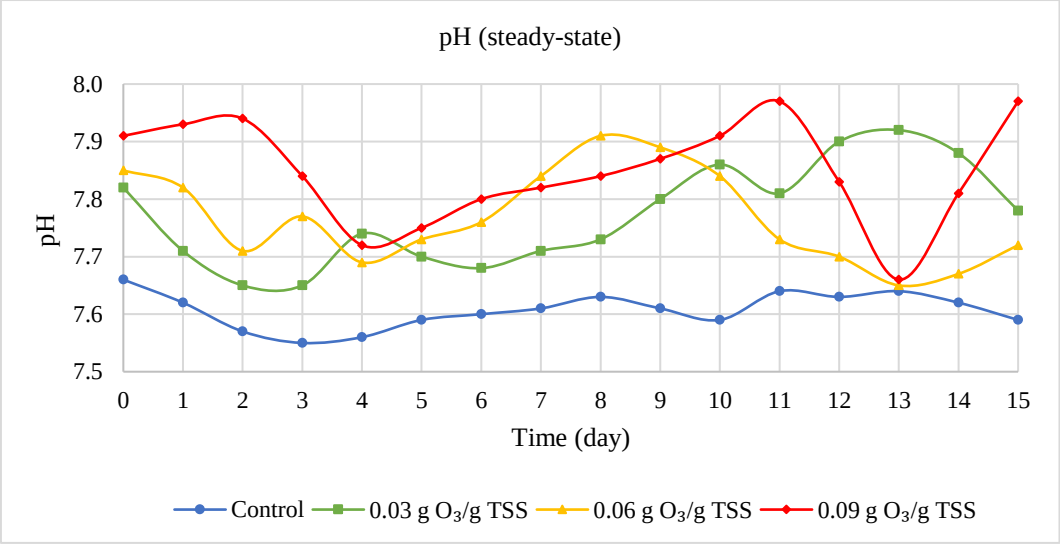

Figure 4.2. pH changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.11, ±0.08, ±0.15 and ±0.09, respectively.

4.2.2. Temperature

Temperature values of anaerobic digesters were monitored on a daily basis throughout the operation. Temperatures varied in the ranges of 31.1-36.9°C and 33.2-36.5°C during unsteady and steady-state conditions, respectively (Figure 4.3 and Figure 4.4). Cinar and Kuchta (2020) indicated that an adaptation of the digesters to 5°C and 10°C temperature increases were achievable. Mara and Horan (2003) noted that temperature variations should be as small as ±1-2°C. More recently, Westerholm and Schnürer (2019) stated that temperature fluctuations within ±2-3°C might be experienced without causing any instability. Daily temperature fluctuations in this study were no more than ±3.0°C. These variations were caused by precision of thermostat system in the hot room and ambient temperature differences due to the time elapsed until the measurements were done in the laboratory.

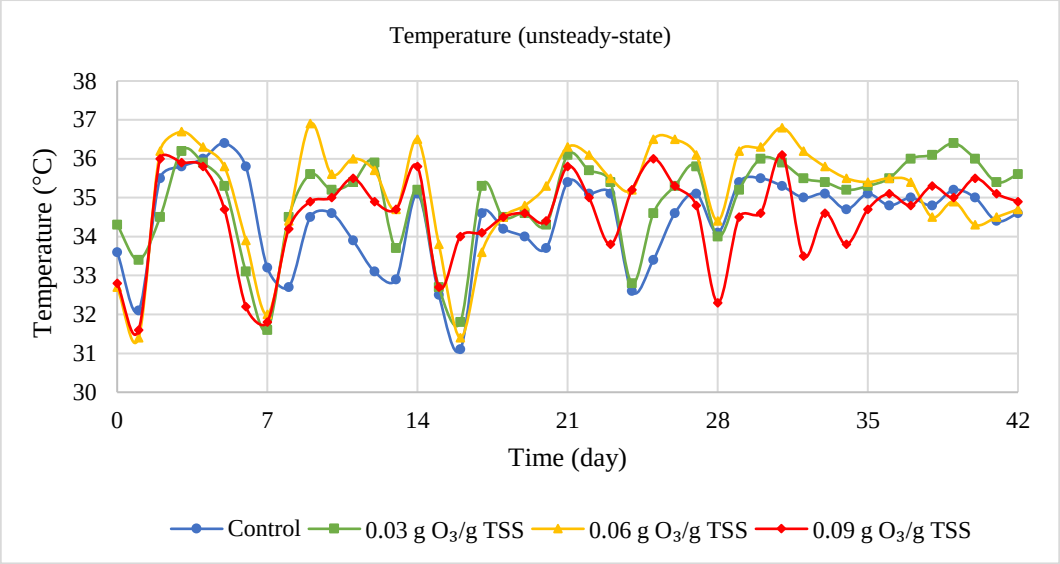

Figure 4.3. Temperature changes during unsteady-state operation without ozone pretreatment

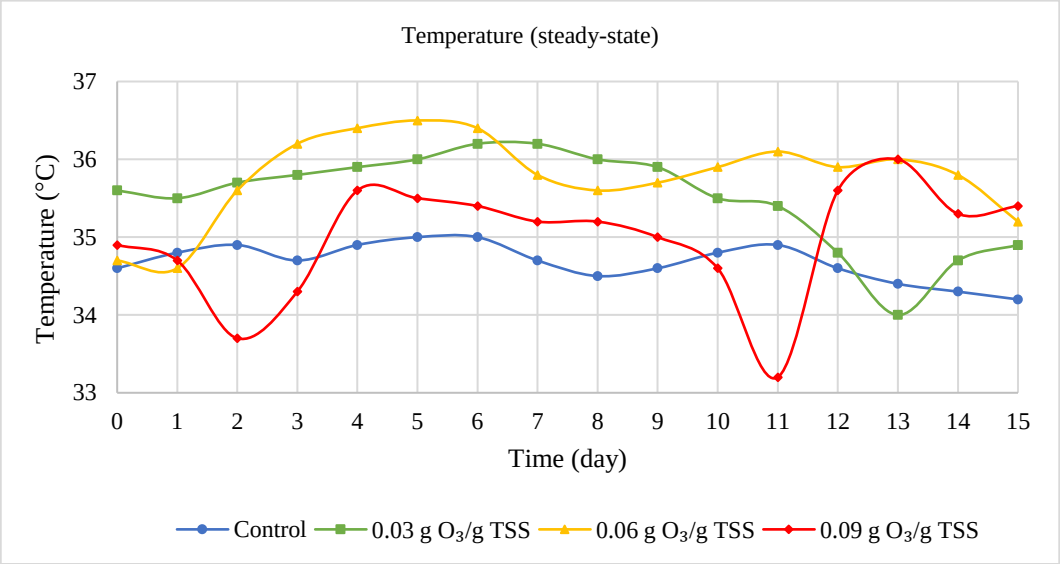

Figure 4.4. Temperature changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.80, ±0.90, ±1.00 and ±0.90, respectively.

4.2.3. COD

COD concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. COD concentrations of unstable digesters varied between 17.83 and 32.90 g/L (Figure 4.5). COD values decreased gradually from the start-up to the end of unsteady-state operation. At the end of unsteady-state operation, COD removal efficiencies for control, 0.03, 0.06 and 0.09 g O_3/g TSS were observed as 36%, 46%, 42% and 44%, respectively. Similarly, Dinh and Le et al. (2020) also stated 41% increase in COD removal for pilot-scale semi-batch digesters operated at unsteady-state. Recently, Caillet and Adelard (2021) noted that COD removal efficiencies of semi-batch digesters ranged from 49% to 82%. With ozone pretreatment, COD concentrations of stable digesters varied between 11.36 and 22.45 g/L (Figure 4.6). Pretreatment for 15 days achieved average COD removals of 30%, 34%, 35% and 28% for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Table 4.2). These COD removal efficiencies were found reasonable when evaluated together with methane production efficiencies given in section 4.2.11. Weemaes et al. (2000) showed that COD degradation was 36% for untreated sludge whereas 54%, 64% and 47% for 0.05, 0.1 and 0.2 g O_3/g TS, respectively. According to studies by Bougrier et al. (2006) and (2007), ozone pretreatment did not affect total COD for ozone doses below 0.15 g O_3/g TS but soluble portion of COD increased when ozone dose was increased. Chacana et al. (2017) accomplished 18% COD reduction for 0.192 g O_3/g COD. Otieno et al. (2019) reported 42% decrease in COD for 2 h ozonolysis. In this study, ozone pretreatment led to a slight increase in COD removals except for 0.09 g O_3/g TSS dose. The highest COD removal of 35% was observed for the digester pretreated with 0.06 g O_3/g TSS.

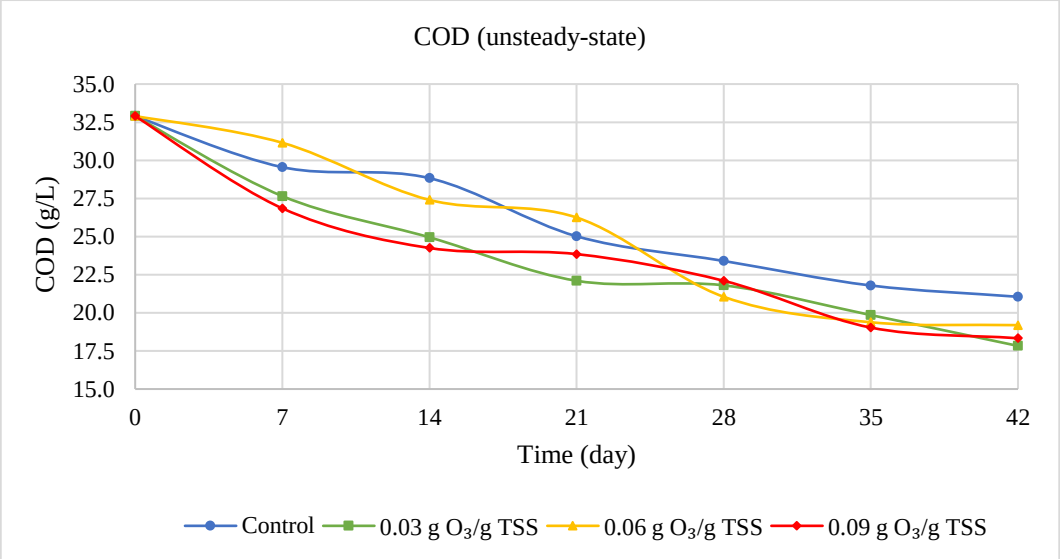

Figure 4.5. COD changes during unsteady-state operation without ozone pretreatment

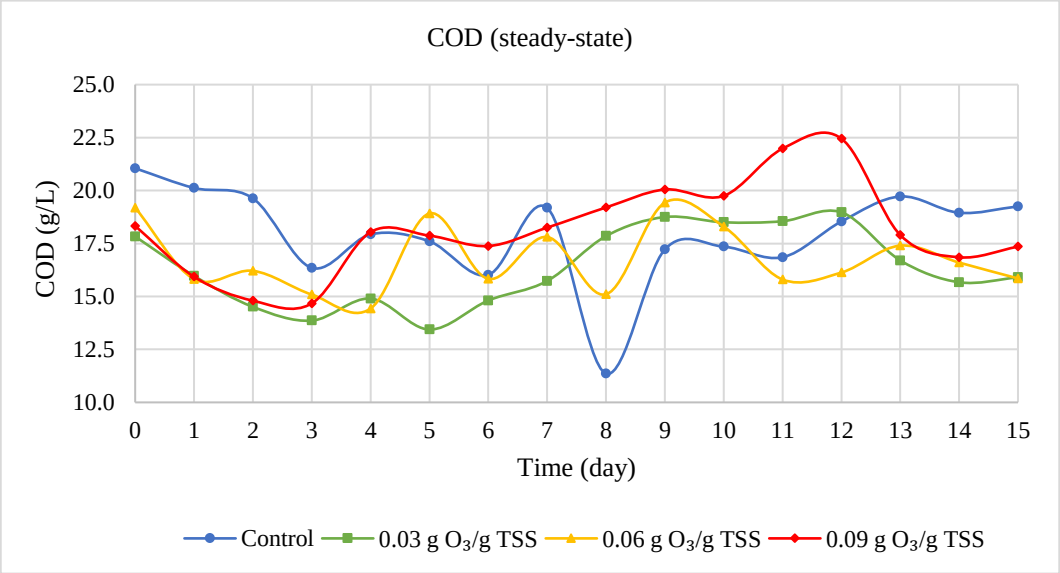

Figure 4.6. COD changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.09, ±0.15, ±0.08 and ±0.11, respectively.

4.2.4. TN

TN concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. TN concentrations of unstable digesters varied between 1.08 and 14.90 g/L (Figure 4.7). For the first three weeks, TN values dramatically increased to very high levels above 8 g/L. This increase was accounted for N_2 purging of seed sludge at the start-up. After the third week, TN values decreased and stabilized. Similar trends were observed for all digesters for the last week. With ozone pretreatment, TN concentrations of stable digesters varied between 1.74 and 3.75 g/L (Figure 4.8). According to Harrison and Ndegwa (2020), TN concentration did not change during AD but its organic portion was mineralized. Although daily fluctuations were observed due to purging of feed sludge, general trend in TN was constant in this study. Thus, it was concluded that ozone pretreatment had no additional effect on this TN concept.

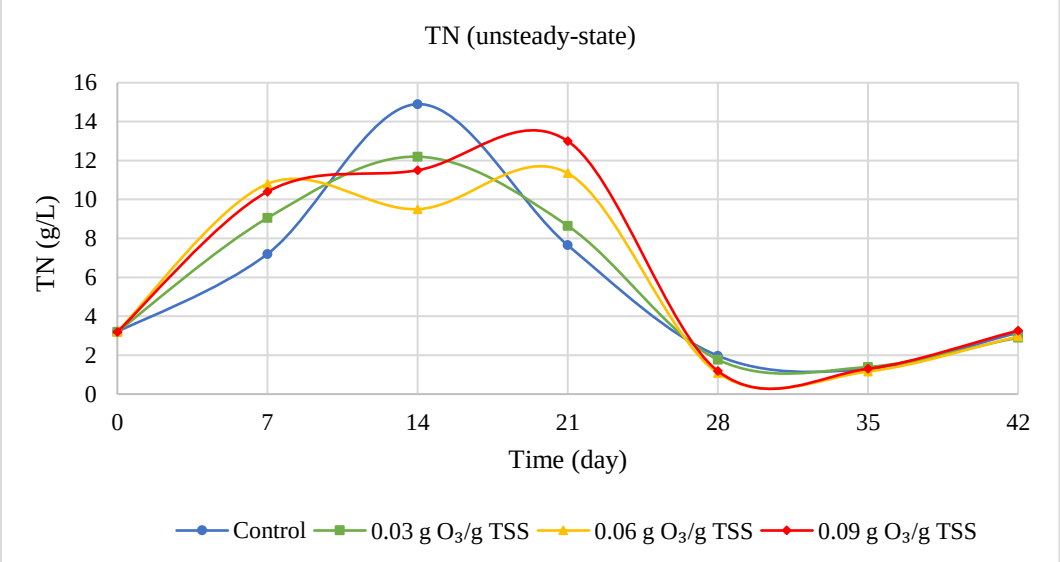

Figure 4.7. TN changes during unsteady-state operation without ozone pretreatment

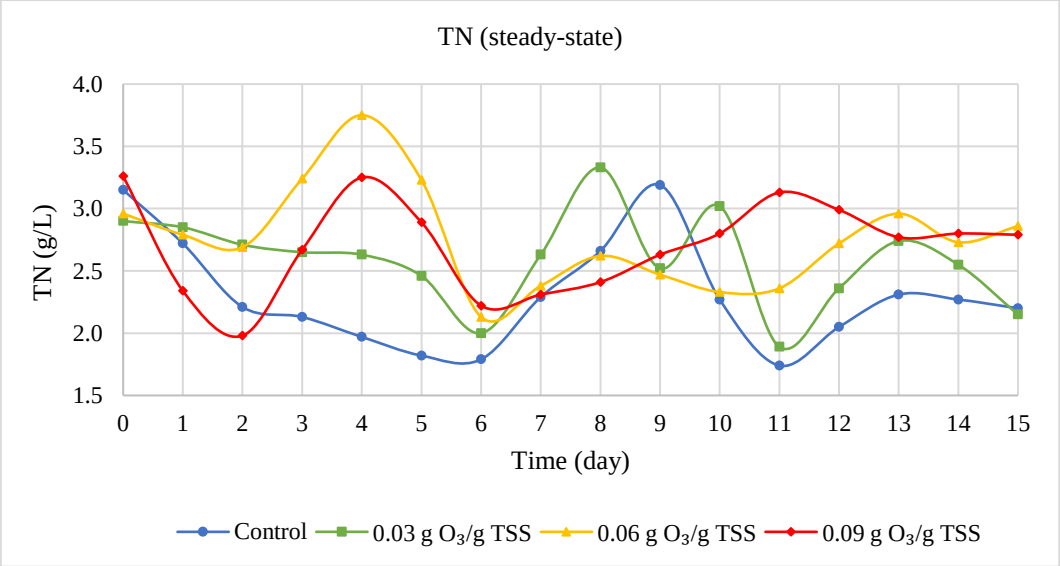

Figure 4.8. TN changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.15, ±0.09, ±0.11 and ±0.08, respectively.

4.2.5. TS

TS concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. TS concentrations of unstable digesters varied between 27.84 and 85.89 g/L (Figure 4.9). At the end of unsteady-state operation, TS removal efficiencies for control, 0.03, 0.06 and 0.09 g O_3/g TSS were observed as 60%, 62%, 64% and 61%, respectively. Digested sludge is about half as concentrated as feed sludge in single-stage anaerobic digesters so, TS are reduced by 45-50% in general (Metcalf & Eddy, 2003). Dohdoh and Aboulfotoh (2017) observed 30.5% removal in TS for unstable semi-batch digesters. Hence, the removals obtained in this study were found higher. This was accounted for high TS of feed sludge in the first week as given in Table 4.1. With ozone pretreatment, TS concentrations of stable digesters varied between 12.35 and 34.21 g/L (Figure 4.10). Pretreatment for 15 days achieved average TS removals of 23%, 28%, 32% and 27% for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Table 4.2). Goel and Takutomi et al. (2003) achieved 28% TS destruction with ozone pretreatment. In another study by Goel and Yasui et al. (2003), 61% TS removal with respect to 39-

43% of the controls was obtained. Bougrier et al. (2007) demonstrated that ozone pretreatment did not affect TS at ozone doses below 0.15 g O_3/g TS. Moussavi et al. (2008) increased TS removals from 15.4% to 80.7% for ozone doses ranging from 0.125 to 2 g O_3/g TS. In this study, ozone pretreatment led to lower TS removals than of the literature. The highest removal of 32% was observed for the digester pretreated with 0.06 g O_3/g TSS.

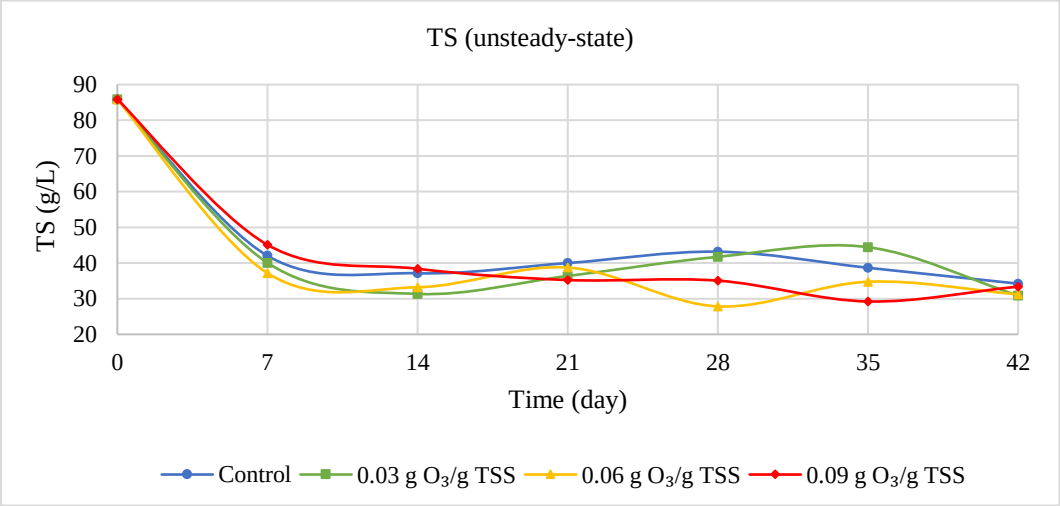

Figure 4.9. TS changes during unsteady-state operation without ozone pretreatment

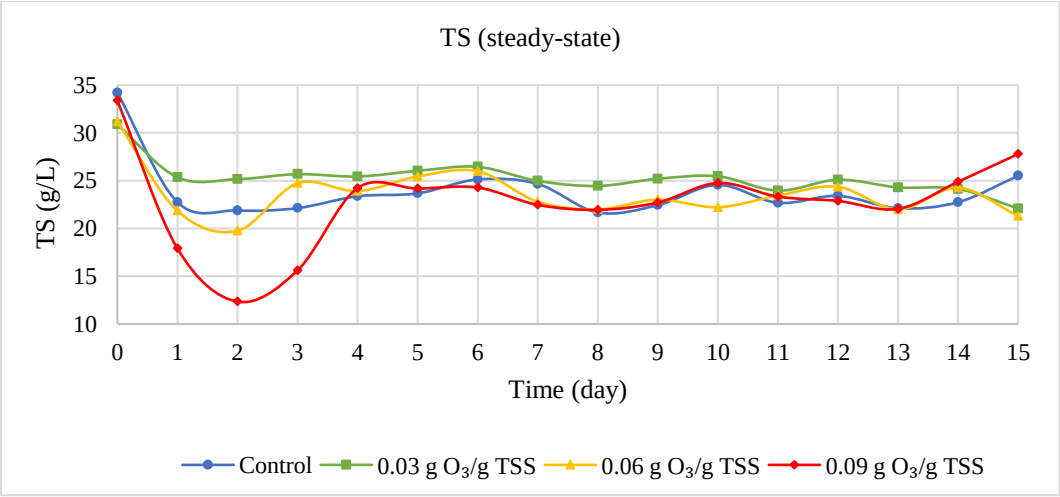

Figure 4.10. TS changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.14, ±0.17, ±0.23 and ±0.20, respectively.

4.2.6. VS

VS concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. VS profiles showed similar trends to TS profiles (Figure 4.9 and Figure 4.10) except for some minor fluctuations. Additionally, VS removals were close to removals obtained for TS. VS concentrations of unstable digesters varied between 18.26 and 57.16 g/L (Figure 4.11). At the end of unsteady-state operation, VS removal efficiencies for control, 0.03, 0.06 and 0.09 g O_3/g TSS were observed as 62%, 65%, 68% and 59%, respectively. Theoretical VS removal for mesophilic digesters with 15 days SRT was estimated as 56% (Metcalf & Eddy, 2003). Dohdoh and Aboulfotoh (2017) found that VS removal for semi-batch digesters was 32%, which was lower than the removals obtained in this study. This might be explained with the high VS of feed sludge in the first week as given in Table 4.1. With ozone pretreatment, VS concentrations of stable digesters varied between 12.35 and 34.21 g/L (Figure 4.12). Pretreatment for 15 days achieved average VS removals of 37%, 36%, 42% and 34% for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Table 4.2). In the literature, a large number of VS removals ranging from 31% to 143% were stated depending on ozone doses (Chiavola et al., 2019). According to Goel et al. (2003), VS removals for untreated sludge were found as 25-35% and removals were increased by only 10-30% for the sludge pretreated with 0.015 g O_3/g TS. Moussavi et al. (2008) increased VS removals from 5.8% to 45.9% for ozone doses ranging from 0.125 to 2 g O_3/g TS. In this study, ozone pretreatment increased VS removals in parallel with the literature although ozone doses were different. The highest removal of 42% was observed for the digester pretreated with 0.06 g O_3/g TSS.

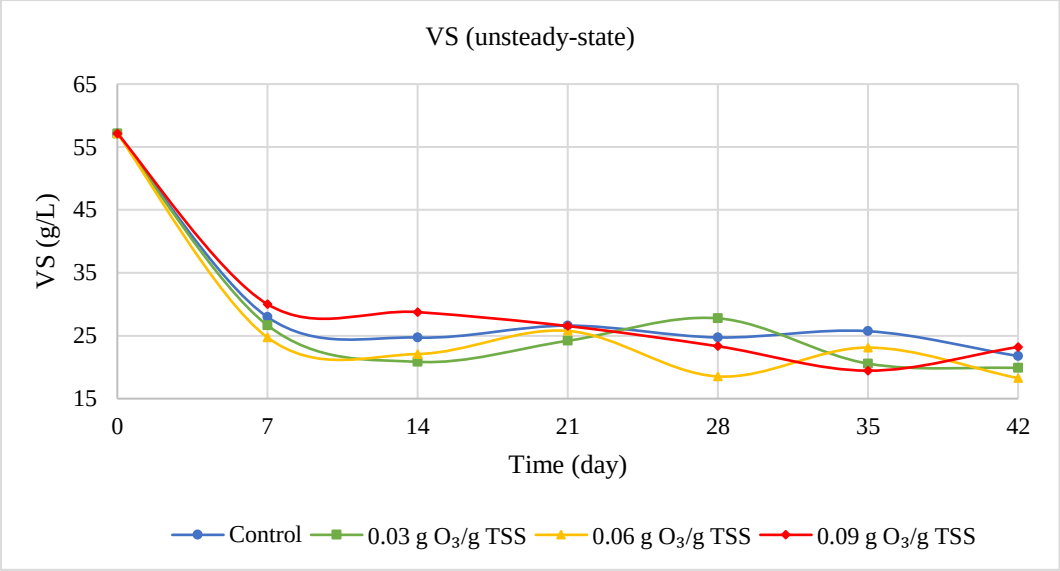

Figure 4.11. VS changes during unsteady-state operation without ozone pretreatment

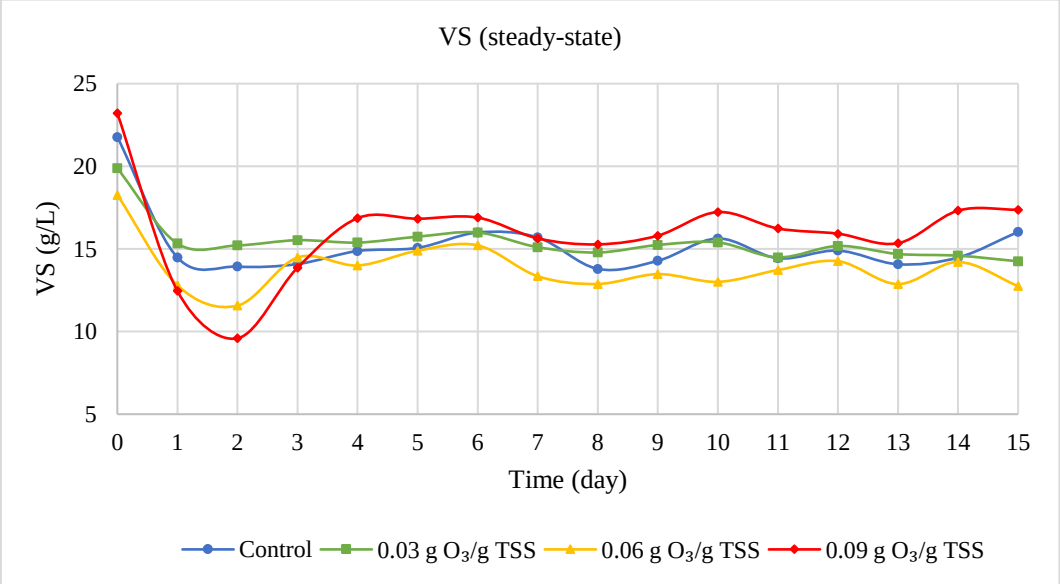

Figure 4.12. VS changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.17, ±0.14, ±0.14 and ±0.23, respectively.

4.2.7. TSS

TSS concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. TSS profiles were similar to TS profiles (Figure 4.9 and Figure 4.10) except for some fluctuations. TSS concentrations of unstable digesters varied between 10.80 and 68.80 g/L (Figure 4.13). At the end of unsteady-state operation, TSS removal efficiencies for control, 0.03, 0.06 and 0.09 g O_3/g TSS were observed as 48%, 53%, 55% and 46%, respectively. Since TSS reductions for conventional mesophilic digesters were within the range of 45-50%, these removals were probable as concluded by Shi (2011). With ozone pretreatment, TSS concentrations of stable digesters varied between 7.03 and 26.20 g/L (Figure 4.14). Pretreatment for 15 days achieved average TSS removals of 46%, 58%, 60% and 51% for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Table 4.2). Weemaes et al. (2000) also indicated that TSS removals were 35%, 43% and 64% for the sludges ozonated with 0.05, 0.1 and 0.2 g O_3/g COD. Battimelli et al. (2003) recorded 22% decrease in TSS for 0.16 g O_3/g TS. Moussavi et al. (2008) increased TSS removals from 8.3% to 47.9% for ozone doses ranging from 0.125 to 2 g O_3/g TS. Otieno et al. (2019) stated approximately 50% TS reduction for different ozonation times. Considering the literature, increase in TSS removals due to ozone doses used in this study was found reasonable. The highest removal of 60% was observed for the digester pretreated with 0.06 g O_3/g TSS.

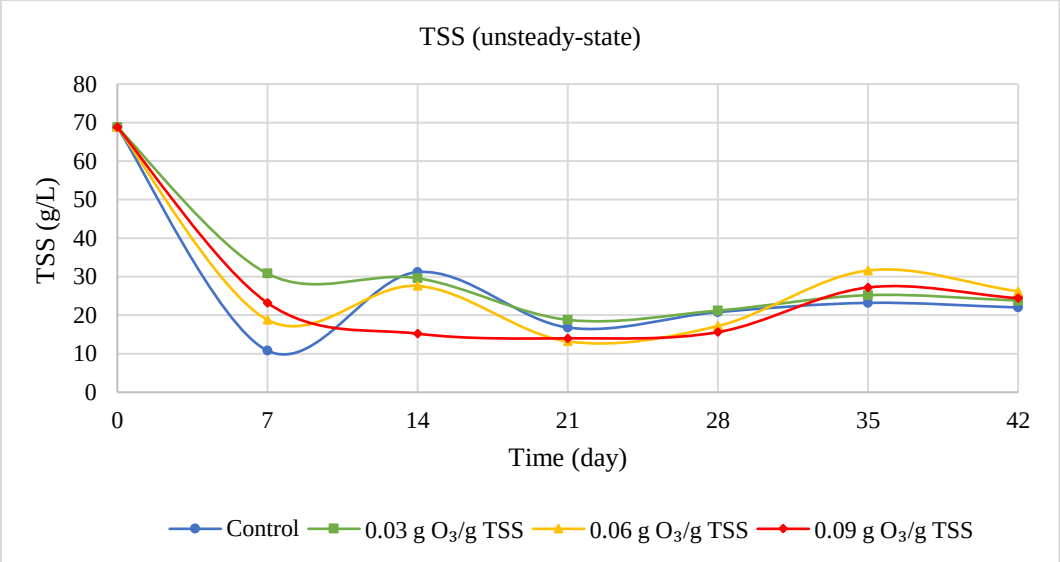

Figure 4.13. TSS changes during unsteady-state operation without ozone pretreatment

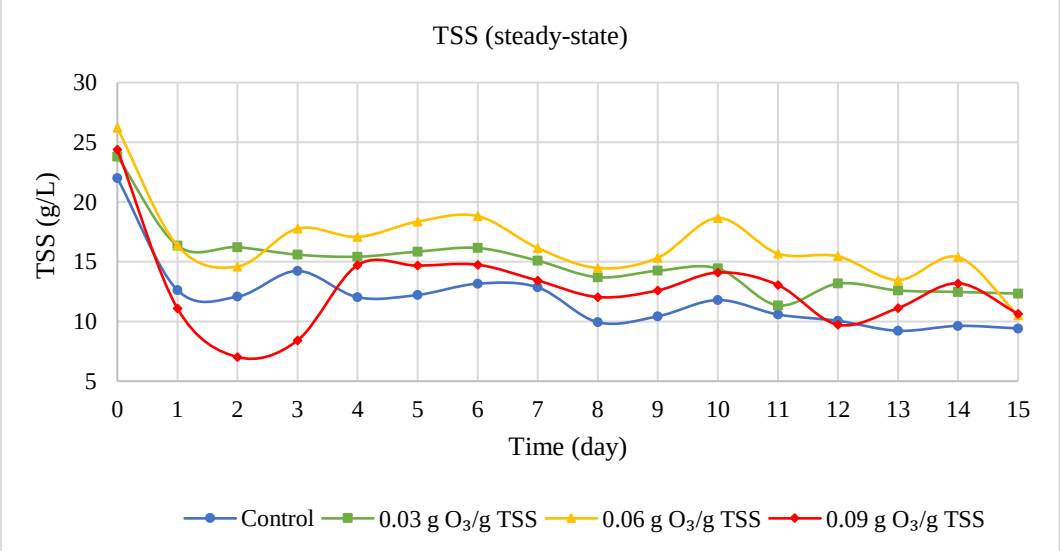

Figure 4.14. TSS changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.20, ±0.18, ±0.17 and ±0.14, respectively.

4.2.8. VSS

VSS concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. VSS profiles were similar to TSS profiles (Figure 4.13 and Figure 4.14) except for some fluctuations. VSS concentrations of unstable digesters varied between 12.60 and 36.40 g/L (Figure 4.15). At the end of unsteady-state operation, VSS removal efficiencies for control, 0.03, 0.06 and 0.09 g O_3/g TSS were observed as 51%, 52%, 57% and 49%, respectively. These removals were found higher than expected since VSS removal was around 40% for conventional mesophilic digesters as stated by Shi (2011). With ozone pretreatment, VSS concentrations of stable digesters varied between 3.92 and 14.80 g/L (Figure 4.16). Pretreatment for 15 days achieved average VSS removals of 66%, 67%, 69% and 64% for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Table 4.2). Weemaes et al. (2000) indicated that VSS removals were 41%, 55% and 72% for the sludges ozonated with 0.05, 0.1 and 0.2 g O_3/g COD. Goel et al. (2003) reported that VSS removal without ozone pretreatment as 35% and with ozone pretreatment as 60%. A full-scale study by Sievers et al. (2004) concluded an increase in VSS removal from 46.6% to 55.5% for 0.05 g O_3/g TSS at 24 days SRT. In this study, ozone pretreatment led to higher VSS removals than of the literature. The highest removal of 69% was observed for the digester pretreated with 0.06 g O_3/g TSS.

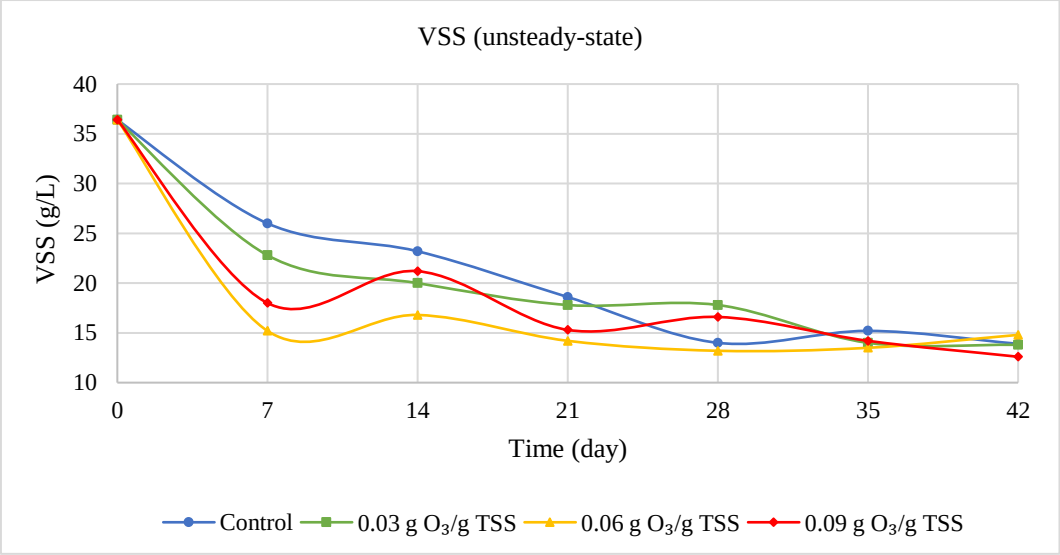

Figure 4.15. VSS changes during unsteady-state operation without ozone pretreatment

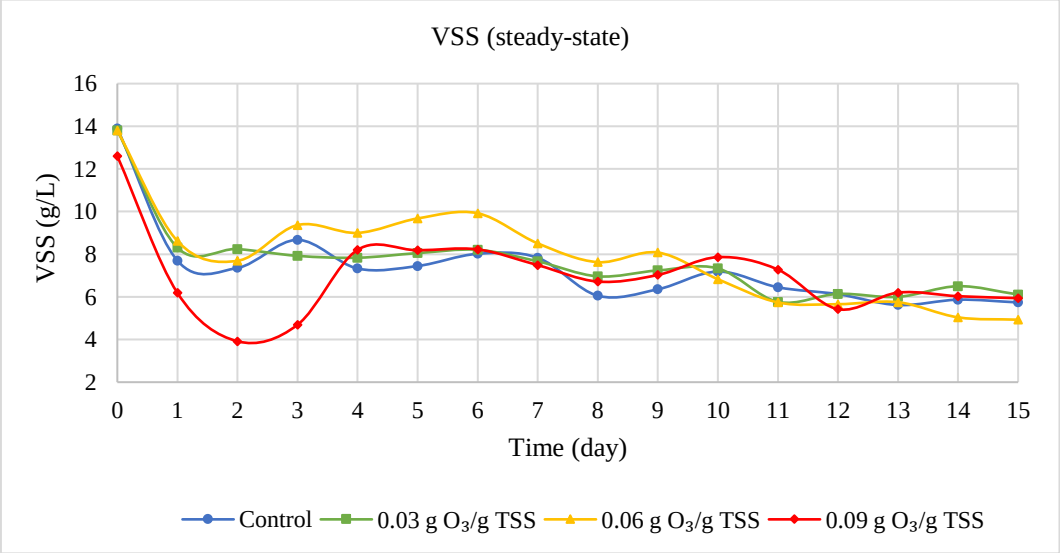

Figure 4.16. VSS changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.18, ±0.20, ±0.14 and ±0.15, respectively.

For all digesters, removal efficiencies and ratios observed at the end of pretreatment were tabularized in Table 4.2.

Table 4.2. Removal efficiencies and ratios after pretreatment

		Digesters			
	Parameters	**Control**	**0.03 g O_3/g TSS**	**0.06 g O_3/g TSS**	**0.09 g O_3/g TSS**
Removals (%)	**COD**	30.02±0.09	34.43±0.15	35.32±0.08	28.24±0.12
	TS	23.45±0.14	28.31±0.17	31.73±0.23	27.45±0.19
	VS	36.35±0.17	35.63±0.18	42.19±0.21	34.12±0.23
	TSS	45.76±0.20	58.26±0.14	60.13±0.17	50.76±0.14
	VSS	65.53±0.18	66.73±0.20	68.67±0.14	64.10±0.15
Ratios	**VS/TS**	0.64±0.01	0.60±0.01	0.59±0.01	0.71±0.06
	VSS/TSS	0.61±0.01	0.51±0.02	0.47±0.08	0.55±0.02

Average removals for 15 days were calculated from daily removals by using corresponding values of feed sludge for that week. Ratios were calculated daily and averaged.

4.2.9. Total VFA

Total VFA concentrations of anaerobic digesters at unsteady- and steady-state conditions were monitored weekly and daily, respectively. Total VFA concentrations of unstable digesters varied between 10.80 and 39.33 mM (Figure 4.17). For the first two weeks, total VFA concentrations increased. This increase was reasonable due to imbalance of methanogenic and acidogenic activity at the adaptation period. Most probably, acidogens were easily adapted and started to produce VFA. On the contrary, sensitive methanogens could not sufficiently utilize that produced VFA because of varying environmental conditions during the adaptation period as stated by Anukam et al. (2019). After the third week, AD balance was established and a stable trend was observed. With ozone pretreatment, total VFA concentrations of stable digesters varied between 7.88 and 30.74 mM (Figure 4.18). It was noted that VFA profile was compatible with pH profile as expected (Figure 4.2). In other words, increasing VFA caused pH decrease and showed similar fluctuations. There is no study in the literature about the effect of ozone pretreatment on VFA because VFA level of each digester was thought to be unique and comparison of VFA levels were not meaningful (Franke-Whittle et al., 2014). Spinosa and Vesilind (2001) noted that total VFA levels in the digesters were in a range of 8-300 mM. For this study, no

inhibitory values of VFA were observed. Ozone pretreatment did not show a significant impact on VFA profile of the digesters.

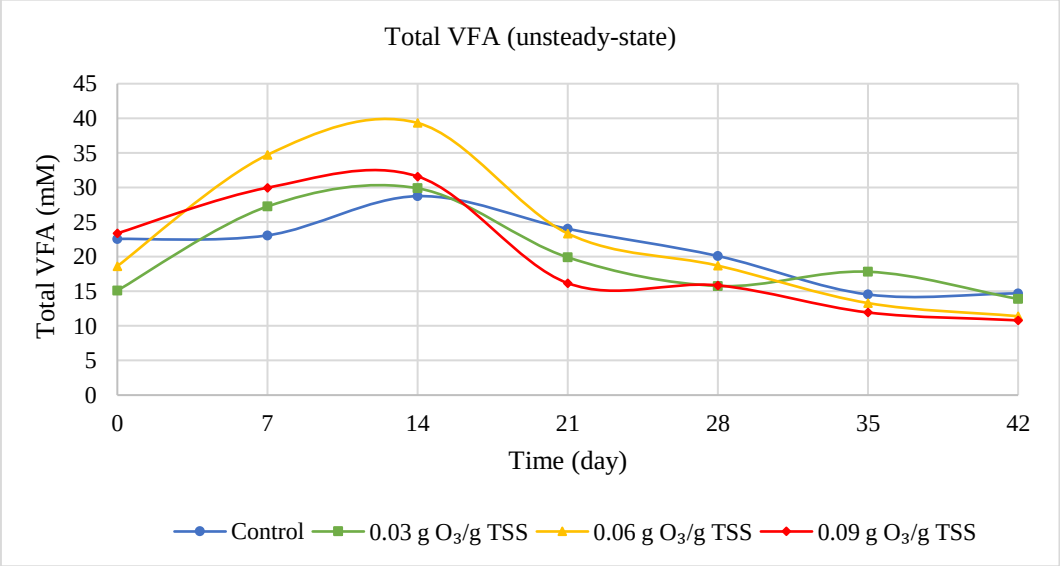

Figure 4.17. Total VFA changes during unsteady-state operation without ozone pretreatment

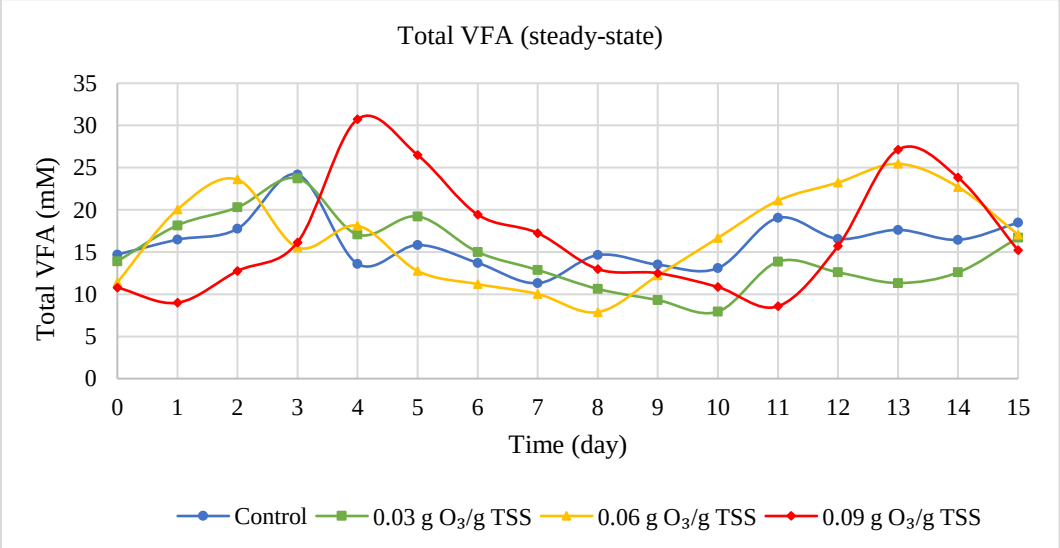

Figure 4.18. Total VFA changes during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±0.23, ±0.22, ±0.17 and ±0.21, respectively.

4.2.10. Biogas production

Biogas productions of anaerobic digesters were monitored on a daily basis throughout the operation. Biogas productions of unstable digesters increased sharply for the first week as expected due to the adaptation of the digesters (Figure 4.19). Biogas productions obtained at unsteady-state conditions varied between 355 and 810 mL irrespective of the first week results. After the fourth week, digesters were begun to reach steady-state. With pretreatment, biogas productions of stable digesters varied between 265 and 2245 mL (Figure 4.20). For the first three days, pretreatment with 0.06 g O_3/g TSS improved daily biogas production while other doses did not show any meaningful increase with respect to control. After day 4, a decreasing trend were observed for all digesters. At steady-state conditions, maximum daily biogas productions for control, 0.03, 0.06 and 0.09 g O_3/g TSS were 1975, 1735, 2245 and 1430 mL whereas maximum daily biogas yields were 455, 500, 517 and 329 mL biogas/g VS, respectively (Figure 4.21). Cumulative biogas productions obtained at unsteady- and steady-state conditions were illustrated in Figure 4.22 and Figure 4.23, respectively. At the end of unsteady-state operation, 25520, 25380, 26110 and 26075 mL biogas was produced for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.22). With pretreatment, 17395, 16140, 18685 and 13130 mL biogas were produced by control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.23). Cumulative biogas yields at steady-state conditions were 3720, 3513, 4026 and 2851 mL biogas/g VS for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.24). The highest biogas production was achieved at 0.06 g O_3/g TSS. 0.03 g O_3/g TSS tracked nearly the same profile with control and the least biogas production was obtained at 0.09 g O_3/g TSS.

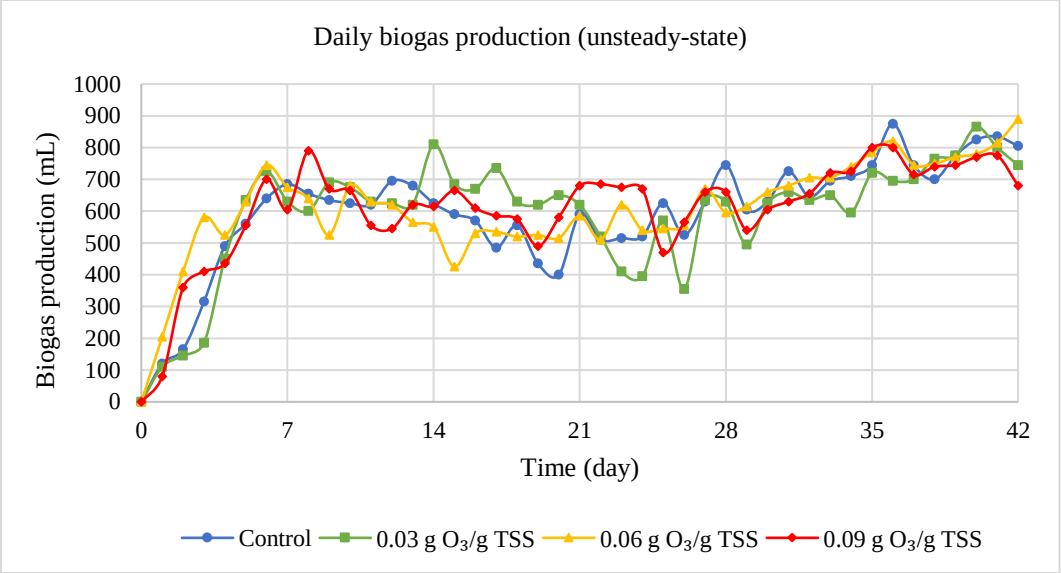

Figure 4.19. Daily biogas production during unsteady-state operation without ozone pretreatment

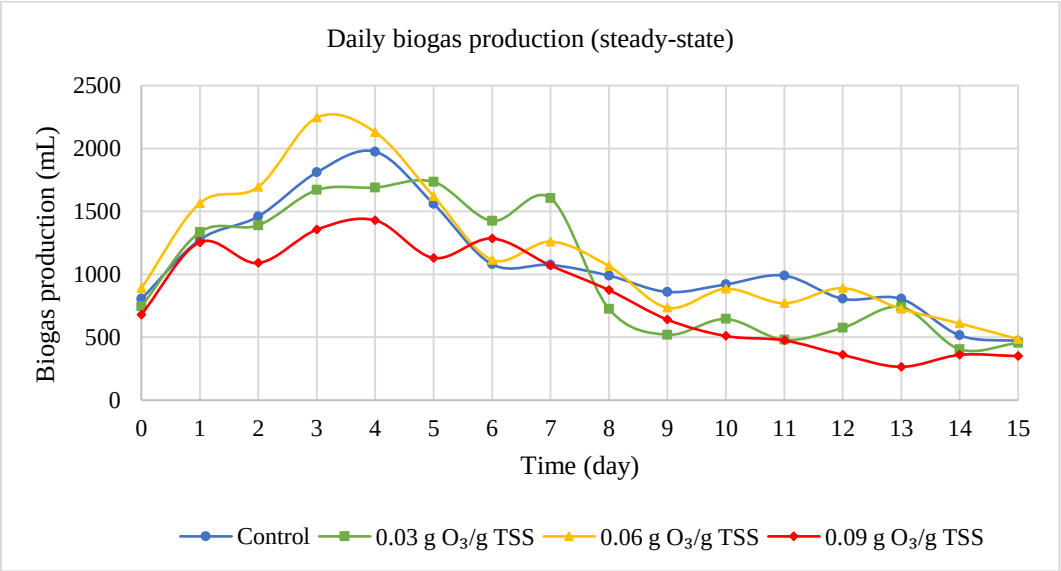

Figure 4.20. Daily biogas production during steady-state operation with ozone pretreatment

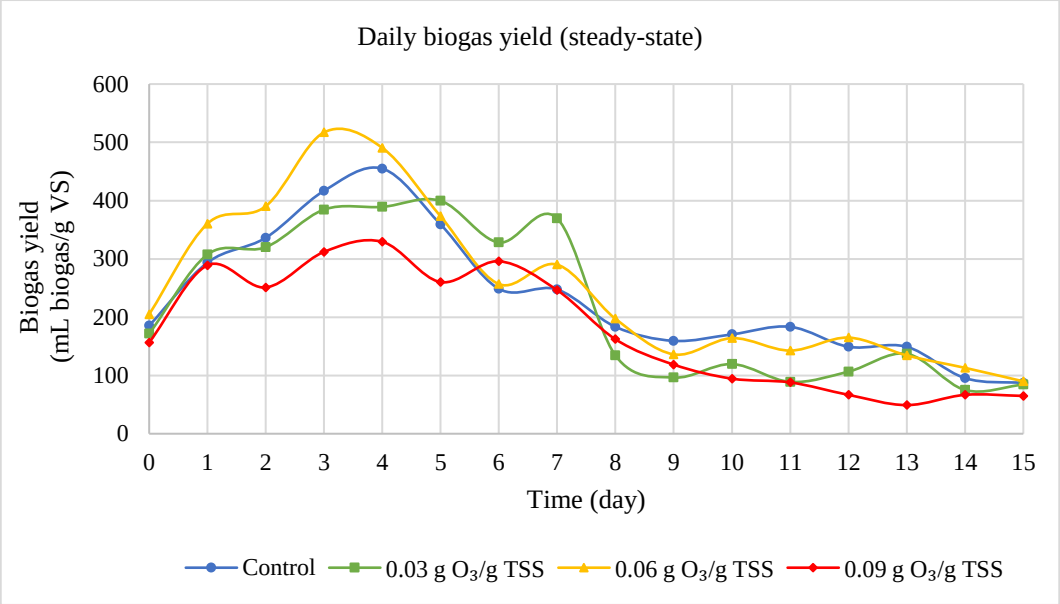

Figure 4.21. Daily biogas yield during steady-state operation with ozone pretreatment

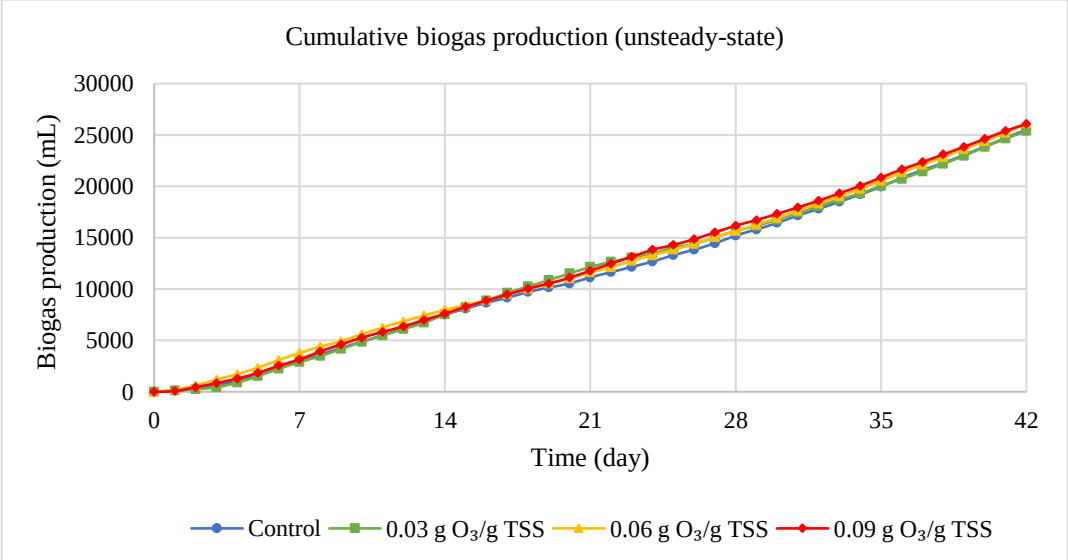

Figure 4.22. Cumulative biogas production during unsteady-state operation without ozone pretreatment

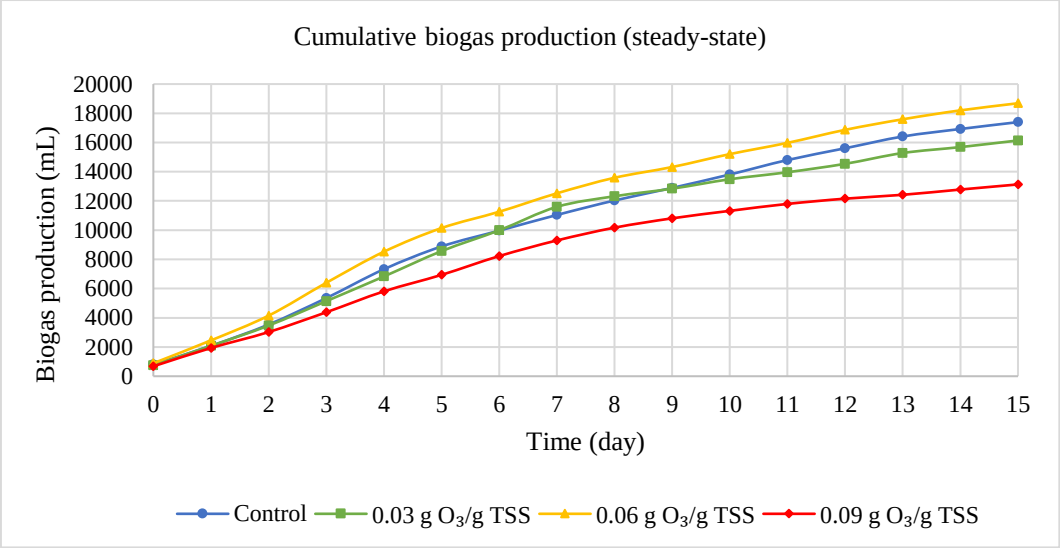

Figure 4.23. Cumulative biogas production during steady-state operation with ozone pretreatment

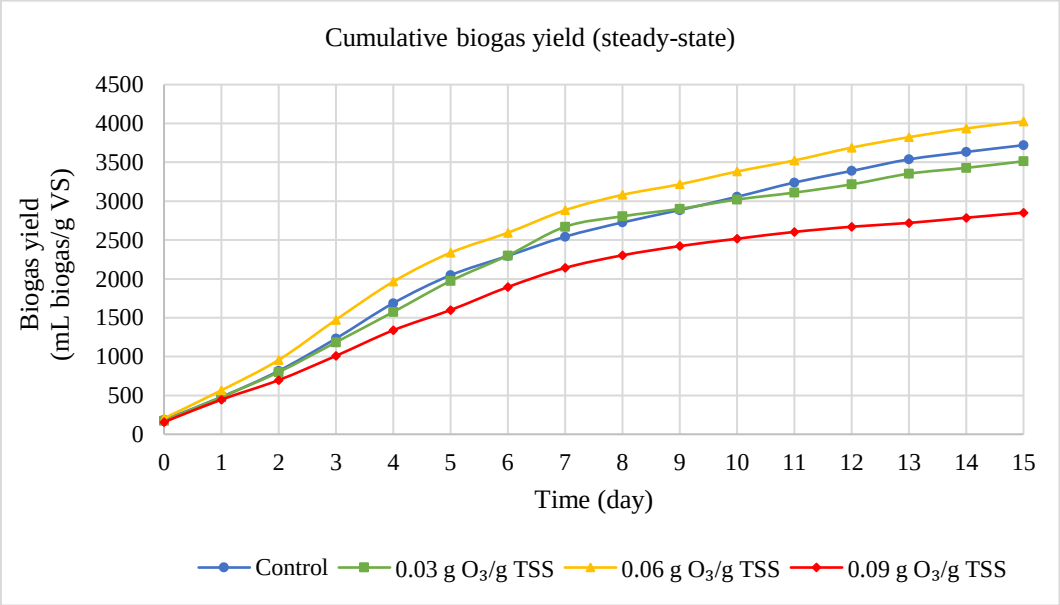

Figure 4.24. Cumulative biogas yield during steady-state operation with ozone pretreatment

In the literature, there were inconsistencies related to biogas production with ozone pretreatment. Bougrier et al. (2006) reported that 0.1 and 0.16 g O_3/g TS doses improved biogas production by 8% and 25%, respectively. In another research by Bougrier et al. (2007), pretreatment with 0.04, 0.06 and 0.09 g O_3/g TS enhanced

biogas production by 14%, 24% and 58%, respectively. Braguglia et al. (2012) found 6% decrease in biogas at 0.05 g O_3/g TS whereas 17% increase at 0.07 g O_3/g TS in the digesters operated as semi-batch. Silvestre et al. (2015) reported that 0.043 and 0.063 g O_3/g TSS doses increased biogas by 5% and 21%, respectively. Although biogas data for higher doses were not presented in that study, it was stated that 0.08 and 0.1 g O_3/g TSS doses decreased biogas. Most of these studies were conducted on batch systems with different doses and sludge types. However, it might be deduced that doses close to 0.06 g O_3/g TSS led to biogas increase no more than 25%. In this study, 0.06 g O_3/g TSS was found to be the most efficient dose for increasing biogas by 7% as compared to control digester. On the other hand, 0.03 and 0.09 g O_3/g TSS resulted in 7% and 25% decrease in biogas. Biogas profile of 0.03 g O_3/g TSS was almost same as control digester even though a slight increase was stated for the doses close to 0.03 g O_3/g TSS in the literature. It was very likely that this ozone dose was insufficient to enable cell lysis and hydrolyze organics. As also indicated in the literature, a considerable drop in biogas was observed for higher doses like 0.09 g O_3/g TSS. This was attributed to inhibition of sensitive methanogens due to toxic effect of ozone.

4.2.11. Methane production

Methane productions of anaerobic digesters were monitored on a daily basis throughout the operation. Methane contents in biogas produced by unstable digesters varied between 13% and 51% (Figure 4.25). For the first two weeks, extreme fluctuations were observed due to the adaptation of digesters but they were stabilized after the fifth week. With pretreatment, methane contents of stable digesters varied between 31% and 78% (Figure 4.26). The highest methane contents obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS were 61%, 78%, 68% and 58%, respectively. Daily methane productions obtained at unsteady- and steady-state conditions were illustrated in Figure 4.27 and Figure 4.28, respectively. Daily methane productions at unsteady-state conditions showed similar trend with methane contents (Figure 4.27). At steady-state conditions, maximum daily methane productions for control,

0.03, 0.06 and 0.09 g O_3/g TSS were 1150, 1263, 1610 and 835 mL whereas maximum daily methane yields were 265, 273, 371 and 192 mL methane/g VS, respectively (Figure 4.29). Cumulative methane productions obtained at unsteady- and steady-state conditions were illustrated in Figure 4.30 and Figure 4.31, respectively. At the end of unsteady-state operation, 8967, 9513, 9457 and 9387 mL methane was produced for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.30). With pretreatment, 12727, 9562, 8643 and 6697 mL methane were produced by control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.31). Cumulative methane yields at steady-state conditions were 1856, 2089, 2751 and 1460 mL methane/g VS for control, 0.03, 0.06 and 0.09 g O_3/g TSS, respectively (Figure 4.32). The highest methane production was achieved at 0.06 g O_3/g TSS. Although 0.03 g O_3/g TSS did not outperform in biogas, it improved methane production.

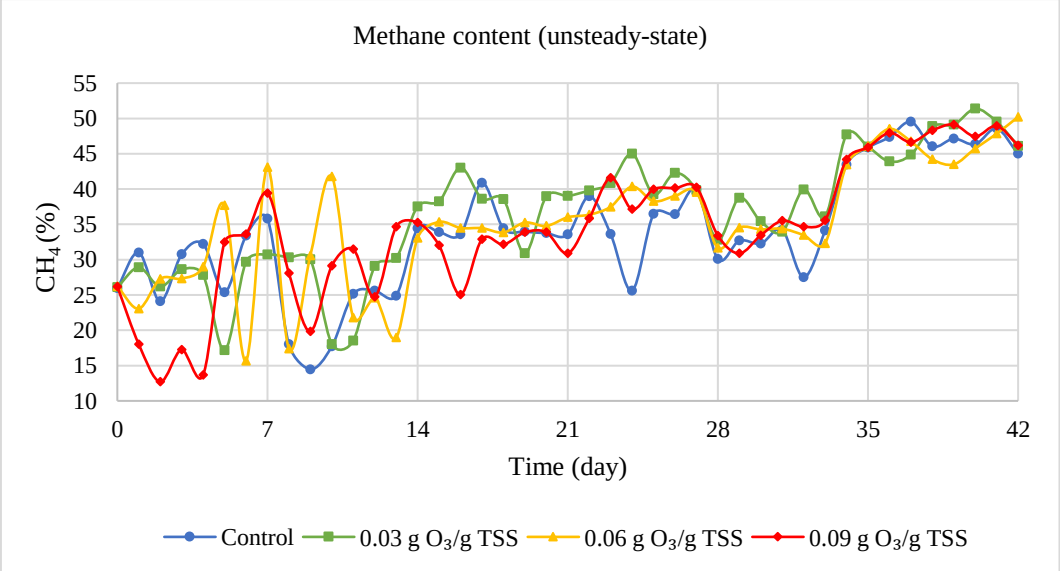

Figure 4.25. Methane content of biogas during unsteady-state operation without ozone pretreatment

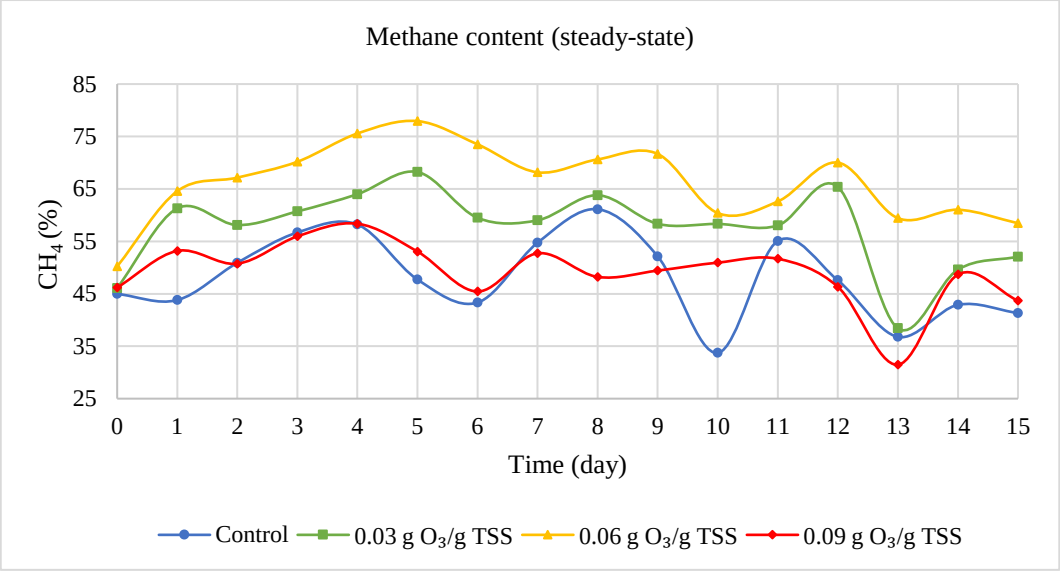

Figure 4.26. Methane content of biogas during steady-state operation with ozone pretreatment. Standard deviations obtained for control, 0.03, 0.06 and 0.09 g O_3/g TSS did not exceed ±6.11, ±7.55, ±5.98 and ±6.01, respectively.

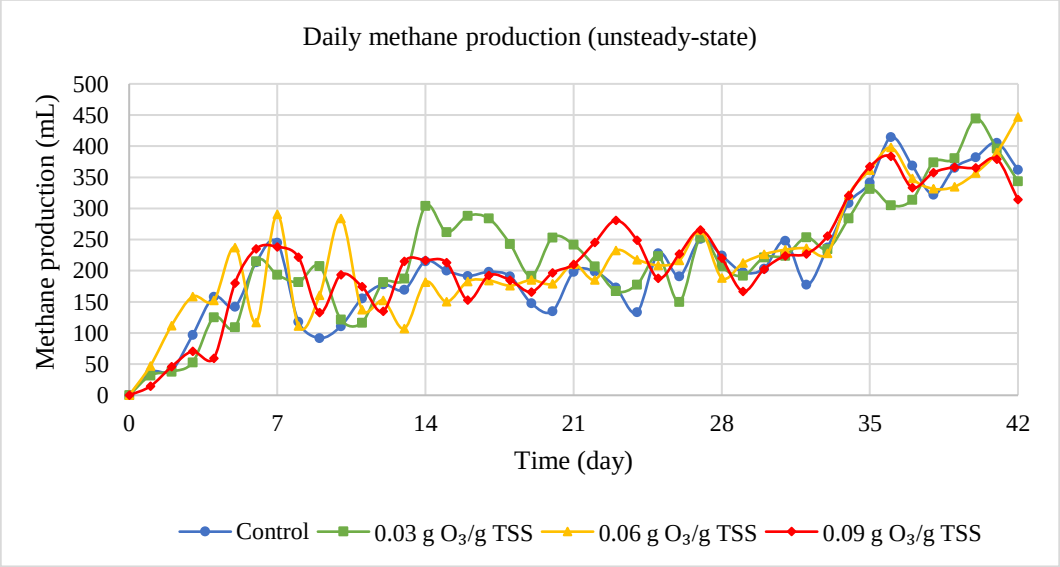

Figure 4.27. Daily methane production during unsteady-state operation without ozone pretreatment

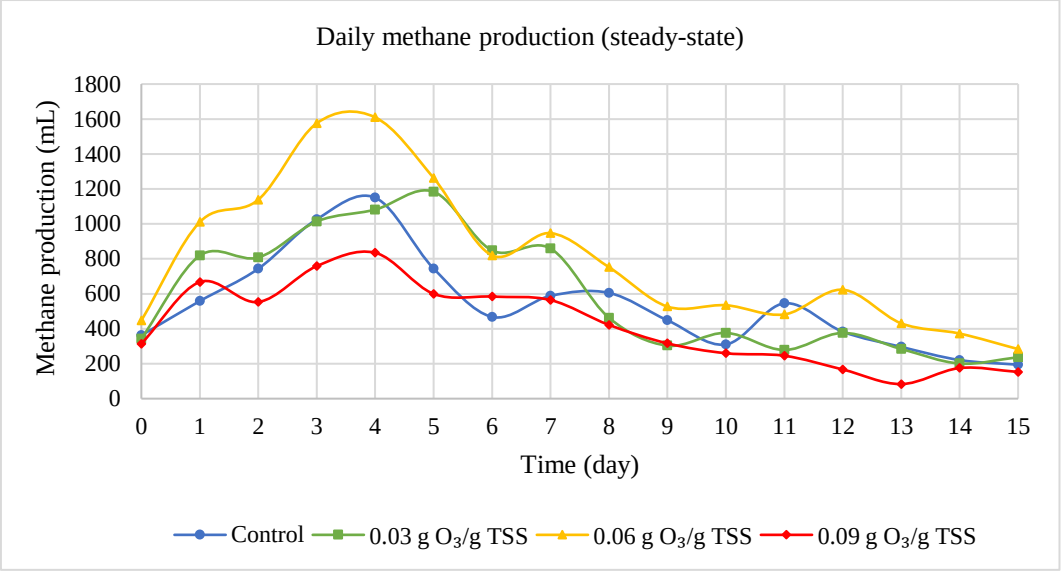

Figure 4.28. Daily methane production during steady-state operation with ozone pretreatment

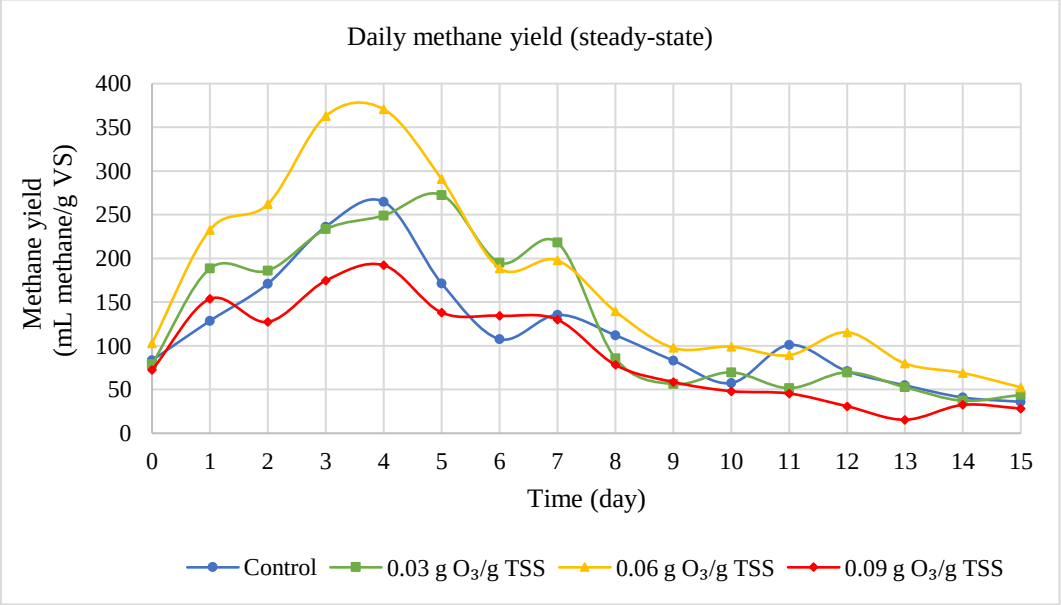

Figure 4.29. Daily methane yield during steady-state operation with ozone pretreatment

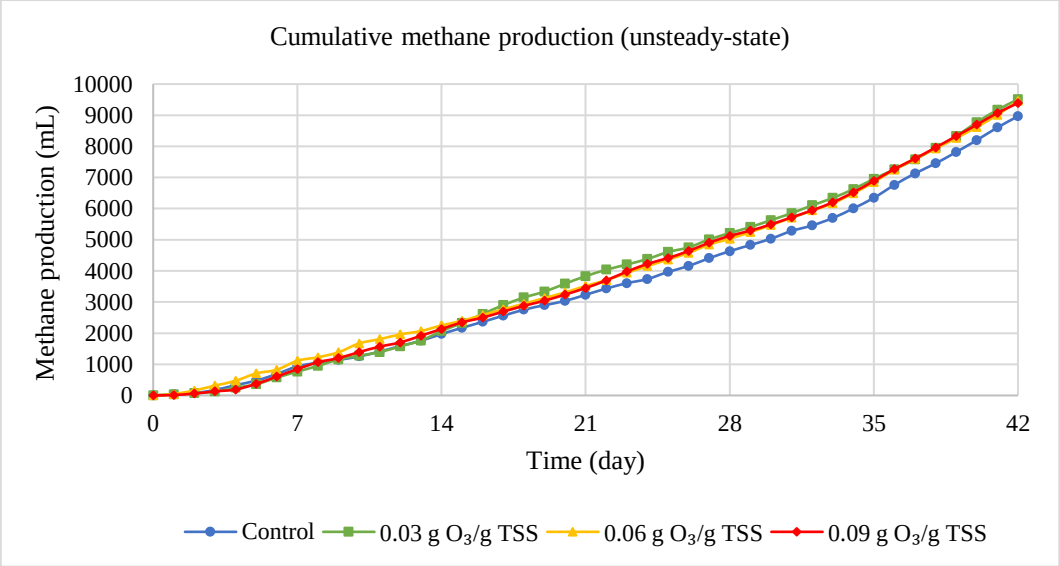

Figure 4.30. Cumulative methane production during unsteady-state operation without ozone pretreatment

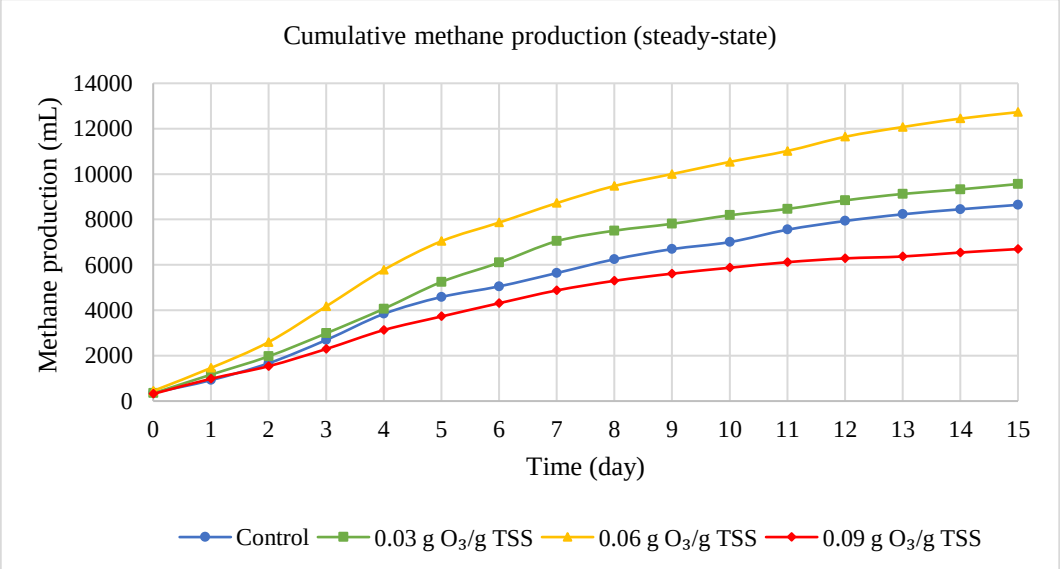

Figure 4.31. Cumulative methane production during steady-state operation with ozone pretreatment

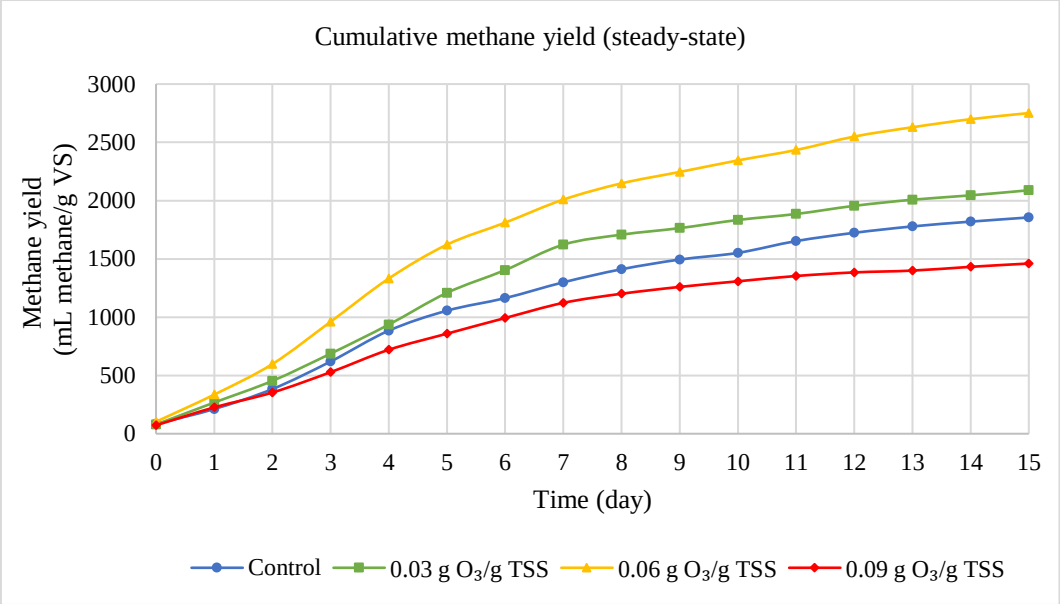

Figure 4.32. Cumulative methane yield during steady-state operation with ozone pretreatment

According to the literature, Weemaes et al. (2000) reported 50%, 80% and 30% increase in methane for 0.05, 0.1 and 0.2 g O_3/g COD doses, respectively. Yeom et al. (2002) found that methane productions were increased by 26%, 75%, 114%, 130%, 135% for the digesters pretreated with 0.02, 0.05, 0.1, 0.2 and 0.5 g O_3/g TSS doses, respectively. Goel et al. (2003) observed 17% and 109% increase for the digesters operated with 14 days SRT as semi-batch and pretreated with 0.015 and 0.05 g O_3/g TS doses, respectively. Bougrier et al. (2006) revealed that methane yields improved 11% and 23% at 0.1 and 0.16 g O_3/g TS, respectively. The same study showed that methane content decreased from 77% to 74% when the dose was increased. In another study by Bougrier et al. (2007), 0.15 g O_3/g TS achieved 144% increase in methane. Erden and Filibeli (2011) investigated 0.1 g O_3/g TS dose and acquired 25% increase. Chacana et al. (2017) stated that methane content was not impacted during ozonation. Most recently, Chiavola et al. (2019) examined low ozone doses on mixed sludge and WAS as semi-batch. For mixed sludge, 4.8 mg O_3/g TS dose achieved 6% increase in methane production but 9.5 and 73.2 mg O_3/g TS decreased methane by 14% and 21%, respectively. For WAS, methane production was enhanced by 30%, 16% and 5% for 3.5, 7.7 and 53.6 mg O_3/g TS

doses, respectively. In this study, pretreatment with 0.06 g O_3/g TSS accomplished the highest increase of 47% in methane production as compared to control digester. Although 0.03 g O_3/g TSS did not exhibit any significant increase for biogas, it provided only 11% increase in methane production. However, similar to the findings by Chiavola et al. (2019), toxic effect of ozone raised for 0.09 g O_3/g TSS which led to 23% decrease in methane production as in case of biogas. Despite all doses did not differ quantitatively in biogas production, they improved the quality of biogas by increasing its methane content.

4.3. Microbial community structure of anaerobic digesters

4.3.1. Optimization of probes

FISH analyses were performed for the optimization of probes used. Each probe was hybridized with their corresponding pure cultures and *E. coli* (DH5α) as positive and negative controls, respectively, under varying FA and NaCl concentrations as described in section 3.5.1. As an example, MX825 probe was hybridized with *Methanosaeta* spp. (DSM17206) as positive and *E. coli (*DH5α) as negative control at 45, 50 and 55% FA and 0.040, 0.028 and 0.020 M NaCl, respectively. Optimization images and the results obtained from image analyses for MX825 probe were given in Figure 4.33 and Table 4.3, respectively. MX825 probe was found highly efficient as 84.92% for the detection of *Methanosaeta* spp. at 50% FA and 0.028 M NaCl. The specifity of MX825 probe was justified with non-target *E. coli* having the intensity of 2.51%. Accordingly, optimal hybridization stringency conditions for each probe used in this study were determined separately (Table 4.4).

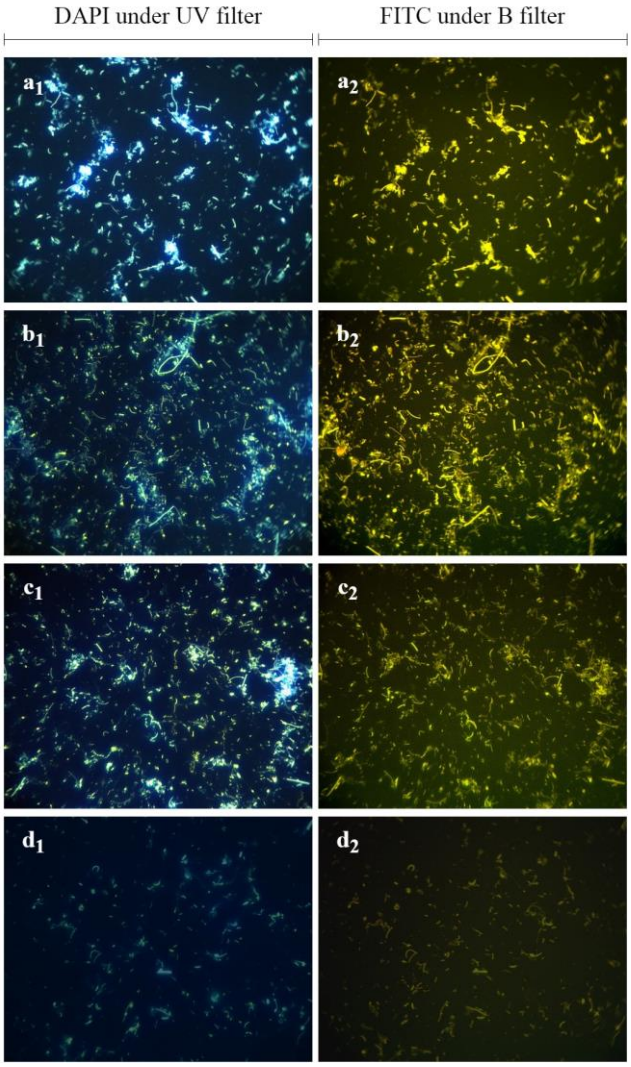

Figure 4.33. Representative optimization images for MX825 probe hybridized with pure culture of *Methanosaeta* spp. (DSM17206) as positive control at 45% FA and 0.040 M NaCl (a_1-a_2), 50% FA and 0.028 M NaCl (b_1-b_2), 55% FA and 0.020 M NaCl (c_1-c_2); and with pure culture of *E. coli* (DH5α) as negative control at 50% FA and 0.028 M NaCl (d_1-d_2). Total microorganisms stained with DAPI (a_1, b_1, c_1, d_1) and FITC-labeled MX825 probe applied to pure cultures for corresponding areas (a_2, b_2, c_2, d_2)

Table 4.3. Optimization measurements of signal intensities for MX825 probe

		Positive control	**Negative control**
[FA] (%)	**[NaCl] (M)**	***Methanosaeta* spp. (DSM17206) (% signal intensity)**	***E. coli* (DH5α) (% signal intensity)**
45	0.040	71.27±1.36	3.24±0.97
50	0.028	84.92±0.45	2.51±0.36
55	0.020	75.60±1.18	3.79±1.54

[FA], formamide concentration; [NaCl], sodium chloride concentration; M, molar; ±, standard deviation

Table 4.4. Optimal stringency conditions of the probes used in the study

Microbial community	Probe	Target microorganism	[FA] (%)	[NaCl] (M)
Acidogens	HoAc1402	*Acidobacteria*	15	0.318
	SS_HOL1400	*Acidobacteria*	25	0.159
	Clost I	*Clostridium* spp.	25	0.159
	Actino221	*Actinobacteria*	35	0.080
	CFB563	*Flavobacterium*	20	0.225
Acetogens	DSBAC355	*Syntrophobacterales*	35	0.080
	GTAG992	*Thermacetagenium*	45	0.040
	SYN835	*Syntrophobacter*	35	0.080
	GTE1002	*Tepidanaerobacter*	50	0.028
Sulfate reducers	DSV687	*Desulfovibrio* spp.	20	0.225
	DSB129	*Desulfobacter* spp.	15	0.318
	DBB660	*Desulfobulbus* spp.	55	0.020
	DSC193	*Desulfosarcina variabilis*	40	0.056
Denitrifiers	Pae997	*Pseuodomonas* spp.	0	0.900
	Bmy843	*Bacillus* spp.	35	0.080
	DEN124	Acetate-denitrifying cluster	35	0.080
Methanogens	MS1414	*Methanosarcina* spp.	55	0.020
	MG1200	*Methanomicrobiales*	20	0.225
	MX825	*Methanosaeta* spp.	50	0.028
	MB1174	*Methanobacteriales*	45	0.040
Archaea	ARC915	*Archaea*	30	0.112
Bacteria	EUBmix*	*Bacteria*	35	0.080
	NONEUB	Negative control	0	0.900

*EUBmix, equal mixtures of EUB338, EUB338 II and EUB338 III; [FA], optimized formamide concentration used in hybridization buffer; [NaCl], optimized sodium chloride concentration used in washing buffer; M, molar

4.3.2. Determination of microbial changes

After steady-state conditions were provided, sludge samples of 0, 7 and 15 days were taken from each pretreated anaerobic digester and fixed. According to the FISH procedure mentioned in section 3.5, samples were hybridized with the probes depending on corresponding optimal stringency conditions (Table 4.4). Hybridization images for methanogenic and archaeal probes were given representatively (Figure 4.34). Accordingly, hybridization images for each probe used in this study were taken and analyzed.

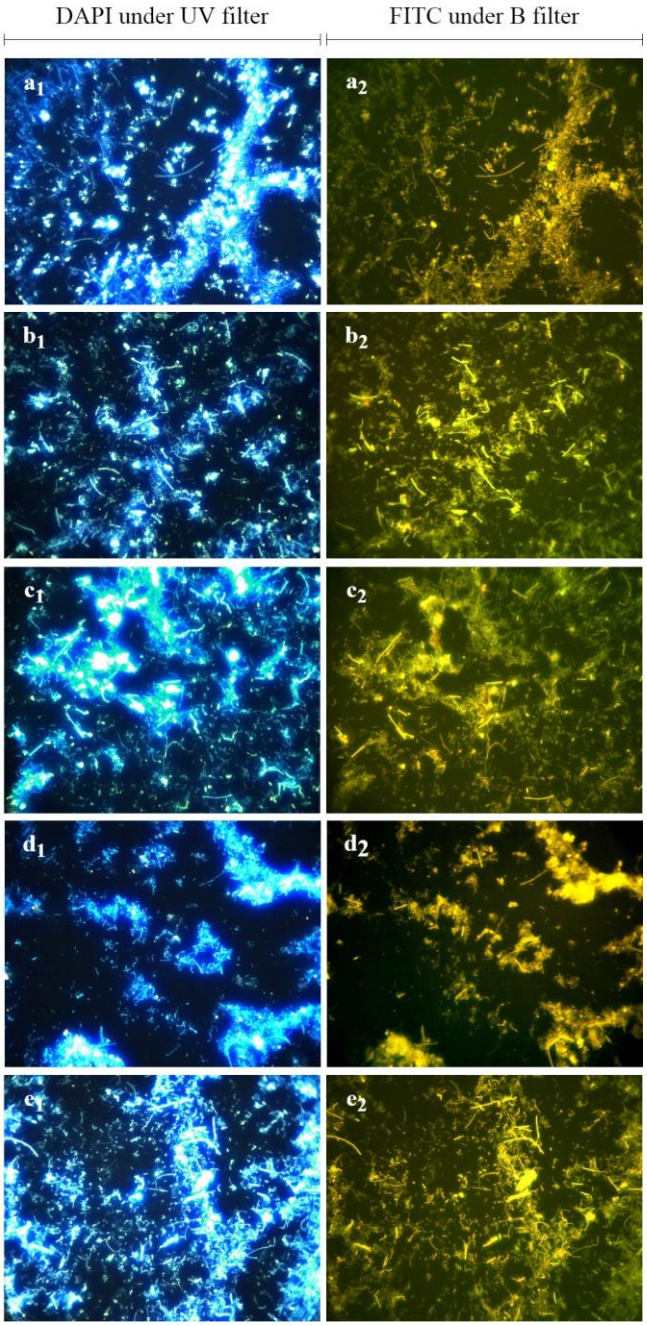

Figure 4.34. Representative hybridization images for methanogenic probes of MX825 (a_1-a_2), MS1414 (b_1-b_2), MG1200 (c_1-c_2), MB1174 (d_1-d_2) and ARC915 (e_1-e_2) at optimal stringency conditions. Total microorganisms stained with DAPI (a_1, b_1, c_1, d_1, e_1) and target microorganisms hybridized with FITC-labeled probes for corresponding areas (a_2, b_2, c_2, d_2, e_2)

As a result of image analyses mentioned in section 3.5.6, microbial changes were monitored on domain and microbial community basis (Figure 4.35 and Figure 4.36). Only mean values of replicate experimental results were illustrated in the graphs. Detailed results from the processing of image data were provided with standard deviations in Appendix C. It should be noted that all methanogens belong to *Archaea* domain while other communities belong to *Bacteria* domain.

On domain basis, the populations of *Bacteria* and *Archaea* varied in a range of 39.4-57.8% and 42.2-60.5%, respectively (Figure 4.35). Control and 0.03 g O_3/g TSS showed nearly same profile in a way that predominating *Archaea* over *Bacteria* gradually throughout ozone pretreatment. On the other hand, 0.09 g O_3/g TSS was predominated *Bacteria* over *Archaea*. In case of 0.06 g O_3/g TSS, the population of *Archaea* peaked at the first week of pretreatment when highest methane production was observed. Swiatczak et al. (2017) also concluded that *Archaea* to *Bacteria* ratio increased during pretreatment.

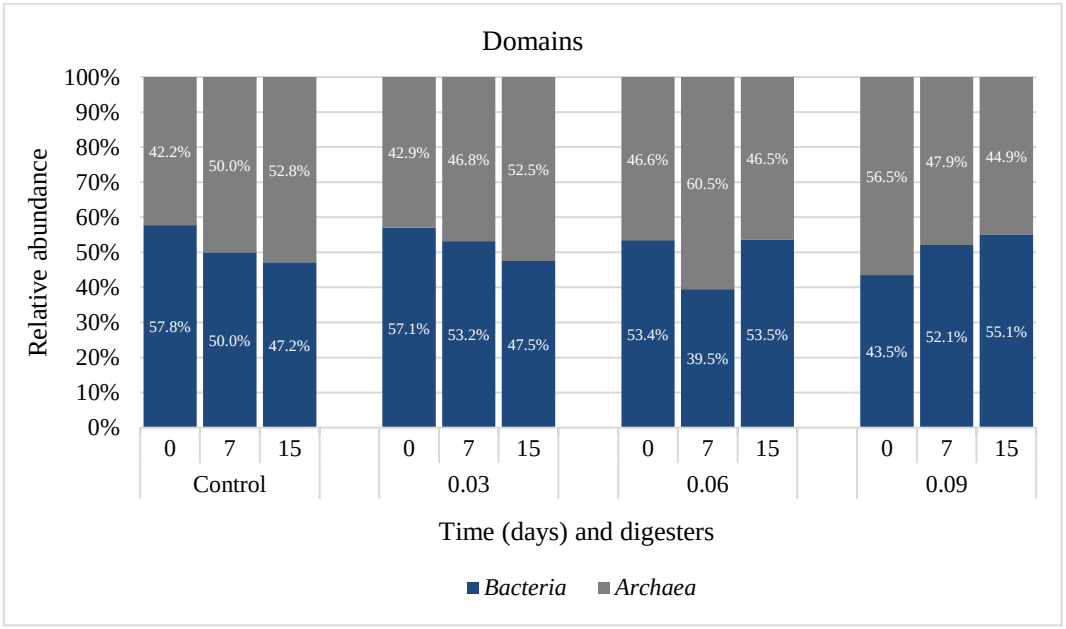

Figure 4.35. Microbial changes in anaerobic digesters on domain basis

On microbial community basis, the populations of acidogens, acetogens, sulfate reducers, denitrifiers and methanogens were determined (Figure 4.36). Acidogenic populations in the digesters varied between 13.3% and 19.5%. Acidogens were generally abundant at the beginning of ozone pretreatment for all digesters except 0.09 g O_3/g TSS. They demonstrated a slight decrease for control and 0.03 g O_3/g TSS. Conversely, acidogens increased for 0.09 g O_3/g TSS. For 0.06 g O_3/g TSS, acidogens decreased at the first week but increased again at the end of pretreatment. Acetogenic populations in the digesters varied between 9.4% and 16.5%. Acetogens in control digester lowered at the first week and unchanged until the end of pretreatment. 0.03 and 0.06 g O_3/g TSS reduced acetogens at the first week and increased at the end of pretreatment. However, 0.09 g O_3/g TSS raised acetogens progressively. It was considered that both acidogens and acetogens were suppressed by ozone pretreatment except for 0.09 g O_3/g TSS because of high sensitivity of methanogens to elevated ozone dose.

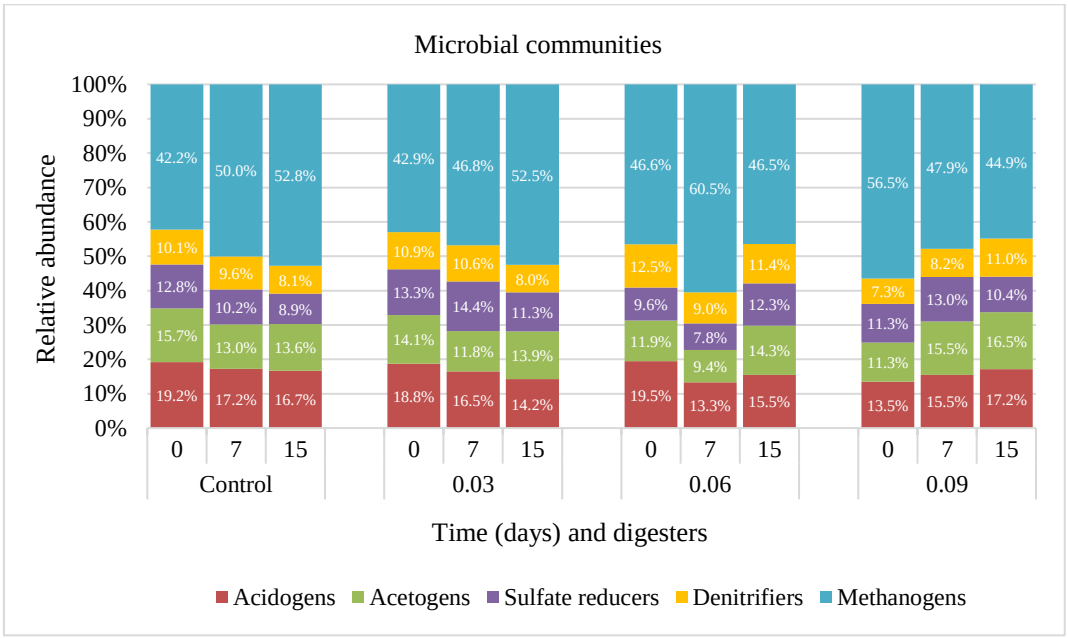

Figure 4.36. Microbial changes in anaerobic digesters on microbial community basis

The populations of sulfate reducers and denitrifiers in the digesters varied in a range of 7.8-14.4% and 7.3-12.5%, respectively. Sulfate reducers and denitrifiers were slightly reduced for control. For 0.03 and 0.09 g O_3/g TSS, sulfate reducers increased at the first week and decreased at the end of pretreatment. Denitrifiers stayed constant at 0.03 g O_3/g TSS during the first week, but declined at the end of pretreatment. 0.09 g O_3/g TSS increased denitrifiers moderately. On the contrary, both sulfate reducers and denitrifiers were decreased by 0.06 g O_3/g TSS at the first week, but they were increased at the end of pretreatment.

Methanogenic populations in the digesters varied between 42.2% and 60.5%. Methanogens increased for control and 0.03 g O_3/g TSS during ozone pretreatment. However, 0.09 g O_3/g TSS affected methanogens adversely. 0.06 g O_3/g TSS increased methanogens to their maximum level of 60.5% at the first week and then decreased until the end of pretreatment. It is observed that highest methane production obtained for the first week of pretreatment was due to this increase (Figure 4.28). 0.03 g O_3/g TSS was not found effective enough for boosting methanogens for higher methane production. 0.09 g O_3/g TSS demonstrated a toxic effect on methanogens resulting a decrease in methane production. When considering the competition among methanogens, sulfate reducers and denitrifiers, methanogens predominated over others for 0.06 g O_3/g TSS so, methane production was increased in this digester. It was deduced that inadequate methane production performance of 0.03 g O_3/g TSS was caused by the dominancy of sulfate reducers. Moreover, 0.09 g O_3/g TSS did not show any toxic effect on sulfate reducers and denitrifiers but severe effect of this dose was observed for methanogens.

On genus/species basis, microbial changes for each anaerobic digester were monitored and elaborated in following sections. Since there was no study examining how ozone pretreatment affected microbial communities at genus/species level, it was not possible to compare the findings of this study with the literature.

4.3.3. Microbial changes in control digester

Microbial structure of control digester without ozone pretreatment was illustrated in Figure 4.37. Among acidogens, *Acidobacteria* (HoAc1402) remained almost constant in all days. *Acidobacteria* (SS_HOL1400), *Clostridium* spp., *Actinobacteria* and *Flavobacterium* decreased over time. The most dominant acidogen was found as *Acidobacteria* (SS_HOL1400) with an average population of 3.96%±0.00. In contrast, among acetogens, *Syntrophobacter* increased unlike *Tepidanaerobacter* decreased gradually. *Syntrophobacterales* declined suddenly on day 15. *Thermacetagenium* diminished on day 7 and increased on day 15. The most dominant acetogens were found as *Syntrophobacterales* and *Thermacetagenium* with average populations of 3.74%±0.01 and 3.71%±0.01. Among sulfate reducers including *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus* spp. and *Desulfosarcina variabilis* went down through operation. *Desulfovibrio* spp. were found as the most dominant sulfate reducer as 3.31%±0.01. Among denitrifiers, *Pseuodomonas* and *Bacillus* spp. reduced progressively but acetate-denitrifying cluster raised at first day and then reduced. *Bacillus* spp. were found as the most dominant denitrifier with an average population of 3.31%±0.00. Among methanogens, *Methanosaeta* spp. increased day by day unlike the order of *Methanobacteriales. Methanosarcina* spp. decreased on day 7 and increased again on day 15. Oppositely, the order of *Methanomicrobiales* climbed aggressively on day 7 and fell on day 15. The most dominant methanogen was observed as the order of *Methanomicrobiales* with an average population of 13.95%±0.03. This was found contrary to general perspective of *Methanosaeta* or *Methanosarcina* spp. dominancy in anaerobic digesters (Q. Zhang et al., 2019; Vítěz et al., 2020). However, some studies reported that hydrogenotrophic *Methanomicrobiales* were the most dominant constituting 94% of methanogenic population (Kim, Kim, et al., 2013). Swiatczak et al. (2017) also found the dominancy of *Methanosaeta*, *Methanosarcina* spp. and *Syntrophobacterales* in full-scale anaerobic digesters whereas *Actinobacteria* were less abundant

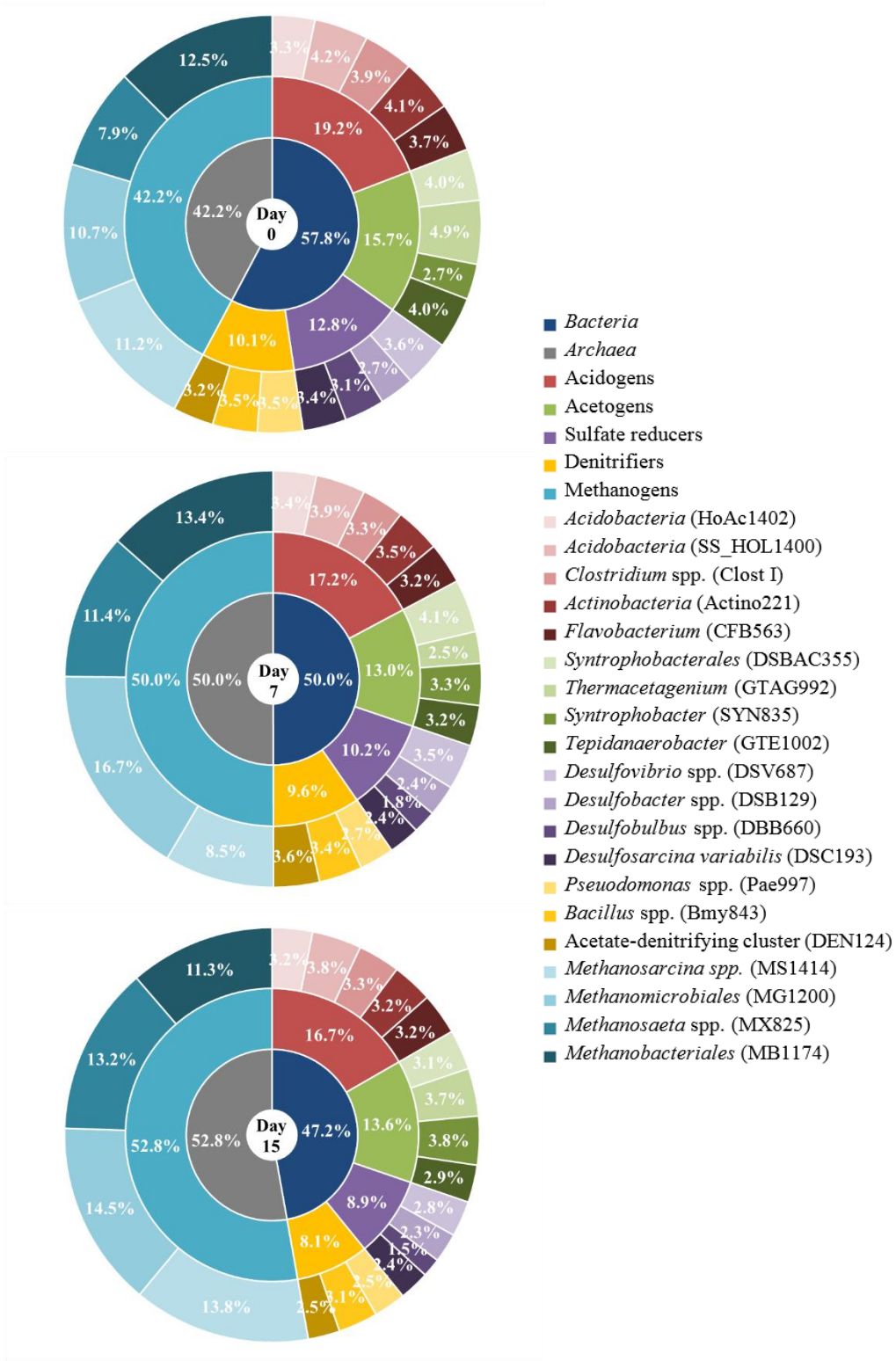

Figure 4.37. Microbial changes with respect to relative abundancies in control digester on day 0 (top), day 7 (middle) and day 15 (bottom) in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

4.3.4. Microbial changes due to pretreatment with 0.03 g O_3/g TSS

Microbial structure of the digester pretreated with 0.03 g O_3/g TSS was illustrated in Figure 4.38. All acidogens decreased over time except *Clostridium* spp. which increased. *Clostridium* spp. were the most dominant acidogen together with *Acidobacteria* (SS_HOL1400) having the average populations of 3.49%±0.00 and 3.47%±0.01, respectively. Among acetogens, *Syntrophobacterales* sharply decreased on day 7 and returned back its initial population level on day 15. *Thermacetagenium* increased slowly. *Syntrophobacter* decreased on day 7 and continued to maintain this population level. *Tepidanaerobacter* increased slightly at first and then decreased. It was found as the most dominant acetogen with an average population of 3.68%±0.01. Among sulfate reducers, the populations of *Desulfobacter*, *Desulfobulbus* spp. and *Desulfosarcina variabilis* raised on day 7, then dropped on day 15. An opposite behavior was observed for *Desulfovibrio* spp. which were the most dominant sulfate reducer with an average population of 3.64%±0.01. Among denitrifiers, *Pseuodomonas* spp. and acetate-denitrifying cluster decreased during pretreatment period. *Bacillus* spp. raised at first day and then reduced. The most dominant denitrifier was observed as *Pseuodomonas* spp. with an average population of 3.77%±0.01. Among methanogens, *Methanosarcina* spp. declined day by day on contrary to the order of *Methanobacteriales*. The order of *Methanomicrobiales* decreased on day 7 and increased again on day 15 unlike *Methanosaeta* spp. The most dominant methanogen was observed as *Methanosarcina* spp. with an average population of 14.64%±0.01. According to (Karakashev et al., 2005), *Methanosarcina* spp. could be dominant by oneself in some anaerobic digesters. Lim et al. (2018) also showed that the order of *Methanosarcinales* were the most dominant methanogens in two different sludge from full-scale anaerobic digesters.

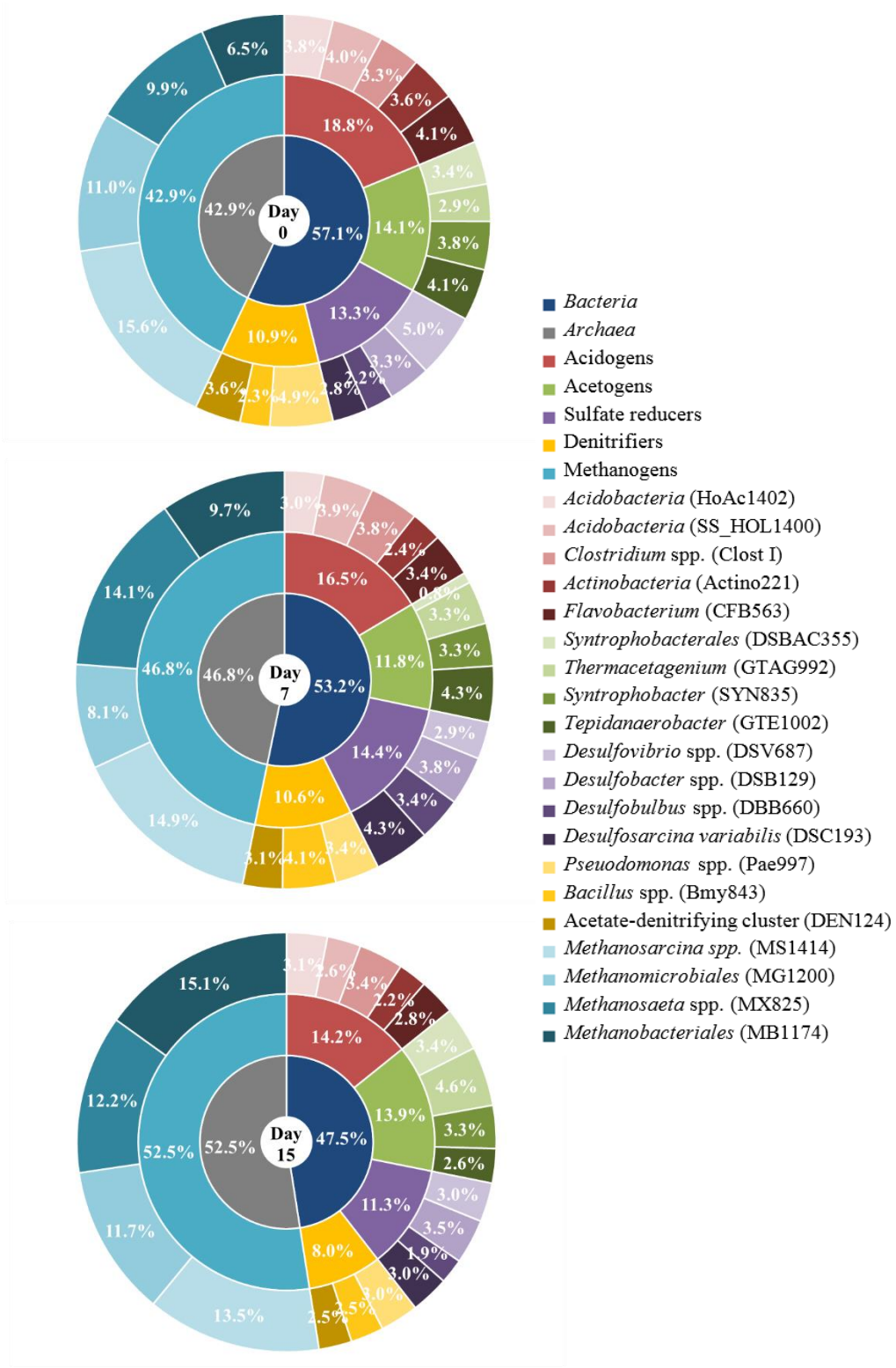

Figure 4.38. Microbial changes with respect to relative abundancies in the digester pretreated with 0.03 g O_3/g TSS on day 0 (top), day 7 (middle) and day 15 (bottom) in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

4.3.5. Microbial changes due to pretreatment with 0.06 g O_3/g TSS

Microbial structure of the digester pretreated with 0.06 g O_3/g TSS was illustrated in Figure 4.39. Among acidogens, *Acidobacteria* (HoAc1402) decreased on day 7 and did not change after that. *Acidobacteria* (SS_HOL1400) and *Clostridium* spp. declined on day 7 and approached their initial population levels on day 15. *Actinobacteria* diminished through the pretreatment. *Flavobacterium*, which was found as the most dominant acidogen with an average population of 3.77%±0.01, decreased and increased on day 7 and 15, respectively. Among acetogens, *Syntrophobacterales* increased substantially day by day. *Thermacetagenium*, *Syntrophobacter* and *Tepidanaerobacter* decreased on day 7 then increased on day 15. *Syntrophobacter* was observed as the most dominant acetogen with average population of 3.33%±0.02. Among sulfate reducers, *Desulfobulbus* spp. increased gradually during pretreatment. *Desulfovibrio*, *Desulfobacter* spp., and *Desulfosarcina variabilis* went down on day 7 and went up on day 15. *Desulfovibrio* spp. were found as the most dominant sulfate reducer with average population of 3.32%±0.01. Although all denitrifiers reduced on day 7 and raised on day 15, the most dominant denitrifier was determined as acetate-denitrifying cluster with an average population of 3.89%±0.01. Among methanogens, *Methanosarcina*, *Methanosaeta* spp. and the order of *Methanobacteriales* increased on day 7 and decreased again on day 15. After increasing on day 7, the order of *Methanomicrobiales* stayed constant. The most dominant methanogens were observed as *Methanosaeta* spp. with an average population of 13.45%±0.02. This was in parallel with the finding of Khan et al. (2018) that *Methanosaeta* spp. were more responsible for methane production.

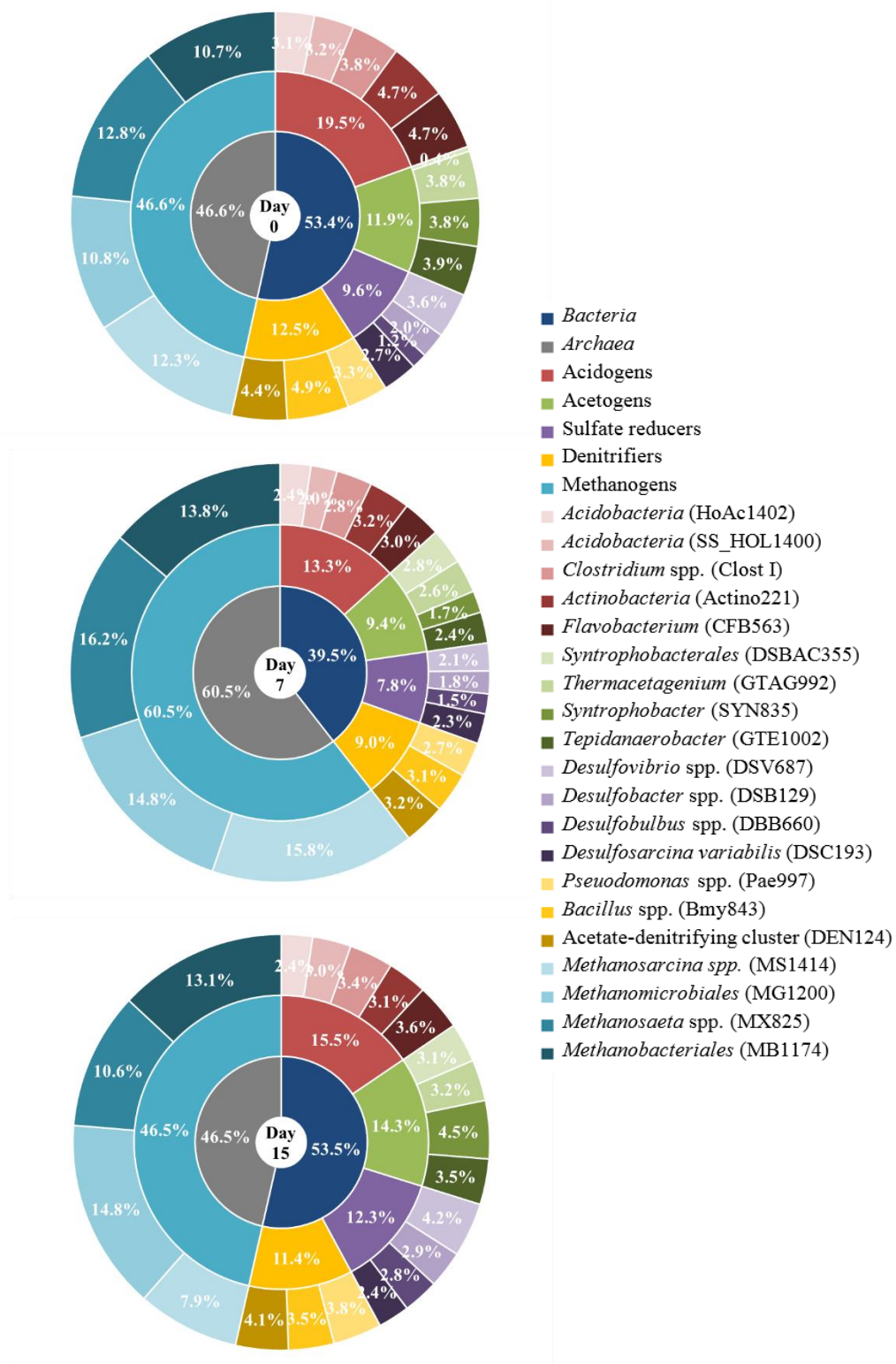

Figure 4.39. Microbial changes with respect to relative abundancies in the digester pretreated with 0.06 g O_3/g TSS on day 0 (top), day 7 (middle) and day 15 (bottom) in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

4.3.6. Microbial changes due to pretreatment with 0.09 g O_3/g TSS

Microbial structure of the digester pretreated with 0.09 g O_3/g TSS was illustrated in Figure 4.40. Among acidogens, *Acidobacteria* (HoAc1402) decreased on day 7 and increased after that. *Acidobacteria* (SS_HOL1400) and *Clostridium* spp. increased gradually in all days unlike *Actinobacteria* diminished through the pretreatment. As the most dominant acidogen with an average population of 3.64%±0.01, *Flavobacterium* increased and decreased on day 7 and 15, respectively. Among acetogens, *Syntrophobacterales* decreased on day 7 then sharply increased on day 15. Conversely, *Thermacetagenium*, *Syntrophobacter* and *Tepidanaerobacter* increased on day 7 an decreased on day 15. *Tepidanaerobacter* was observed as the most dominant acetogen with average population of 4.61%±0.01. Among sulfate reducers, *Desulfovibrio* spp. increased on day 15 after dropped on day 7. The population level of *Desulfobacter* spp. were almost steady for all days. *Desulfobulbus* spp. and *Desulfosarcina variabilis* raised on day 7 and fell on day 15. *Desulfovibrio* spp. were found as the most dominant sulfate reducer with average population of 3.89%±0.01. Among denitrifiers, *Pseuodomonas* and *Bacillus* spp. increased progressively but acetate-denitrifying cluster decreased on day 7 before increased on day 15. The most dominant denitrifier was determined as acetate-denitrifying cluster with an average population of 3.43%±0.01. Among methanogens, *Methanosaeta* spp., the orders of *Methanomicrobiales* and *Methanobacteriales* decreased over time while *Methanosarcina* spp. were the only methanogens showing an increase. The most dominant methanogen was observed as the order of *Methanobacteriales* with an average population of 15.39%±0.04.

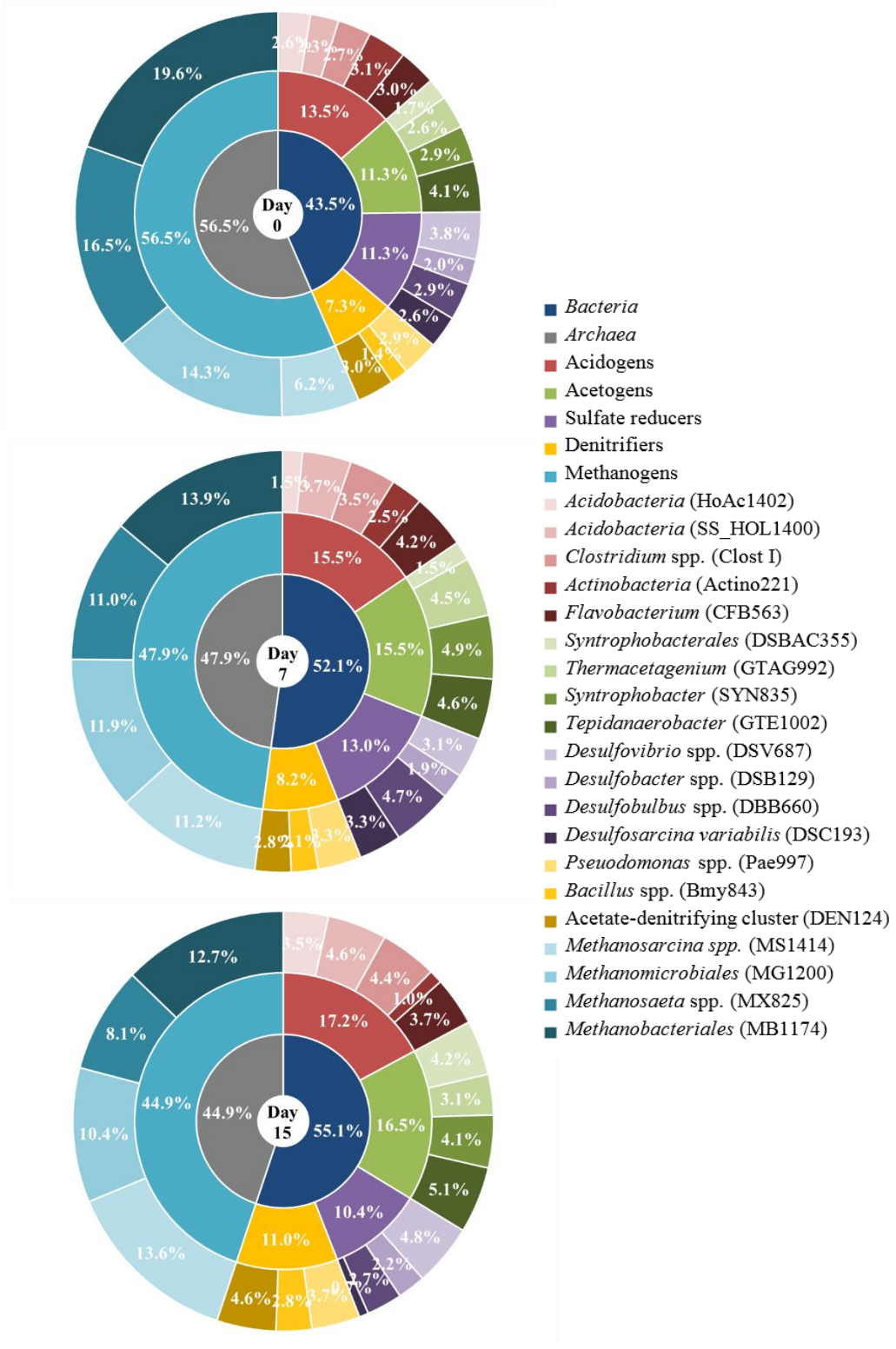

Figure 4.40. Microbial changes with respect to relative abundancies in the digester pretreated with 0.09 g O_3/g TSS on day 0 (top), day 7 (middle) and day 15 (bottom) in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

4.3.7. Comparison of methanogens

Distribution of methanogens is crucial for understanding of their role in methane production (Figure 4.28). In this context, methanogenic populations of the digesters were illustrated in Figure 4.41.

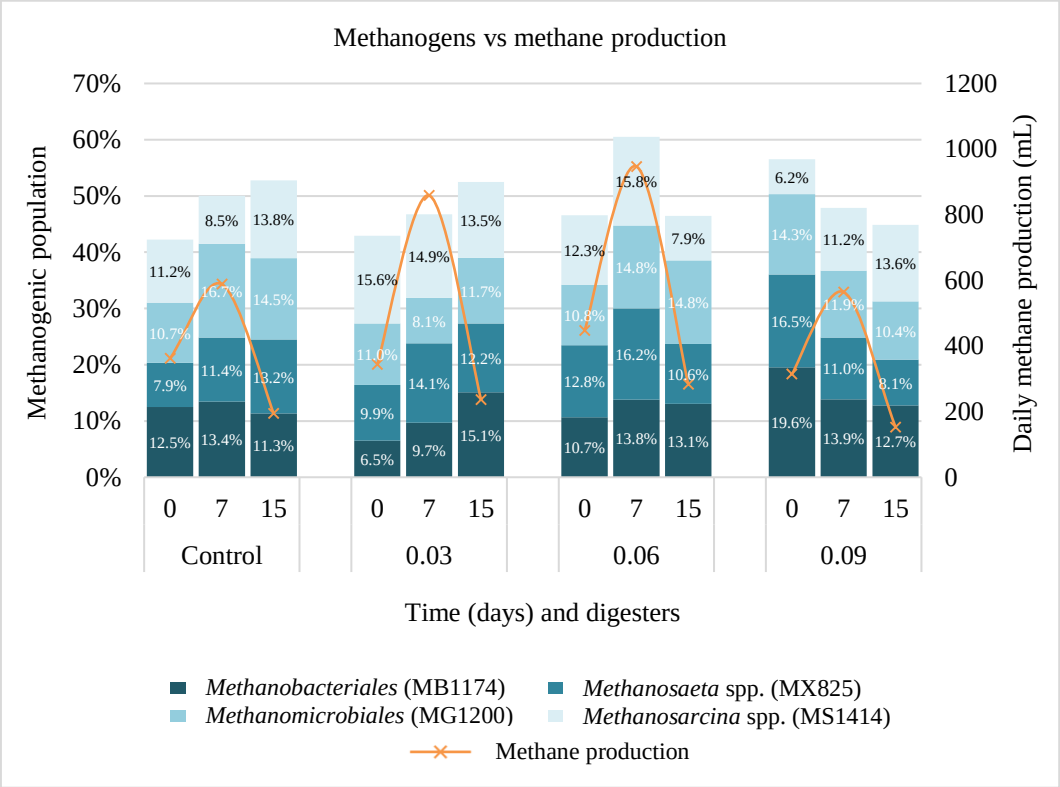

Figure 4.41. Changes in methanogenic population and methane production in anaerobic digesters

At the beginning of ozone pretreatment (day 0), the highest and the lowest methanogenic population were observed for 0.09 g O_3/g TSS and control, respectively. Methanogenic population levels were found similar for 0.03 g O_3/g TSS and control, but distribution of individual methanogens was different. Control digester was scarce in *Methanosaeta* spp. Digesters pretreated with 0.03 and 0.09 g O_3/g TSS doses were scarce in the order of *Methanobacteriales* and *Methanosarcina* spp. All methanogens were evenly distributed in the digester pretreated with 0.06 g O_3/g TSS dose.

At the first week (day 7), the effects of ozone pretreatment were started to be observed as increasing both methane production and total methanogenic population at 0.03 and 0.06 g O_3/g TSS. For control digester, the population of *Methanomicrobiales* significantly increased when the highest methane production was observed. Zhang et al. (2019) found that the order of *Methanomicrobiales* showed strong, positive correlations with methane production although *Methanosaeta* spp. were found as the most abundant methanogens in a full-scale digester. Lim et al. (2020) also stated that the order of *Methanomicrobiales* could replace the order of *Methanobacteriales* and *Methanosaeta* spp. in anaerobic digesters treating municipal sludge.

Although the most dominant methanogens were *Methanosarcina* spp. at 0.03 g O_3/g TSS dose throughout the pretreatment, increase in methane production was correlated with increase in the population of *Methanosaeta* spp. 0.09 g O_3/g TSS dose affected total methanogenic population and individual methanogens negatively except *Methanosarcina* spp. The most dominant methanogens during this pretreatment were the order of *Methanobacteriales* which were found inefficient in methane production as compared to control. This finding was in parallel with the finding of Sun et al. (2015) who observed that hydrogenotrophic *Methanobacteriales* functioned better in thermophilic environment rather than mesophilic. *Methanosarcina* spp. were observed to be more resistant to this high ozone dose than other methanogens. Some *Methanosarcina* spp. were also reported to be resistant to ozone by Anderson et al. (2012). Additionally, *Methanosarcina* spp. generally prefer higher temperatures of 55-60°C (Swiatczak et al., 2017). Increase in the population of *Methanosarcina* spp. throughout the pretreatment with 0.09 g O_3/g TSS dose did not show significant methane production as compared to control. Therefore, *Methanosarcina* spp. were not found as effective as *Methanosaeta* spp. in methane production at 0.03 and 0.09 g O_3/g TSS doses. Moreover, *Methanosaeta* spp. were the most suppressed methanogens by 0.09 g O_3/g TSS dose which decreased methane production. It was known that *Methanosaeta* spp. were adversely affected under

elevated ozone doses (Bal et al., 2018). This case supported the idea of toxic effect of high ozone doses (Kosowski et al., 2020).

Among all ozone doses, the highest methanogenic population was observed at the first week of pretreatment with 0.06 g O_3/g TSS which also ended up with the highest methane production. Additionally, all individual methanogens were increased by this ozone dose. However, it was revealed that this increase caused by the dominancy of *Methanosaeta* spp. Similar results were also reported by Swiatczak et al. (2017), Khan et al. (2018) and Lim et al. (2020).

To conclude, the most dominant methanogens were found different for each digester operated in this study as; *Methanomicrobiales* for control, *Methanosarcina* spp. for 0.03 g O_3/g TSS, *Methanosaeta* spp. for 0.06 g O_3/g TSS and *Methanobacteriales* for 0.09 g O_3/g TSS.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The ultimate goal of this study was to state the impact of ozone pretreatment with varying doses on methane production and microbial community structure in mesophilic single-stage anaerobic digesters. Within the scope of the study, anaerobic digesters were pretreated with varying ozone doses of 0.03, 0.06 and 0.09 g O_3/g TSS. Sludge characteristics, methane productions and microbial community structures of the digesters were monitored during semi-batch operation with 15 days SRT. Sludge characteristics were tracked with the operational parameters of pH, temperature, COD, TN, solids content and total VFA. Biogas and methane productions were determined by GC. Microbial community structure was examined by FISH method in terms of acidogens, acetogens, sulfate reducers, denitrifiers and methanogens.

This study concluded that:

- For methane production, the effectiveness of ozone doses from the highest to the lowest were found as 0.06, 0.03 and 0.09 g O_3/g TSS.
- Although ozone doses did not show significant increase in biogas production, a noteworthy improvement in biogas quality was observed in terms of methane. The highest methane content of 78% was obtained at 0.06 g O_3/g TSS dose.
- 0.06 g O_3/g TSS was found as the most effective ozone dose leading to 47% increase in methane production.
- *Methanosaeta* spp. were the most dominant methanogens found in the digester pretreated with 0.06 g O_3/g TSS.

- The most dominant methanogens in the digesters differed depending on ozone dose applied as; *Methanomicrobiales* for control, *Methanosarcina* spp. for 0.03 g O_3/g TSS, *Methanosaeta* spp. for 0.06 g O_3/g TSS and *Methanobacteriales* for 0.09 g O_3/g TSS.
- All ozone doses improved sludge characteristics. The highest removals of COD (35%), TS (32%), VS (42%), TSS (60%) and VSS (69%) were observed at 0.06 g O_3/g TSS dose.
- Pretreatment with 0.06 g O_3/g TSS resulted in the dominancy of *Flavobacterium* among acidogens, *Syntrophobacter* among acetogens, *Desulfovibrio* spp. among sulfate reducers and acetate-denitrifying cluster among denitrifiers.
- Different ozone doses dominated different genus/species among microbial communities except *Desulfovibrio* spp. which were found as the most dominant sulfate reducers in all digesters. They need to be controlled to avoid inhibition of methane production due to sulfate reduction.

5.2. Recommendations for future studies

- Different ozone doses and sludge types other than those used in this study should be investigated extensively for a complete optimization of ozone pretreatment in AD.
- Different anaerobic digester types and configurations need to be pretreated with those ozone doses and examined under changing operational conditions.
- Combination of ozone pretreatment with other pretreatment methods need to be evaluated. Co-digestion options should be considered.
- For pilot- and full-scale application of ozone pretreatment in AD, feasibility studies should be carried out.
- Due to high cost of ozone, cost/benefit analyses should be performed as compared to other pretreatment methods.

- Microbiology of AD should be investigated at metagenomic level for detailed functional activities.
- Methanogens and their effective members in methane production need to be explored in detail for possible biostimulation and bioaugmentation practices for further increase in methane and biogas.

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APPENDICES

A. Calibration curves

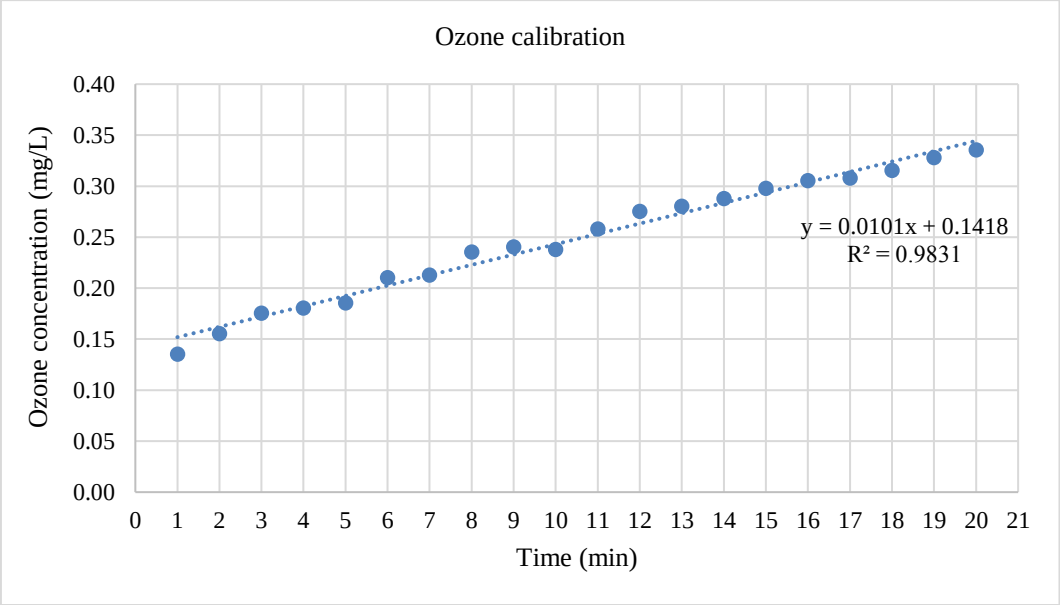

Figure A.1. Ozone calibration curve

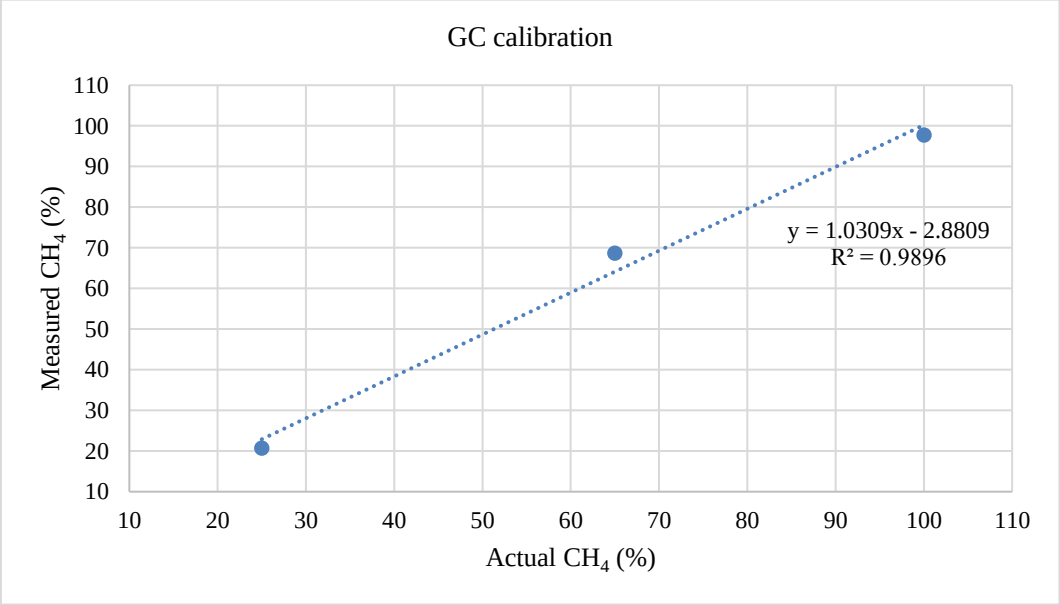

Figure A.2. GC calibration curve

Table B.1. Characterization analyses of control digester

Day	pH	Temperature (°C)	COD (g/L)	TN (g/L)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	Total VFA (mM)	Biogas (mL)	Methane content (%)
0	7.66±0.07	34.60±0.50	21.05±0.06	3.15±0.01	34.21±0.08	21.77±0.08	22.00±0.10	13.90±0.02	14.70±0.05	805	44.97±3.99
1	7.62±0.03	34.80±0.20	20.12±0.03	2.72±0.08	22.76±0.13	14.48±0.16	12.64±0.08	7.70±0.08	16.47±0.08	1275	43.80±4.80
2	7.57±0.06	34.90±0.30	19.63±0.04	2.21±0.06	21.89±0.03	13.93±0.01	12.08±0.03	7.36±0.18	17.75±0.06	1460	50.91±5.03
3	7.55±0.04	34.70±0.10	16.35±0.08	2.13±0.12	22.13±0.05	14.08±0.09	14.23±0.02	8.67±0.04	24.19±0.16	1810	56.69±0.87
4	7.56±0.03	34.90±0.80	17.93±0.03	1.97±0.03	23.37±0.10	14.87±0.07	12.03±0.14	7.33±0.14	13.60±0.08	1975	58.21±0.75
5	7.59±0.02	35.00±0.60	17.60±0.05	1.82±0.15	23.67±0.07	15.06±0.08	12.22±0.20	7.45±0.11	15.83±0.07	1560	47.72±1.96
6	7.60±0.06	35.00±0.70	16.02±0.06	1.79±0.08	25.13±0.14	15.99±0.03	13.16±0.01	8.02±0.07	13.72±0.08	1080	43.32±1.91
7	7.61±0.02	34.70±0.50	19.18±0.02	2.29±0.05	24.66±0.02	15.69±0.12	12.86±0.12	7.84±0.08	11.31±0.23	1075	54.73±2.08
8	7.63±0.10	34.50±0.70	11.36±0.09	2.66±0.03	21.67±0.08	13.79±0.17	9.94±0.03	6.05±0.13	14.64±0.08	990	61.12±5.01
9	7.61±0.03	34.60±0.20	17.22±0.01	3.19±0.09	22.44±0.01	14.28±0.04	10.43±0.05	6.36±0.06	13.50±0.14	860	52.14±3.02
10	7.59±0.09	34.80±0.10	17.35±0.03	2.27±0.05	24.56±0.02	15.63±0.07	11.79±0.09	7.19±0.04	13.09±0.04	920	33.72±2.45
11	7.64±0.11	34.90±0.40	16.85±0.02	1.74±0.08	22.68±0.05	14.43±0.06	10.59±0.02	6.45±0.02	19.04±0.02	990	55.08±6.11
12	7.63±0.08	34.60±0.80	18.54±0.06	2.05±0.01	23.41±0.03	14.90±0.04	10.05±0.07	6.13±0.06	16.56±0.12	805	47.57±4.00
13	7.64±0.06	34.40±0.20	19.72±0.07	2.31±0.07	22.11±0.03	14.07±0.03	9.22±0.05	5.62±0.05	17.63±0.09	805	36.79±5.05
14	7.62±0.03	34.30±0.50	18.95±0.08	2.27±0.04	22.75±0.11	14.48±0.06	9.63±0.05	5.87±0.02	16.45±0.20	515	42.89±1.56
15	7.59±0.01	34.20±0.40	19.25±0.02	2.20±0.05	25.51±0.09	16.03±0.02	9.42±0.03	5.74±0.07	18.47±0.11	470	41.32±2.01
Removal (%)	-	-	30.02±0.09	-	23.45±0.14	36.35±0.17	45.76±0.20	65.53±0.18	-	-	-

COD, chemical oxygen demand; TN, total nitrogen; TS, total solids, VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; Total VFA, total volatile fatty acids; mM, millimolar; ±, standard deviation. Analyses were performed as duplicates except biogas. Average removals for 15 days were calculated from daily removal efficiencies by using corresponding values of feed sludge for that week.

Table B.2. Characterization analyses of digester pretreated with 0.03 g O_3/g TSS

Day	pH	Temperature (°C)	COD (g/L)	TN (g/L)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	Total VFA (mM)	Biogas (mL)	Methane content (%)
0	7.82±0.05	35.60±0.70	17.83±0.01	2.90±0.06	32.87±0.08	19.88±0.08	23.89±0.02	13.80±0.10	13.90±0.14	745	46.10±0.27
1	7.71±0.02	35.50±0.30	15.97±0.08	2.85±0.03	25.35±0.16	15.33±0.13	16.36±0.08	8.31±0.08	18.16±0.04	1335	61.29±6.36
2	7.65±0.03	35.70±0.60	14.51±0.06	2.71±0.04	25.16±0.01	15.21±0.03	16.22±0.18	8.24±0.03	20.29±0.02	1390	58.12±0.33
3	7.65±0.01	35.80±0.40	13.86±0.12	2.65±0.08	25.68±0.09	15.53±0.05	15.59±0.04	7.92±0.02	23.73±0.12	1670	60.71±5.57
4	7.74±0.08	35.90±0.30	14.90±0.03	2.63±0.03	25.44±0.07	15.38±0.10	15.42±0.14	7.84±0.14	17.07±0.09	1690	63.99±2.98
5	7.70±0.06	36.00±0.20	13.44±0.15	2.46±0.05	26.03±0.08	15.74±0.07	15.85±0.11	8.05±0.20	19.22±0.20	1735	68.23±4.66
6	7.60±0.06	36.20±0.60	14.81±0.08	2.00±0.06	26.45±0.03	15.99±0.18	16.15±0.07	8.21±0.01	15.00±0.08	1425	59.49±4.59
7	7.71±0.05	36.20±0.20	15.73±0.05	2.63±0.02	24.98±0.12	15.10±0.02	15.09±0.08	7.67±0.12	12.88±0.06	1605	59.01±4.57
8	7.73±0.07	36.00±0.10	17.85±0.03	3.33±0.09	24.45±0.17	14.78±0.08	13.70±0.13	6.96±0.03	10.63±0.16	725	63.79±4.26
9	7.80±0.02	35.90±0.30	18.75±0.09	2.52±0.01	25.20±0.04	15.24±0.01	14.25±0.06	7.24±0.05	9.31±0.08	520	58.34±5.71
10	7.86±0.01	35.50±0.90	18.50±0.05	3.02±0.03	25.46±0.07	15.39±0.02	14.43±0.04	7.33±0.09	7.94±0.07	645	58.32±0.28
11	7.81±0.04	35.40±0.10	18.55±0.08	1.89±0.02	23.96±0.06	14.49±0.05	11.35±0.02	5.77±0.02	13.86±0.08	480	58.02±2.78
12	7.90±0.08	34.80±0.80	18.97±0.01	2.36±0.06	25.10±0.04	15.18±0.03	13.17±0.06	6.14±0.07	12.60±0.22	575	65.39±4.06
13	7.92±0.02	34.00±0.60	16.70±0.07	2.74±0.07	24.30±0.03	14.69±0.03	12.59±0.05	6.00±0.05	11.33±0.09	740	38.42±2.24
14	7.88±0.05	34.70±0.30	15.67±0.04	2.55±0.08	24.13±0.06	14.59±0.11	12.47±0.02	6.50±0.05	12.59±0.2	405	49.61±3.76
15	7.78±0.04	34.90±0.10	15.90±0.05	2.15±0.02	24.09±0.02	14.25±0.09	12.33±0.07	6.11±0.03	16.70±0.11	455	52.08±7.55
Removal (%)	-	-	34.43±0.15	-	28.31±0.17	35.63±0.18	58.26±0.14	66.73±0.20	-	-	-

COD, chemical oxygen demand; TN, total nitrogen; TS, total solids, VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; Total VFA, total volatile fatty acids; mM, millimolar; ±, standard deviation. Analyses were performed as duplicates except biogas. Average removals for 15 days were calculated from daily removals by using corresponding values of feed sludge for that week.

Table B.3. Characterization analyses of digester pretreated with 0.06 g O_3/g TSS

Day	pH	Temperature (°C)	COD (g/L)	TN (g/L)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	Total VFA (mM)	Biogas (mL)	Methane content (%)
0	7.85±0.01	34.70±0.60	19.19±0.05	2.96±0.07	31.20±0.14	18.26±0.10	26.20±0.08	13.80±0.08	11.40±0.08	890	50.22±1.13
1	7.82±0.08	34.60±0.30	15.82±0.02	2.79±0.03	21.86±0.04	12.80±0.08	16.36±0.16	8.62±0.13	20.03±0.16	1565	64.56±1.91
2	7.71±0.06	35.60±0.40	16.21±0.03	2.69±0.06	19.76±0.02	11.57±0.03	14.59±0.01	7.69±0.03	23.59±0.01	1695	67.16±2.58
3	7.77±0.12	36.20±0.80	15.09±0.01	3.24±0.04	24.74±0.12	14.48±0.02	17.78±0.09	9.37±0.05	15.54±0.09	2245	70.18±5.57
4	7.69±0.03	36.40±0.30	14.43±0.08	3.75±0.03	23.91±0.09	14.00±0.14	17.08±0.07	9.00±0.10	18.12±0.07	2130	75.57±1.25
5	7.73±0.15	36.50±0.50	18.92±0.06	3.23±0.02	25.44±0.20	14.89±0.21	18.36±0.08	9.68±0.07	12.76±0.08	1620	77.94±3.99
6	7.60±0.06	36.40±0.60	15.83±0.06	2.13±0.06	25.99±0.08	15.21±0.01	18.82±0.03	9.92±0.14	11.20±0.03	1115	73.51±1.60
7	7.84±0.05	35.80±0.20	17.81±0.05	2.38±0.02	22.80±0.06	13.35±0.12	16.15±0.12	8.51±0.02	10.03±0.12	1260	68.16±1.36
8	7.91±0.03	35.60±1.00	15.10±0.07	2.62±0.10	22.00±0.16	12.88±0.03	14.47±0.17	7.63±0.08	7.88±0.17	1065	70.63±2.81
9	7.89±0.09	35.70±0.10	19.43±0.02	2.47±0.03	23.02±0.08	13.48±0.05	15.33±0.04	8.08±0.01	12.22±0.04	735	71.68±4.87
10	7.84±0.05	35.90±0.30	18.30±0.01	2.33±0.09	22.22±0.07	13.01±0.09	18.66±0.07	6.83±0.02	16.67±0.07	885	60.39±5.98
11	7.73±0.08	36.10±0.20	15.80±0.04	2.36±0.11	23.44±0.08	13.72±0.02	15.68±0.06	5.74±0.05	21.09±0.06	770	62.60±5.34
12	7.70±0.01	35.90±0.60	16.13±0.08	2.72±0.08	24.36±0.23	14.26±0.07	15.46±0.04	5.66±0.03	23.23±0.04	890	70.02±5.46
13	7.65±0.07	36.00±0.70	17.40±0.02	2.96±0.06	21.99±0.09	12.87±0.05	13.47±0.03	5.75±0.03	25.45±0.03	725	59.43±5.62
14	7.67±0.04	35.80±0.80	16.60±0.05	2.73±0.03	24.27±0.20	14.21±0.05	15.38±0.06	5.04±0.11	22.71±0.06	610	61.03±1.01
15	7.72±0.05	35.20±0.20	15.85±0.04	2.86±0.01	21.32±0.11	12.75±0.03	10.51±0.02	4.93±0.09	17.05±0.02	485	58.46±2.47
Removal (%)	-	-	35.32±0.08	-	31.73±0.23	42.19±0.21	60.13±0.17	68.67±0.14	-	-	-

COD, chemical oxygen demand; TN, total nitrogen; TS, total solids, VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; Total VFA, total volatile fatty acids; mM, millimolar; ±, standard deviation. Analyses were performed as duplicates except biogas. Average removals for 15 days were calculated from daily removals by using corresponding values of feed sludge for that week.

Table B.4. Characterization analyses of digester pretreated with 0.09 g O_3/g TSS

Day	pH	Temperature (°C)	COD (g/L)	TN (g/L)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	Total VFA (mM)	Biogas (mL)	Methane content (%)
0	7.91±0.06	34.90±0.10	18.33±0.07	3.26±0.05	33.38±0.10	23.21±0.14	24.40±0.08	12.60±0.08	10.80±0.02	680	46.19±2.28
1	7.93±0.03	34.70±0.80	15.93±0.03	2.34±0.02	17.92±0.08	12.46±0.04	11.10±0.13	6.19±0.13	8.99±0.08	1255	53.18±1.58
2	7.94±0.04	33.70±0.60	14.80±0.06	1.98±0.03	12.35±0.03	9.59±0.02	7.03±0.03	3.92±0.03	12.75±0.18	1090	50.74±0.30
3	7.84±0.08	34.30±0.20	14.67±0.04	2.67±0.01	15.61±0.02	13.86±0.12	8.41±0.05	4.69±0.05	16.15±0.04	1355	55.95±5.84
4	7.72±0.03	35.60±0.30	18.04±0.03	3.25±0.08	24.23±0.14	16.86±0.09	14.71±0.10	8.20±0.10	30.74±0.14	1430	58.37±3.96
5	7.75±0.05	35.50±0.10	17.87±0.02	2.89±0.06	24.18±0.19	16.83±0.20	14.68±0.07	8.18±0.07	26.50±0.11	1130	53.06±2.12
6	7.60±0.06	35.40±0.80	17.38±0.06	2.22±0.06	24.28±0.01	16.89±0.08	14.75±0.14	8.23±0.15	19.44±0.07	1285	45.44±0.57
7	7.82±0.02	35.20±0.50	18.25±0.02	2.31±0.05	22.47±0.12	15.64±0.06	13.43±0.02	7.49±0.02	17.25±0.08	1070	52.73±1.84
8	7.84±0.09	35.20±0.30	19.20±0.10	2.41±0.07	21.95±0.03	15.27±0.16	12.04±0.08	6.72±0.08	12.98±0.13	875	48.22±4.43
9	7.87±0.01	35.00±0.90	20.05±0.03	2.63±0.02	22.71±0.05	15.80±0.08	12.60±0.01	7.03±0.01	12.50±0.06	640	49.43±3.15
10	7.91±0.03	34.60±0.50	19.75±0.09	2.80±0.01	24.76±0.09	17.23±0.07	14.10±0.02	7.86±0.02	10.86±0.04	510	50.94±5.04
11	7.97±0.02	33.20±0.80	21.98±0.12	3.13±0.04	23.33±0.02	16.23±0.08	13.05±0.05	7.28±0.05	8.57±0.21	475	51.68±6.01
12	7.83±0.06	35.60±0.10	22.45±0.08	2.99±0.08	22.88±0.07	15.92±0.23	9.72±0.03	5.42±0.03	15.70±0.06	360	46.33±4.71
13	7.66±0.07	36.00±0.70	17.90±0.06	2.77±0.02	22.05±0.05	15.34±0.09	11.12±0.03	6.20±0.03	27.14±0.05	265	31.47±1.56
14	7.81±0.08	35.30±0.20	16.85±0.03	2.80±0.05	24.89±0.05	17.32±0.20	13.19±0.11	6.03±0.11	23.82±0.02	360	48.66±2.14
15	7.97±0.02	35.40±0.50	17.35±0.01	2.79±0.04	27.79±0.03	17.37±0.11	10.64±0.09	5.94±0.09	15.21±0.07	350	43.68±0.39
Removal (%)	-	-	28.24±0.12	-	27.45±0.19	34.12±0.23	50.76±0.14	64.10±0.15	-	-	-

COD, chemical oxygen demand; TN, total nitrogen; TS, total solids, VS, volatile solids; TSS, total suspended solids; VSS, volatile suspended solids; Total VFA, total volatile fatty acids; mM, millimolar; ±, standard deviation. Analyses were performed as duplicates except biogas. Average removals for 15 days were calculated from daily removals by using corresponding values of feed sludge for that week.

Table C.1. Intensity analyses for images of control digester

Microbial community	Target microorganism and probe	Raw data obtained from ImageJ*			Relative abundancies from processed data**		
		Day 0	Day 7	Day 15	Day 0	Day 7	Day 15
Acidogens	*Acidobacteria* (HoAc1402)	61.16%±0.18	71.87%±0.23	70.19%±0.20	3.30%±0.00	3.35%±0.01	3.15%±0.01
	Acidobacteria (SS_HOL1400)	72.59%±0.22	80.50%±0.26	82.55%±0.29	4.18%±0.01	3.85%±0.01	3.85%±0.03
	Clostridium spp. (Clost I)	68.56%±0.25	70.83%±0.21	72.48%±0.24	3.87%±0.00	3.29%±0.01	3.28%±0.02
	Actinobacteria (Actino221)	71.19%±0.33	74.63%±0.21	70.42%±0.24	4.07%±0.02	3.51%±0.01	3.17%±0.02
	Flavobacterium (CFB563)	66.96%±0.15	69.34%±0.12	71.65%±0.25	3.75%±0.02	3.20%±0.02	3.24%±0.01
Acetogens	*Syntrophobacterales* (DSBAC355)	70.19%±0.11	85.00%±0.38	69.56%±0.21	3.99%±0.01	4.12%±0.01	3.12%±0.00
	Thermacetagenium (GTAG992)	82.37%±0.19	56.77%±0.09	80.44%±0.30	4.93%±0.03	2.47%±0.02	3.73%±0.01
	Syntrophobacter (SYN835)	53.97%±0.09	70.18%±0.23	81.78%±0.33	2.75%±0.00	3.25%±0.02	3.80%±0.02
	Tepidanaerobacter (GTE1002)	70.37%±0.13	68.47%±0.11	65.93%±0.17	4.01%±0.01	3.15%±0.00	2.92%±0.01
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	65.32%±0.27	75.01%±0.15	63.43%±0.19	3.62%±0.01	3.53%±0.02	2.78%±0.01
	Desulfobacter spp. (DSB129)	53.23%±0.15	56.13%±0.20	54.89%±0.11	2.69%±0.01	2.43%±0.01	2.30%±0.01
	Desulfobulbus spp. (DBB660)	58.58%±0.10	45.15%±0.19	39.96%±0.09	3.10%±0.01	1.79%±0.01	1.46%±0.00
	Desulfosarcina variabilis (DSC193)	61.90%±0.26	55.81%±0.11	55.86%±0.11	3.36%±0.02	2.41%±0.01	2.35%±0.01
Denitrifiers	*Pseuodomonas* spp. (Pae997)	64.08%±0.24	60.33%±0.16	58.55%±0.15	3.53%±0.01	2.68%±0.01	2.50%±0.02
	Bacillus spp. (Bmy843)	63.29%±0.21	72.09%±0.24	69.02%±0.26	3.46%±0.01	3.36%±0.02	3.09%±0.03
	Acetate-denitrifying cluster (DEN124)	59.22%±0.18	75.54%±0.25	57.95%±0.14	3.15%±0.01	3.56%±0.03	2.47%±0.02
Methanogens	*Methanosarcina* spp. (MS1414)	59.12%±0.15	49.34%±0.10	55.25%±0.17	11.20%±0.05	8.52%±0.05	13.83%±0.06
	Methanomicrobiales (MG1200)	57.18%±0.14	82.77%±0.31	57.25%±0.18	10.67%±0.04	16.70%±0.08	14.49%±0.05
	Methanosaeta spp. (MX825)	46.93%±0.10	60.99%±0.15	53.22%±0.12	7.86%±0.03	11.37%±0.07	13.15%±0.07
	Methanobacteriales (MB1174)	63.86%±0.16	69.43%±0.2	47.77%±0.10	12.50%±0.07	13.44%±0.09	11.33%±0.09
Archaea	*Archaea* (ARC915)	54.76%±0.22	63.07%±0.14	69.52%±0.23	42.24%±0.13	50.03%±0.17	52.80%±0.18
Bacteria	*Bacteria* (EUBmix)	68.19%±0.29	63.01%±0.16	63.61%±0.22	57.76%±0.19	49.97%±0.15	47.20%±0.13
	Negative control (NONEUB)	18.21%±0.07	14.46%±0.04	13.81%±0.03	-	-	-

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; **Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation

Table C.2. Intensity analyses for images of digester pretreated with 0.03 g O_3/g TSS

Microbial community	Target microorganism and probe	Raw data obtained from ImageJ*			Relative abundancies from processed data**		
		Day 0	Day 7	Day 15	Day 0	Day 7	Day 15
Acidogens	*Acidobacteria* (HoAc1402)	68.41%±0.23	61.04%±0.20	66.60%±0.25	3.82%±0.01	3.04%±0.00	3.14%±0.01
	Acidobacteria (SS_HOL1400)	70.33%±0.24	72.34%±0.28	57.95%±0.19	3.98%±0.01	3.86%±0.01	2.59%±0.00
	Clostridium spp. (Clost I)	61.31%±0.15	71.21%±0.23	71.24%±0.23	3.26%±0.00	3.78%±0.01	3.44%±0.01
	Actinobacteria (Actino221)	65.90%±0.16	52.27%±0.13	52.23%±0.17	3.62%±0.01	2.40%±0.01	2.22%±0.00
	Flavobacterium (CFB563)	71.56%±0.27	66.06%±0.21	61.50%±0.14	4.07%±0.02	3.40%±0.01	2.82%±0.00
Acetogens	*Syntrophobacterales* (DSBAC355)	62.89%±0.18	30.62%±0.12	70.35%±0.26	3.38%±0.01	0.82%±0.00	3.39%±0.00
	Thermacetagenium (GTAG992)	56.93%±0.14	65.16%±0.27	88.91%±0.30	2.91%±0.00	3.34%±0.01	4.58%±0.02
	Syntrophobacter (SYN835)	67.90%±0.23	64.80%±0.23	69.32%±0.26	3.78%±0.01	3.31%±0.01	3.32%±0.01
	Tepidanaerobacter (GTE1002)	71.50%±0.29	78.65%±0.20	58.88%±0.23	4.07%±0.01	4.32%±0.01	2.65%±0.00
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	83.75%±0.34	58.92%±0.16	64.55%±0.12	5.04%±0.02	2.88%±0.00	3.01%±0.00
	Desulfobacter spp. (DSB129)	62.12%±0.16	71.33%±0.24	71.36%±0.23	3.32%±0.01	3.79%±0.01	3.45%±0.01
	Desulfobulbus spp. (DBB660)	47.33%±0.02	66.03%±0.24	47.62%±0.13	2.15%±0.00	3.40%±0.01	1.92%±0.00
	Desulfosarcina variabilis (DSC193)	55.11%±0.18	78.28%±0.22	63.60%±0.15	2.77%±0.00	4.30%±0.02	2.95%±0.00
Denitrifiers	*Pseuodomonas* spp. (Pae997)	82.33%±0.23	66.22%±0.23	63.67%±0.17	4.93%±0.03	3.41%±0.00	2.96%±0.00
	Bacillus spp. (Bmy843)	49.74%±0.12	75.81%±0.29	57.36%±0.18	2.34%±0.00	4.12%±0.01	2.55%±0.00
	Acetate-denitrifying cluster (DEN124)	65.65%±0.15	61.50%±0.18	57.18%±0.16	3.60%±0.01	3.07%±0.01	2.54%±0.00
Methanogens	*Methanosarcina* spp. (MS1414)	84.90%±0.46	72.05%±0.26	73.51%±0.28	15.58%±0.07	14.88%±0.06	13.45%±0.05
	Methanomicrobiales (MG1200)	65.71%±0.10	48.00%±0.13	66.20%±0.23	10.96%±0.04	8.08%±0.04	11.69%±0.04
	Methanosaeta spp. (MX825)	61.23%±0.18	69.27%±0.28	68.52%±0.24	9.88%±0.04	14.09%±0.06	12.25%±0.04
	Methanobacteriales (MB1174)	47.27%±0.16	53.79%±0.15	80.28%±0.29	6.52%±0.02	9.72%±0.03	15.09%±0.08
Archaea	*Archaea* (ARC915)	64.09%±0.25	54.19%±0.13	64.96%±0.22	42.95%±0.11	46.77%±0.13	52.48%±0.18
Bacteria	*Bacteria* (EUBmix)	78.51%±0.29	58.99%±0.12	60.50%±0.17	57.05%±0.16	53.23%±0.16	47.52%±0.17
	Negative control (NONEUB)	20.21%±0.11	19.44%±0.09	17.76%±0.08	-	-	-

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; **Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation

Table C.3. Intensity analyses for images of digester pretreated with 0.06 g O_3/g TSS

Microbial community	Target microorganism and probe	Raw data obtained from ImageJ*			Relative abundancies from processed data**		
		Day 0	Day 7	Day 15	Day 0	Day 7	Day 15
Acidogens	*Acidobacteria* (HoAc1402)	62.30%±0.13	67.62%±0.16	57.35%±0.15	3.09%±0.01	2.37%±0.00	2.43%±0.00
	Acidobacteria (SS_HOL1400)	63.12%±0.19	61.44%±0.17	66.23%±0.14	3.16%±0.01	2.03%±0.00	2.99%±0.01
	Clostridium spp. (Clost I)	70.35%±0.18	74.70%±0.23	72.92%±0.28	3.83%±0.01	2.76%±0.00	3.41%±0.01
	Actinobacteria (Actino221)	79.60%±0.26	82.42%±0.29	67.59%±0.10	4.68%±0.01	3.18%±0.01	3.07%±0.00
	Flavobacterium (CFB563)	80.15%±0.26	78.35%±0.25	76.11%±0.22	4.73%±0.02	2.96%±0.00	3.61%±0.00
Acetogens	*Syntrophobacterales* (DSBAC355)	32.94%±0.06	74.92%±0.26	67.44%±0.15	0.39%±0.00	2.77%±0.00	3.06%±0.01
	Thermacetagenium (GTAG992)	69.53%±0.12	71.30%±0.20	69.42%±0.11	3.75%±0.01	2.57%±0.01	3.19%±0.00
	Syntrophobacter (SYN835)	69.92%±0.16	55.17%±0.11	90.56%±0.42	3.79%±0.01	1.68%±0.00	4.52%±0.02
	Tepidanaerobacter (GTE1002)	71.47%±0.14	68.10%±0.12	75.02%±0.24	3.93%±0.02	2.40%±0.00	3.54%±0.00
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	67.92%±0.18	63.38%±0.17	85.58%±0.36	3.60%±0.01	2.14%±0.00	4.21%±0.01
	Desulfobacter spp. (DSB129)	50.78%±0.11	56.49%±0.18	64.56%±0.14	2.03%±0.01	1.76%±0.00	2.88%±0.00
	Desulfobulbus spp. (DBB660)	42.02%±0.06	52.67%±0.19	62.93%±0.16	1.22%±0.01	1.55%±0.00	2.78%±0.00
	Desulfosarcina variabilis (DSC193)	58.59%±0.11	66.71%±0.15	57.65%±0.15	2.74%±0.01	2.32%±0.01	2.44%±0.00
Denitrifiers	*Pseuodomonas* spp. (Pae997)	64.28%±0.12	73.36%±0.22	78.74%±0.22	3.27%±0.01	2.68%±0.01	3.78%±0.01
	Bacillus spp. (Bmy843)	81.78%±0.35	80.75%±0.28	74.79%±0.23	4.88%±0.02	3.09%±0.01	3.53%±0.00
	Acetate-denitrifying cluster (DEN124)	76.23%±0.24	83.04%±0.30	83.76%±0.29	4.37%±0.02	3.22%±0.01	4.09%±0.01
Methanogens	*Methanosarcina* spp. (MS1414)	73.40%±0.20	77.41%±0.23	53.21%±0.10	12.34%±0.06	15.76%±0.07	7.93%±0.05
	Methanomicrobiales (MG1200)	67.73%±0.26	74.05%±0.22	83.00%±0.35	10.77%±0.04	14.76%±0.06	14.83%±0.07
	Methanosaeta spp. (MX825)	75.09%±0.24	78.88%±0.24	64.90%±0.14	12.80%±0.05	16.20%±0.07	10.64%±0.06
	Methanobacteriales (MB1174)	67.30%±0.29	70.90%±0.22	75.33%±0.24	10.65%±0.04	13.82%±0.06	13.06%±0.06
Archaea	*Archaea* (ARC915)	72.32%±0.22	80.40%±0.25	71.86%±0.23	46.56%±0.12	60.53%±0.18	46.46%±0.13
Bacteria	*Bacteria* (EUBmix)	78.75%±0.26	60.97%±0.17	79.92%±0.26	53.44%±0.15	39.47%±0.12	53.54%±0.14
	Negative control (NONEUB)	28.75%±0.12	24.57%±0.11	18.97%±0.10	-	-	-

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; **Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation

Table C.4. Intensity analyses for images of digester pretreated with 0.09 g O_3/g TSS

Microbial community	Target microorganism and probe	Raw data obtained from ImageJ*			Relative abundancies from processed data**		
		Day 0	Day 7	Day 15	Day 0	Day 7	Day 15
Acidogens	*Acidobacteria* (HoAc1402)	55.10%±0.17	37.91%±0.11	52.83%±0.20	2.57%±0.00	1.53%±0.00	3.46%±0.01
	Acidobacteria (SS_HOL1400)	50.44%±0.14	60.44%±0.25	61.65%±0.24	2.25%±0.00	3.74%±0.01	4.56%±0.02
	Clostridium spp. (Clost I)	56.56%±0.16	58.43%±0.16	60.24%±0.22	2.67%±0.00	3.54%±0.01	4.38%±0.02
	Actinobacteria (Actino221)	62.65%±0.23	47.32%±0.13	33.25%±0.16	3.09%±0.01	2.45%±0.00	1.04%±0.00
	Flavobacterium (CFB563)	60.73%±0.24	65.51%±0.24	55.07%±0.13	2.96%±0.00	4.23%±0.02	3.74%±0.01
Acetogens	*Syntrophobacterales* (DSBAC355)	42.54%±0.14	37.29%±0.10	58.97%±0.16	1.71%±0.00	1.47%±0.00	4.22%±0.01
	Thermacetagenium (GTAG992)	56.09%±0.15	68.47%±0.21	50.07%±0.15	2.64%±0.00	4.52%±0.02	3.12%±0.01
	Syntrophobacter (SYN835)	59.62%±0.16	71.90%±0.27	57.62%±0.13	2.88%±0.00	4.86%±0.02	4.06%±0.01
	Tepidanaerobacter (GTE1002)	76.68%±0.21	69.75%±0.28	66.16%±0.24	4.05%±0.02	4.65%±0.02	5.11%±0.02
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	72.55%±0.29	54.30%±0.16	63.41%±0.21	3.77%±0.01	3.14%±0.00	4.77%±0.02
	Desulfobacter spp. (DSB129)	47.44%±0.10	41.88%±0.17	42.30%±0.17	2.05%±0.00	1.92%±0.01	2.16%±0.00
	Desulfobulbus spp. (DBB660)	60.56%±0.22	69.95%±0.28	46.99%±0.17	2.95%±0.00	4.67%±0.02	2.74%±0.00
	Desulfosarcina variabilis (DSC193)	55.17%±0.16	55.56%±0.19	30.62%±0.13	2.58%±0.00	3.26%±0.00	0.71%±0.00
Denitrifiers	*Pseuodomonas* spp. (Pae997)	60.54%±0.28	56.11%±0.18	54.82%±0.18	2.95%±0.00	3.31%±0.00	3.71%±0.01
	Bacillus spp. (Bmy843)	38.40%±0.09	43.35%±0.18	47.07%±0.19	1.43%±0.00	2.06%±0.00	2.75%±0.00
	Acetate-denitrifying cluster (DEN124)	60.67%±0.22	50.66%±0.11	61.61%±0.23	2.95%±0.00	2.78%±0.00	4.55%±0.01
Methanogens	*Methanosarcina* spp. (MS1414)	35.04%±0.08	48.68%±0.10	63.60%±0.27	6.20%±0.03	11.17%±0.06	13.64%±0.04
	Methanomicrobiales (MG1200)	57.68%±0.13	50.33%±0.23	54.28%±0.16	14.25%±0.06	11.87%±0.06	10.36%±0.05
	Methanosaeta spp. (MX825)	64.02%±0.14	48.17%±0.12	48.01%±0.15	16.51%±0.08	10.96%±0.05	8.15%±0.05
	Methanobacteriales (MB1174)	72.58%±0.29	55.06%±0.14	61.06%±0.26	19.55%±0.09	13.88%±0.04	12.75%±0.06
Archaea	*Archaea* (ARC915)	56.82%±0.16	63.11%±0.26	57.70%±0.12	56.51%±0.19	47.88%±0.13	44.89%±0.15
Bacteria	*Bacteria* (EUBmix)	47.78%±0.15	66.72%±0.27	65.17%±0.27	43.49%±0.17	52.12%±0.18	55.11%±0.18
	Negative control (NONEUB)	17.60%±0.08	22.31%±0.13	24.85%±0.13	-	-	-

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; **Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation