EFFECT OF OZONE PRETREATMENT ON BIOGAS PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF TWO-STAGE ANAEROBIC DIGESTERS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MERVE AKÇAKAYA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
ENVIRONMENTAL ENGINEERING

FEBRUARY 2021

Approval of the thesis:

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ABSTRACT

EFFECT OF OZONE PRETREATMENT ON BIOGAS PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF TWO-STAGE ANAEROBIC DIGESTERS

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February 2021, 138 pages

The amount of sludge produced in treatment plants is increasing depending on the population growth and water consumption. Anaerobic digestion (AD) is a widely used sludge stabilization method in wastewater treatment plants, as it provides volume reduction and methane-rich biogas production. In single-stage anaerobic digestions, the process fails due to volatile fatty acid accumulation and pH changes. To solve this problem, two-stage anaerobic digestion (TSAD) process has emerged through physical separation of methane production stages. Various pretreatment applications are also used in AD to accelerate the rate limiting stage, hydrolysis. Of these, with its strong oxidizing ability ozonation stands out. Therefore, in this study, the effect of ozone pretreatment on TSAD was evaluated in terms of biogas production and microbial population dynamics. For this purpose, two-stage anaerobic digesters were operated after treatment with previously determined ozone dose of 0.06 g O_3/g TSS and monitored in terms of pH, chemical oxygen demand, total nitrogen, solids content, total volatile fatty acids, biogas/methane production and microbial community structure. Biogas and methane production were monitored

through water displacement method and gas chromatography, microbial consortium was monitored through fluorescent *in situ* hybridization method. Methane and biogas productions were found to be increased 69% and 38%, respectively. This increase was accounted for the dominance of *Methanosaeta* spp.

Keywords: Two-stage anaerobic digestion, Ozone pretreatment, Biogas production, Methane production, Microbial community

ÖZ

OZON ÖN ARITIMININ İKİ AŞAMALI ANAEROBİK ÇÜRÜTÜCÜLERDEKİ BİYOGAZ ÜRETİMİ VE MİKROBİYAL KONSORSİYUM ÜZERİNDEKİ ETKİSİ

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Şubat 2021, 138 sayfa

Arıtma tesislerinde üretilen çamur miktarı, artan nüfus ve su tüketimine bağlı olarak her geçen gün artmaktadır. Anaerobik çürütme (AÇ), atık su arıtma tesislerinde çamur hacmini azalttığı ve biyogaz üretimi sağladığı için yaygın olarak kullanılan bir çamur stabilizasyon yöntemidir. Tek aşamalı AÇ sistemlerinde, çürütücülerde uçucu yağ asidi birikimi ve pH değişiklikleri nedeniyle proses başarısızlıkları gözlenir. Bu sorunu çözmek için metan üretim aşamalarının fiziksel olarak ayrılmasıyla oluşturulan iki aşamalı anaerobik çürütme (İAAÇ) sistemleri kullanılmaya başlanmıştır. Ayrıca, AÇ'de hız sınırlayıcı basamak olarak bilinen hidroliz aşamasını hızlandırmak için çeşitli ön arıtım uygulamaları kullanılmaktadır. Bu uygulamalardan ozonlama, güçlü oksitleme kabiliyeti ile ön plana çıkmaktadır. Bu nedenle bu çalışmada ozon ön işleminin İAAÇ üzerindeki etkisi biyogaz üretimi ve mikrobiyal popülasyon dinamikleri açısından değerlendirilmiştir. Bu amaçla, iki aşamalı anaerobik çürütücülere, labımızca önceden belirlenen optimum 0,06 g O_3/g TAKM ozon dozu ile ön arıtım uygulanarak çalıştırılmıştır. Çürütücüler süreç boyunca, pH, kimyasal oksijen ihtiyacı, toplam nitrojen, katı içeriği, toplam uçucu

yağ asitleri, biyogaz/metan üretimi ve mikrobiyal konsorsiyum açısından izlenmiştir. Biyogaz ve metan üretimi su yer değiştirme yöntemi ve gaz kromatografisi ile mikrobiyal konsorsiyum ise floresan *in situ* hibridizasyon yöntemi ile takip edilmiştir. Sonuç olarak, metan ve biyogaz üretiminde sırasıyla %69 ve %38 artış gözlemlenmiştir. Çürütücülerde gözlenen bu artış, *Methanosaeta* türünün baskınlığı ile ilişkilendirilmiştir.

Anahtar Kelimeler: İki aşamalı anaerobik çürütücü, Ozon ön arıtımı, Biyogaz üretimi, Metan üretimi, Mikrobiyal konsorsiyum

To my family

ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my advisor Prof. Dr. Bülent İçgen for his guidance, advice, trust, encouragement, and continuous support throughout this research.

Secondly, I would like to thank examining committee members for their comments and contributions.

I am very thankful to İçgen Team members; Serkan Küçükünsal, Sera Tuncay, Osman Kayalı, and Aslı Onursal for being best labmates ever. I would also like to thank my dearest friend Çağrı Gürbüz. Thank you all for being my family in METU.

Additionally, I am thankful to my besties Kübra Uyanık and İpek Girgin for their support and for asking in every speech "When does the thesis end?".

I would like to present my deepest gratitude to my wonderful family Şefika and Kurtuluş Akçakaya for their endless love and support. Especially I thank to my grandmother Emine Erdem for raising me.

Lastly, I would like to thank to my soul mate İsmail Ekşici, for being there for me in this difficult process with his love and patient.

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) with the project number 116Y181.

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ABBREVIATIONS

AD: Anaerobic Digestion

COD: Chemical Oxygen Demand

CSTR: Continuously Stirred Tank Reactor

DAPI: 4′,6-Diamidino-2-phenylindole dihydrochloride

DGGE: Denaturing Gradient Gel Electrophoresis

FA: Formamide

FISH: Fluorescence in situ Hybridization

FITC: Fluorescein isothiocyanate

GC: Gas Chromatograph

HRT: Hydraulic Retention Time

MAR-FISH: Microautoradiography combined with FISH

NGS: Next Generation Sequencing

OLR: Organic Loading Rate

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

PFA: Paraformaldehyde

RAS: Return Activated Sludge

SAD: Single-stage anaerobic digestion

SIP: Stable Isotope Probing

SRT: Solids Retention Time

SSCP: Single-Strand Conformation Polymorphism

TN: Total Nitrogen

T-RFLP: Terminal-Restriction Fragment Length Polymorphism

TS: Total Solids

TSAD: Two-stage Anaerobic Digestion

TSS: Total Suspended Solids

VFA: Volatile Fatty Acids

VS: Volatile Solids

VSS: Volatile Suspended Solids

WAS: Waste Activated Sludge

WWTP: Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

The demand for the energy in the industries is on significant rise parallel to the population growth to supply basic needs of people such as food, water, and transportation. Today vast majority of energy needs are met by fossil fuels. Energy researches have shifted to renewable energy sources and biofuels because fossil fuels are limited to reserves, and that emissions from these fuels have a large share in global warming (United Nations, 2019). Sludge, which is produced in the wastewater treatment plants (WWTPs) as a by-product during various treatment processes, has been considered as an alternative renewable energy source (Zsirai, 2011).

The sludge amount produced in treatment facilities is increasing day by day as another important result of the increasing population and water consumption accordingly. This situation makes sludge management more important due to its risks to environment and public health (Zsirai, 2011). Many sludge disposal methods have been used in WWTPs (Andreoli & Fernandes, 2007a). Among these methods, anaerobic sludge digestion process is widely used since it provides volume reduction of sludge and biogas formation (Gavala et al., 2003).

Anaerobic sludge digestion process (AD) is based on the biochemical decomposition of organic materials with microbial activities in anaerobic condition. The general process can be explained by the chemical reaction in which organic material such as glucose is biochemically digested into carbon dioxide (CO_2) and methane (CH_4) by anaerobic microorganisms (Tabatabaei et al., 2010). AD takes place through four stages as hydrolysis, acidogenesis, acetogenesis and methanogenesis (Jeongsik Kim et al., 2003). In conventional single-stage AD reactors, all the four stages occur in single reactor, in other words, acid- and methane-producing microorganism are kept in the same reactor (Göblös et al., 2008). These microorganisms differ significantly

from each other in terms of growth kinetics and sensitivity to environmental conditions (Pham Van et al., 2020). Controlling and ensuring stability in single-stage anaerobic reactors pose serious difficulties. Therefore, it has become necessary to develop new solutions to overcome the drawbacks of single-stage reactors (De Gioannis et al., 2017). For this purpose, the use of two-stage anaerobic digestion reactors was proposed firstly by Pohland and Ghosh in 1971. In two stage anaerobic reactors, separation of acid- and methane-producing bacteria is aimed. In this way, suitable environmental conditions can be provided for their growth, and methane production efficiency (Pohland & Ghosh, 1971).

Many studies indicate that the first step of the anaerobic sludge digestion process, hydrolysis, plays a rate limiting role for the AD processes (Ariunbaatar et al., 2014; Yang et al., 2018; M. Park et al., 2021). Some pre-treatment approaches such as mechanical, chemical, thermal, biological, or a combination of these are applied to speed up this stage and to make the organic content in the sludge more accessible to microorganisms (Rafique et al., 2010). Ozone is one of the most used chemical pre-treatment techniques in AD processes studies. It makes the sludge more biodegradable with strong oxidizing ability (Bougrier et al., 2007). In many studies, it has been proven that ozone pre-treatment application increases methane production and decreases the amount of solids in the sludge (Bougrier et al., 2007; Silvestre et al., 2015; Chiavola et al., 2019).

Molecular techniques, which have been widely used to increase our understanding on microorganisms, have been also employed to identify microbial community of AD (Dinova et al., 2018). By using molecular methods, it is aimed to prevent malfunctions in AD and to increase methane production (Shah et al., 2014). In this direction, different studies are carried out by using different approaches (Khanal, 2009; Cabezas et al., 2015). One of them is fluorescent *in situ* hybridization analysis (FISH) that allows to determine specific groups of microorganisms and provides opportunity to get information about the structure of consortium (Tabatabaei et al., 2010; Dinova et al., 2018). Methanogens are not culturable microorganisms due to

their complex relationships with other microorganisms. Since FISH technique offers information not only for the culturable but also for the unculturable ones, microorganisms in samples taken from the anaerobic reactors can be easily identified at domain, family, genera, even species level (Kumar et al., 2011).

From the literature reviews, it is seen that studies on single-stage AD processes for sludge treatment is more common than two-stage AD. Startup strategies, operating conditions and reactor configurations for single-stage anaerobic reactor have been already evaluated in previous studies, as well as doses of ozone for pretreatment. However, only a few studies are found to deal with the effect of these applications on two-stage AD reactors. In addition, most of the studies did not focus on to determine the effect of ozone pretreatment on microbial population of two-stage AD. Therefore, the scope of this study is to evaluate the impact of ozone pretreatment on two-stage AD for biogas/methane production and microbial population dynamics. For that purpose, previously determined optimum ozone dose in our lab, was applied to two-stage anaerobic reactor to investigate the changes in biogas/methane production, while monitoring microbial dynamics through FISH method.

CHAPTER 2

LITERATURE REVIEW

2.1 Sludge problems and treatment methods

The world population has grown faster than ever in the last 50 years and it is expected to reach 9.6 billion by 2050 (Werle & Dudziak, 2019). Correspondingly, water usage and the amount of produced wastewater is gradually increasing. In parallel, more sludge is produced in wastewater treatment plants and sludge management has become an issue of growing importance (Chang et al., 2020).

Sewage sludge is generated in WWTPs during treatment processes as by-product and is classified as primary and activated sludge. Primary sludge in other words raw sludge is produced during primary treatment processes whereas activated sludge is produced during secondary treatment processes. Activated sludge, also called as excess sludge, is the activated waste biomass resulting from biological treatments (Murta et al., 2019). Typical configuration of wastewater treatment processes and sludge formation processes is given Figure 2.1.

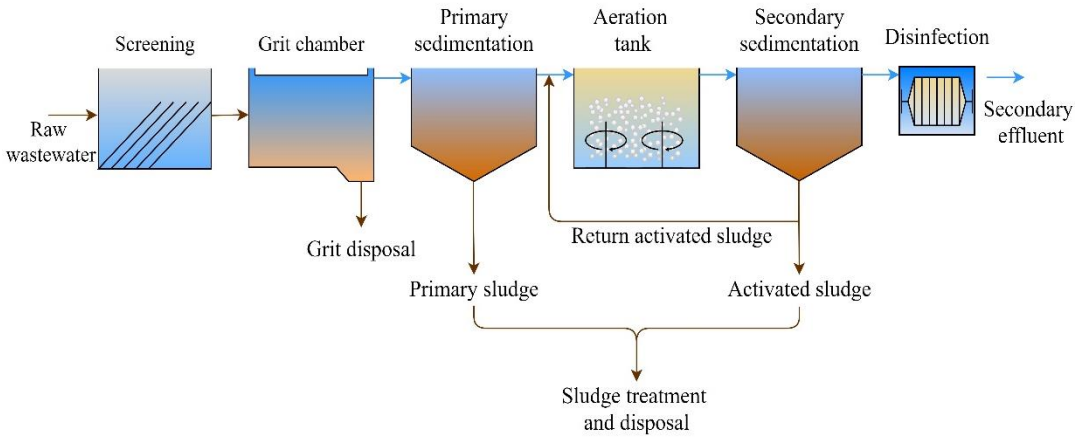

Figure 2.1 Typical Wastewater Tratment Plant Configuration adapted from Ambulkar & Nathanson, 2020

Sludge produced in the WWTPs mainly composed of organic matters, nutrients, heavy metals, and pathogens (Canziani & Spinosa, 2019). Since it contains pathogens such as bacteria and viruses, direct discharge of sewage sludge without any treatment affects the environment and human health negatively. Therefore, the treatment of sewage sludge is crucial before discharging to environment (Dentel, 2014; Chang et al., 2020). In WWTPs, generally sludge treatment includes volume reduction and stabilization methods such as thickening, dewatering, conditioning, anaerobic and aerobic digestion, composting, and alkaline stabilization (Dentel, 2014). In Table 2.1, commonly used processes are listed and described briefly.

Table 2.1 Treatment Methods for Sewage Sludge (Dentel, 2014)

Treatment methods	Description
Thickening	Volume reduction of sludge by removing significant amount of water
Dewatering	Moisture content reduction of biosolids
Aerobic digestion	In presence of oxygen; - stabilize the organic matter in wastewater solids, - reduce the sludge amount, pathogens and odors levels - produce carbon dioxide, nitrate, and sulfur dioxide
Anaerobic digestion	In absence of oxygen; - stabilize the organic matter in wastewater solids, - reduce the sludge amount, pathogens and odors levels - produce biogas contain methane and carbon dioxide
Alkaline stabilization	Alkaline material addition to sludge (commonly lime) - reduce pathogen and parasite content, - eliminate odors - mitigate the potential for further biodegradation of the sludge.
Conditioning	Dewatering preparation process by adding chemical - increases dewatering capability - improves solid capture in sludge
Composting	Mixing sewage sludge with agricultural byproduct sources of carbon such as sawdust or straw in presence of oxygen

Among these sludge treatment methods, AD process is the one of the most widely used sludge stabilization methods in WWTPs, since AD is considered as an effective, economical, and eco-friendly technology (Elagroudy et al., 2020). In addition to these properties, during sludge stabilization, odor and pathogen removal, AD also produces biogas with methane (P. Wang et al., 2020). In other words, anaerobic digesters not only solve the increasing sludge problem but also produce a promising solution for energy problem by producing biogas as an alternative renewable energy source (V. K. Nguyen et al., 2021).

2.2 Anaerobic digestion

Today, organic wastes are converted to renewable energy sources such as biohydrogen or methane in biochemical systems. This conversion has significant role on environmental protection by providing sustainable and renewable energy (Panwar et al., 2011). In this sense, AD is one of the oldest, energy-efficient and environmentally friendly technology (Kay & Li, 2020). AD has been adopted from small-scale to large-scale systems and it is a widely researched topic around the world. Numerous studies have been conducted on the AD process to improve biogas and methane production efficiency (M. J. Park et al., 2010; Karlsson et al., 2014; Li et al., 2019). Although AD offers many important advantages over other processes, it also has some disadvantages. Advantages and disadvantages of AD obtained from the studies in the literature are given in the Table 2.2.

Table 2.2 Advantages and Disadvantages of AD (Abdelgadir et al., 2014)

Advantages	**Disadvantages**
Greenhouse gas emission reduction	High retention time
Volume reduction of sludge	Long start-up period
Methane production as valuable end-product	High sensitivity to environmental conditions
High treatment efficiency	Possible bad odors
Low energy requirements	High initial costs
Low nutrients and chemicals requirement	Ineffective nutrient removal
Lower installation space requirement	
Lower operational cost	

AD is defined as the conversion of organic wastes into biogas and digestate by various microbial species in the absence of oxygen. Biogas, one of the renewable energy sources, is obtained as a result of anaerobic fermentation of plant, animal, domestic and industrial wastes and sewage sludge. Biogas is a gas mixture consisting of 40-70% CH_4, 30-60% CO_2 and 1-5% other gases (Panwar et al., 2011). Biogas has many application areas such as generation of electricity and heat through a combined heat and power unit, natural gas substitution and as transport fuel

(compressed natural gas) and generating liquid fuels (e.g., gasoline, diesel, jet fuel). Another product, generated at the end of AD process, is digestate. Digestate is utilized as fertilizer and soil amendments (V. K. Nguyen et al., 2021). Schematic representation of AD process is shown in Figure 2.2.

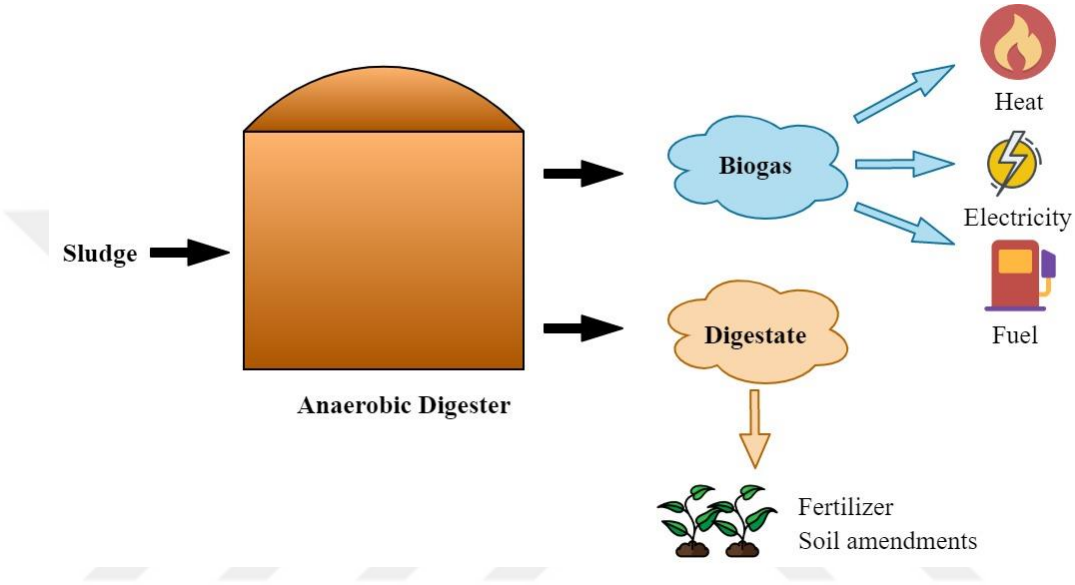

Figure 2.2 Schematic representation of AD process adapted from IEA, 2020

2.2.1 Process description of AD

AD is a series of processes in which a consortium of microorganisms breaks down organic materials in the absence of oxygen. The simplified AD process equation is shown below (Mullai et al., 2020):

$$\text{Organic matter} \rightarrow CH_4 + CO_2 + H_2 + N_2 + H_2S$$

AD is described as a complex fermentation process carried out by the symbiotic association of different bacterial species. The products produced by one group of bacteria in the process, act as the substrate for the next group in coming stage (Bajpai, 2017). Biogas production from organic materials during the anaerobic treatment

process takes place in four successive stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Figure 2.3.

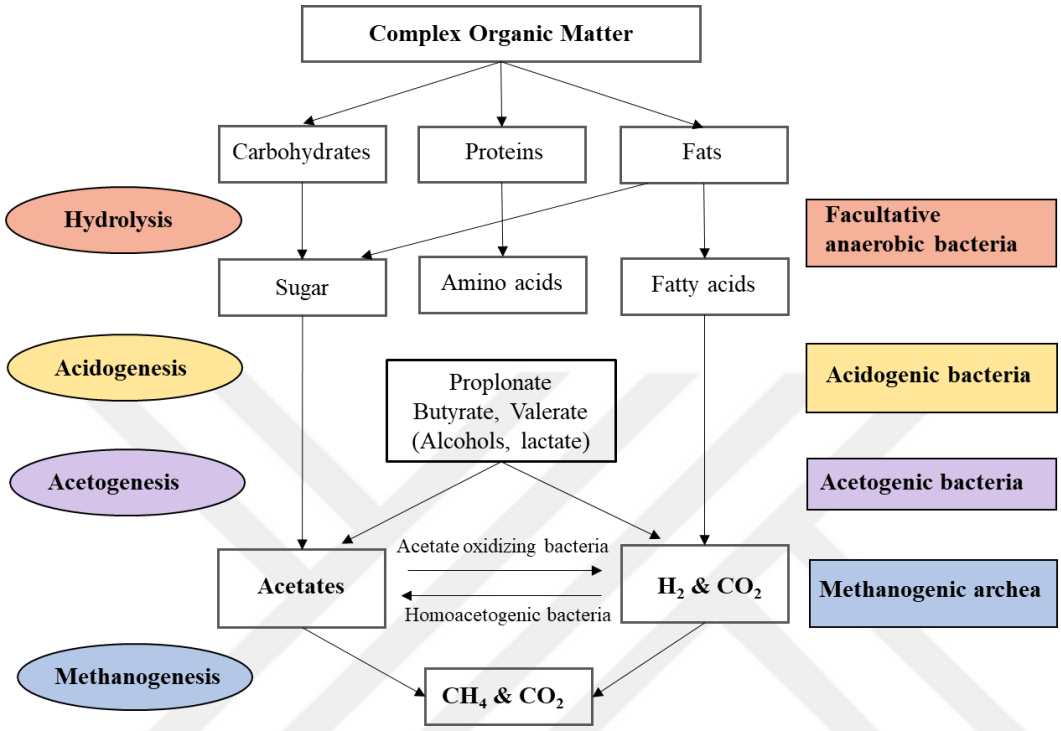

Figure 2.3 Stages of AD process (Kothari et al., 2014)

Hydrolysis: The first stage of AD, hydrolysis, is an essential part of methane production. In this stage, complex organic macromolecules such as proteins, fats and carbohydrates are decomposed by microorganisms into amino acids, fatty acids, and monosaccharides, respectively (Ometto et al., 2019). Hydrolysis is performed by hydrolytic bacteria through hydrolytic enzymes. It is a slow process and, therefore, considered as rate limiting step of methane formation (Bajpai, 2017).

Acidogenesis: In the second stage of the AD process, the products generated in the hydrolysis stage are converted into volatile fatty acids (VFAs), ketones, alcohols, CO_2 and H_2 by acidogenic microorganisms. At this stage, large amounts of CO_2 and H_2 can be produced, depending on the carbon content of the substrate Among the products of acidogenesis stage, H_2, CO_2 and acetic acid can be used directly by

methanogenic bacteria, by passing the third stage of the AD process (Ometto et al., 2019). Acidogenic bacteria can tolerate pH as low as 5-6 and grow rapidly in such an environment. Consequently, if acidic products cannot be metabolized eventually, the entire AD process can be inhibited due to the decrease in pH (Kiran et al., 2016).

Acetogenesis: Acetogenesis is the third step of AD process. At this stage, fatty acids and other organic molecules are converted into acetate, CO_2 and H_2 by acetogenic microorganisms. As in the acidogenesis stage, inhibition of methanogenesis can occur if hydrogen molecules are not metabolized. Acetogenesis takes a long time due to slow growth rate of acetogenic bacteria (Kiran et al., 2016).

Methanogenesis: The final stage of AD process is Methanogenesis. In this stage, methanogenic bacteria metabolized H_2 and acetic acids into CH_4 and CO_2 (Ometto et al., 2019). Methanogenesis is a critical step in AD process, and it is the slowest reaction compared to other steps. Methane-producing bacteria are strict anaerobes and have slow growth rate. They are extremely sensitive to changes in the environment and vulnerable to even small amounts of oxygen (Bajpai, 2017). Methanogenesis is carried out by methanogens which is belong to *Archaea* domain. Methanogens are divided into two groups, hydrogenotrophic and acetoclastic methanogens. Acetoclastic methanogens convert acetate to CH_4 and CO_2, while hydrogenotrophic methanogens convert H_2 or formate to CO_2 and CH_4 (Venkiteshwaran et al., 2015).

2.2.2 Operational process parameters

Anaerobic digestion is considered as the most complicated biological. The process is burdened due to poor operational stability and high sensitivity to changes of environmental conditions. In order to prevent failure of AD, operational parameters should be monitored periodically and maintained within their optimum range (Meegoda et al., 2018).

There are many factors affecting the performances of an anaerobic digesters and these are temperature, pH, solid retention time (SRT) and hydraulic retention time (HRT), organic loading rate (OLR), VFAs, C/N ratio, solid contents, and mixing.

2.2.2.1 Temperature

Temperature is a vital operational parameter in AD process for survival of microbial consortia and continuous biogas production, and determines the process performance (Lin et al., 2017). AD process is usually operated in mesophilic or thermophilic conditions which digester temperature is ranging from 30-35 °C or 50-60 °C, respectively (J. K. Kim et al., 2006). It has been stated that AD continues at temperatures below 20 °C, however, the degradation is three times slower than the mesophilic process (Sarker et al., 2019). At low temperatures, methanogenesis becomes the rate limiting step (Cysneiros et al., 2011). At mesophilic and thermophilic processes, suitable environment for biodegradation is created. At these conditions, high rates of hydrolysis and consequently high biogas yields are obtained in AD (Sarker et al., 2019).

In the AD process, temperature is also important as it affects pH. During process, acidogens are less affected by temperature than methanogens, accordingly low temperature in digesters can lead to an increase in volatile acid concentrations. Due to this increase, the pH of the environment decreases, and methanogens are negatively affected, and methane production is interrupted. Therefore, temperature drop can have serious repercussions on process operation (Amani et al., 2010).

2.2.2.2 pH

The control pH is crucial operational parameter in AD process for methane production since it has an impact on microbial activities. In AD process, optimum pH range is indicated as 6.5-8.2 to facilitate methane production (Lin et al., 2017). Despite this wide range, methanogens are highly sensitive to pH change and they are

generally optimized at a pH close to 7. The pH values below 6.3 or above 7.8 can adversely affect methanogenesis. However, other stages can continue at pH values between 5.5 and 6.5. Due to VFAs produced in the acidogenesis stage, pH values can be dropped under 3 which resulting in the process to collapse. In general, extremely acidic or extremely alkaline pH ranges limit the acidogenesis and hydrolysis steps (Sarker et al., 2019).

2.2.2.3 SRT and HRT

Retention time is an imperative parameter utilized for design and operation of AD (Sarker et al., 2019). The average time that solids spend in the digester is defined as SRT, while the average time the sludge is retained in the digester is defined as HRT. The successive steps of the AD process are directly related to SRT (Appels et al., 2008). In anaerobic processes, relatively long SRTs are preferred due to low specific growth rates of methanogens and long SRTs provide decrease in formation of inhibitory reaction intermediates. The reported minimum SRT values at 20, 25, and 35 °C for methanogenesis are 40, 30, and 15 days respectively (To & Hanjie, 2010).

On the other hand, HRT is an also important parameter since it is used in determination of organic matter and volatile solids amount to be fed into the digester. In WWTPs, most mesophilic anaerobic digesters are operated with 15 to 30 days HRT. Shorter HRTs result in washout of methanogens and pH decrease (Emmanuel Alepu & Li, 2016).

2.2.2.4 OLR

OLR is an important design and operational parameter for AD process and defined as the number of volatile solids fed per unit volume of digester per unit of time (Babaee & Shayegan, 2011). OLR represents the total nutrient demands of microorganisms in system. In case of low OLR, microorganisms suffer from nutritional deficiencies, which may result in a decrease in biogas production

efficiency. Moreover, in this case, due to production of inhibitors such as ammonia, AD process is adversely affected. On the other hand, in the case of high OLR, problems such as VFA accumulation, shock loading and low pH can be observed in reactors (Nsair et al., 2020). In order to obtain optimum conditions for the biogas production OLR should be determined according to type of AD reactor, such as a one- or two-stage and a wet or dry AD. The OLR in wet AD vary within the range of 2–10 kg VS/m^3 d whereas OLR in dry AD vary within the range of 7–15 kg VS/m^3 d (Tassakka et al., 2019).

2.2.2.5 VFAs

In AD process, VFAs are intermediate products of the methane formation process and they formed as a result of the degradation of organic substances such as sugar, amino acids and long chain fatty acids by acidogenic microorganisms. VFAs generally consist of acetic acid, propionic acid, butyric acid and alcohols (Lukitawesa et al., 2020). The majority of the VFAs are degraded to acetate and further to methane during methanogenesis. In AD processes, to achieve equilibrium at different production stages and stable methane production, higher VFA degradation is required. In anaerobic processes, undesirable VFA concentrations can occur in the system due to various operational disturbances caused by OLR, pH, and temperature (Sarker et al., 2019). If there is any operational problem between microorganisms or toxicity problem in the system, VFA concentration gets higher. This causes decrease in pH values and toxic conditions in the reactor especially for the methanogens (Sanin et al., 2011).

2.2.2.6 C/N ratio

C/N ratio is an essential parameter for AD process because it is commonly used for characterization of nutrients. In the AD process, the carbon acts as energy source while nitrogen provides microbial growth enhancement (Mir et al., 2016). Together

these two nutrients often play the role of limiting factor. For these reasons, in the process, it is necessary to provide a substrate with the required carbon and nitrogen concentrations. In the literature, optimum ratio for C/N is determined between 20 and 30 (Puyuelo et al., 2011). In a study with mesophilic and thermophilic digesters, an optimal methane production potential and reduced ammonia inhibition are observed at C/N ratio of 25:1 and 35:1 for mesophilic and thermophilic digesters respectively (Meegoda et al., 2018). C/N ratio is usually calculated through determination of organic carbon and total nitrogen (TN) concentrations in the substrates (Puyuelo et al., 2011). In case of high C/N ratio, biogas production decreases as more N is consumed. In opposite situation where the C/N ratio is low, the pH decreases due to the accumulation of ammonia in the system and a toxic environment for methanogens occurs (Yuan et al., 2019).

2.2.2.7 Solid content

The solids content analyses of sludge include determination of total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS) analyses. TS is a measurement of dry matter including organics and inorganics in a sludge and important parameter for reactor design and mixing (Nsair et al., 2020). Moreover, biogas generation is also associated with the TS concentration of the sludge (J. Yi et al., 2014). VS content is measured as an organic fraction of TS and used for OLR determination (Meegoda et al., 2018). AD performance is generally characterized in terms of the reduction of volatile solids (Arnaiz et al., 2006). In addition, VS/TS ratio is used to determine digestion level in the AD process. The ratio ranges are 0.75-0.80 and 0.60-0.65 for undigested and digested sludges, respectively (Andreoli & Fernandes, 2007b).

2.2.2.8 Mixing

Mixing is one of the parameters that determines the performance and cost of the AD system. It is used to increase contact between microorganisms and substrate and to ensure a homogeneous distribution in the reactor (Sarker et al., 2019). Studies have shown that increase in methane and biogas production can be achieved in AD with a proper mixing system (Kaparaju et al., 2008; Singh et al., 2020).

2.2.3 Anaerobic digester types

Anaerobic digesters essentially perform the same function and provide favorable conditions for the growth of methane-forming microorganisms (EPA, 2019). There are a wide variety of anaerobic digesters, each performing this task in a very different way. The types of reactors used in biogas production are classified considering design mechanisms such as mixing, temperature, feeding mode and fermentation stage (X. Liu et al., 2011). According to this, main digester types are standard and high rate digesters, mesophilic and thermophilic digesters, continuous and batch digesters, and one- and two stage digesters (X. Liu et al., 2011).

Batch processes: Batch digesters are the simplest digester type. In the process, feedstocks mixed with inoculum are loaded into digesters under strict anaerobic conditions and then digestion process is started. During AD process, the reactor is kept closed until degradation of the feedstocks into biogas (CH_4, CO_2 and other gases) is completed. Once the digestion is complete, the residue is removed, and reactor can be used for another run (Patinvoh, 2019).

Semi-continuous process: Semi-continuous processes involve partial withdrawal of degraded substrate from the system followed by re-feeding of the digester (X. Liu et al., 2011). Studies completed by using batch AD process have shown that the processing capacity is limited due to some disadvantages of the system such as longer digestion time and lower organic loads. In contrast, it has been observed that

the treatment capacity of the digester is clearly increased in anaerobic reactors operated in semi-continuous mode. In the study performed by Wang et al., 2020 in a lab-scale system, it is found that semi-continuous process is more effective than batch process. Due to their high capacity and relatively balanced biogas production, semi-continuous AD processes are widely applied in biogas plants (C. Zhang et al., 2015).

Continuous processes: Continuous digesters involve the continuous supply of feedstock to the reactors. The amount of degraded sludge is drawn from the reactor as much as the fed feedstock amount (Glatz et al., 2011). In these systems continuous biogas production is aimed. The continuous process minimizes substrate inhibition and allows a periodic supply of raw materials, preventing changes in microbial community. In continuous systems, the feed and discharge amount are kept in balance during sufficient retention time to achieve steady-state conditions. This type of digesters is suitable for large scale operations (Patinvoh, 2019).

Mesophilic AD: Most of the AD systems used for the stabilization of solids are configured as conventional mesophilic anaerobic digesters. Fermentation temperature range for these digesters is approximately 32–38 °C (Dentel, 2014). To achieve uniform conditions, heating and mixing are employed in the system. Mesophilic anaerobic digesters are suitable for medium and large-scale biogas plants by providing high and stable biogas production rate with lower heating energy requirement compared to thermophilic AD (X. Liu et al., 2011).

Thermophilic AD: Thermophilic AD is the process which operated at 50-60 °C temperature range. The main purpose of this system is to increase biological reaction rates and VS reduction while improving pathogen removal. Thermophilic reactors have some advantages like increased solids reduction, improved dewatering, increased biogas production rate, and smaller digester volume. Besides that, main disadvantages of this reactors compared to mesophilic process include higher energy requirement for heating, extreme sensitivity to temperature changes, and higher odor potential (Dentel, 2014).

Plug flow digestion: No radial mixture is used in the plug-flow digestion and there is a high TS concentration in the feed. It is adopted by mostly biogas plants for processing poultry and livestock manure (X. Liu et al., 2011). Schematic representation of plug flow digestion is shown in Figure 2.4.

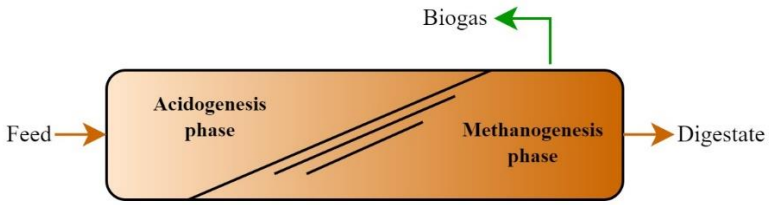

Figure 2.4 Schematic representation of plug flow digestion (Ramatsa et al., 2015)

Complete mixing digestion: In complete mixed digestion, mixing devices are used to ensure homogeneity in the reactor. This provides high biogas production rate and conversion efficiency. It is used by most medium and large biogas plants, as well as for sludge treatment in wastewater treatment plants (X. Liu et al., 2011). Schematic representation of complete- mixing reactor is shown in Figure 2.5.

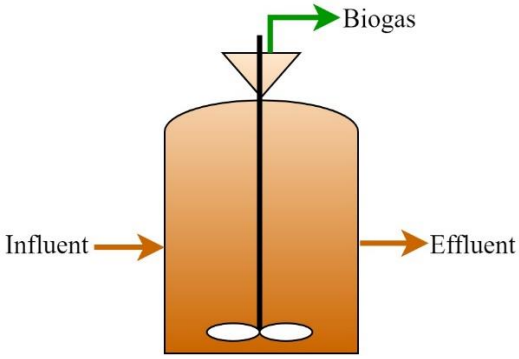

Figure 2.5 Schematic representation of complete- mixing reactor (Hamilton, 2017)

Standard rate digesters: Standard rate digestion process is the simplest AD process types in which digester contents are usually neither heated nor mixing. The digestion period varies from 30 to 60 days (Appels et al., 2008). Schematic representation of standard-rate digester is shown in Figure 2.6.

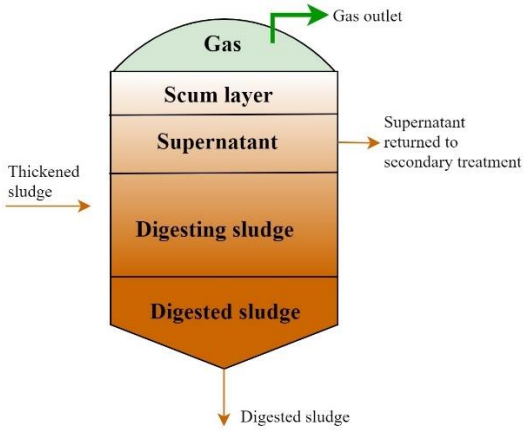

Figure 2.6 Schematic representation of standard-rate digester (Appels et al., 2008)

High rate digester: The high rate digestion process is a significantly enhanced version of standard rate digestion. In the digester, heating and completely mixing are provided. In addition, the raw sludge is thickened in the system. All these elements create a uniform environment in which process stability and efficiency are improved (Appels et al., 2008). Schematic representation of high-rate digester is given below Figure 2.7.

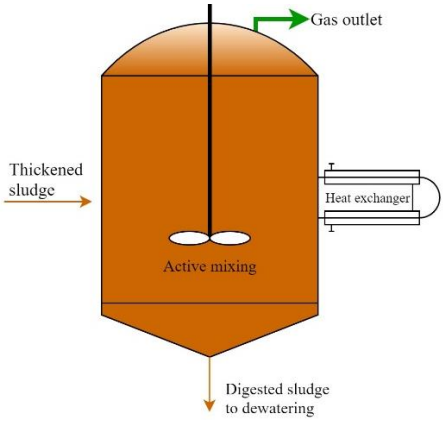

Figure 2.7 Schematic representation of high-rate digester (Appels et al., 2008)

Single stage anaerobic digesters: In AD systems, single-stage anaerobic digesters (SADs) are the simple process where hydrolysis, acidogenesis, acetogenesis and methanogenesis occur in same reactor (Van et al., 2020). SAD can be operated as

standard- and high-rate digesters and in terms of temperature, operation can be mesophilic or thermophilic. SADs are operated in the pH range of 6.5-7.5 in order to obtain maximum biogas yield (Kothari et al., 2014). Schematic representation of high-rate SAD is shown in Figure 2.8.

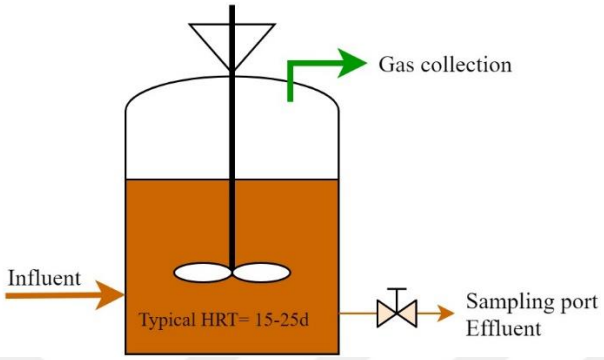

Figure 2.8 Schematic representation of SAD (Rabii et al., 2019)

These systems have a wide application range as they have ability to remove many types of biodegradable waste. SAD is broadly used due to recirculation adaptability, a simple design, less technical breakdowns, and lower cost (Pramanik et al., 2019).

In SAD systems, however, different types of microorganisms are kept together, and they show serious differences in terms of growth kinetics and sensitivity to environmental conditions (Pham Van et al., 2020). Strong group bacteria (acid-generating microorganisms) can easily suppress weak groups (methanogens) while living in the same reactor. Therefore, fluctuations in the organic load, pH and solids concentration of the feedstock can impair the stability of the system and this leads higher acid formation rate than methane formation. In order to solve these problems, two-stage anaerobic reactors have been developed (Kothari et al., 2014; Van et al., 2020).

Two stage anaerobic digesters: In two-stage anaerobic digesters (TSADs), acid-producing and methane-producing microorganisms are physically separated from each other. TSADs have been preferred recently to overcome the limitations of traditional SADs (De Gioannis et al., 2017).

The basic principle of TSAD is to separate the microbiological processes of AD into hydrolysis/acidogenesis and acetogenesis/methanogenesis. Acidogens and methanogens involved in these processes are two dominant groups of syntrophic microorganisms and their microbial growth characteristics differ (Van et al., 2020). The optimum pH range for facultative anaerobes perform the hydrolysis/acidogenesis stage is in the range of 5.5-6.5, whereas acetogens and methanogens are obligatory anaerobes, and their optimum pH is between 7-8. Besides optimum conditions, methanogens are sensitive to VFAs produced in the acidogenesis step. For these reasons, it is difficult to maintain the balance of microbial growth between these steps. Therefore, the idea of TSAD aims to provide the specific optimal environmental and operational conditions required for these organisms (Pham Van et al., 2020).

In TSAD processes, two bioreactors are operated in series and the operational characteristics of each stage are adjusted to support bacteria. In the system, initial reactor is operated with shorter HRT with 5-6 pH range to enhance the acidogenic bacteria. On the other hand, the second reactor is operated with longer HRT, generally over 15 days with 6-8 pH range for the methane producing microorganisms which have a slow growth rate (Voelklein et al., 2016).

As mentioned in the process parameters section, pH is one of the most important process factors in AD processes and needs to be kept in the optimum range (7-8) for biogas production. It was observed that decreasing pH level below 6.5 in reactors inhibits methanogenic activity (B. Zhang et al., 2005). In SAD process, due to VFA accumulation, the pH values drop to levels below 6.5 which are not suitable for methanogenic activity. This means that external pH control is needed in the system. To solve this problem, by using a TSADs, biogas production rate and methane efficiency can be increased without external pH control. In two-stage processes, the feed from initial reactor neutralizes second reactor, and it created the optimum condition for methanogens (Koç, 2015). Schematic representation of TSAD is shown in Figure 2.9.

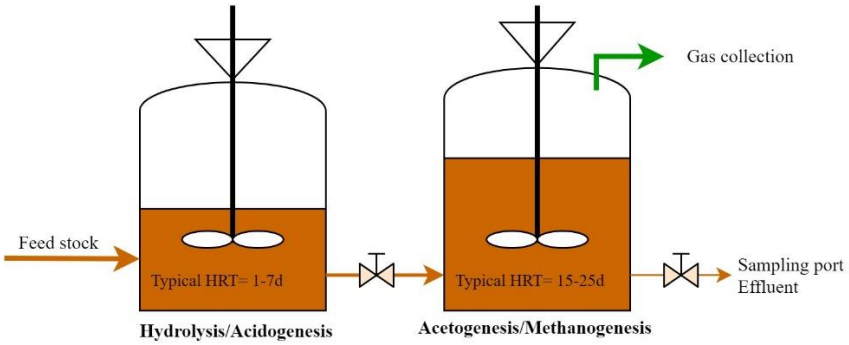

Figure 2.9 Schematic representation of TSAD (Rabii et al., 2019)

The TSAD process is firstly proposed by Ghosh and Poland. They used two sequential reactors and obtained 17% higher methane production rate (Pohland & Ghosh, 1971). Following the results of Pohland and Ghosh, a lot of study has been conducted in the literature to improve biogas and methane production via TSAD (Demirel & Yenigu, 2002; Budiastuti et al., 2018). The results of some studies comparing SAD to TSAD are given in Table 2.3. As seen in the table, methane production and methane content of biogas increase in TSADs.

Table 2.3 Comparison of SAD and TSAD in terms of methane yield and methane content in biogas

References	Results
Pham Van et al., 2020	In the study conducted, it was observed that the SAD system converts only 17.8-22.3% of the initial carbon into biogas with a low methane content (44.1-48.7%), whereas the TSAD system converts 41.67% of the starting carbon into biogas with high methane content (71.68-81.0%).
Baldi et al., 2019	Total biogas production increased by 36% in TSAD compared to SAD and methane content increased from 61.2% to 70.1%.
De Gioannis et al., 2017	As a result of the study, the TSAD system exhibited 20% higher energy recovery efficiency than the SAD system. VS removal also increased by 25% in two stage system.
Voelklein et al., 2016	The two-stage system produced 23% more methane than the one-stage system, the methane content of the produced biogas increased by 14% to 71% in the two-stage system.
Bertin et al., 2013	In the study, the two-stage system reached a methane value more than twice the value obtained with the one-stage process (258 mL CH4g.vs-1) and methane content increased from 48% to 63%.
Nasr et al., 2012	Methane production is increased from 294 (mL/g COD added) to 372 (mL/g COD added) by TSAD

2.3 Pretreatment methods for AD

The AD process is a well-established technology; however, the efficiency of the AD process depends on factors such as longer HRT, low degradation efficiency of organic solids, presence of extracellular polymeric substances and various inhibitory compounds (Montgomery & Bochmann, 2014; Anjum et al., 2016). All these limiting factors are related to the first stage of AD, hydrolysis (Anjum et al., 2016). In order to overcome these limitations and enhance the digestion rate, many pretreatment methods have been studied in the literature (Appels et al., 2008; Amin et al., 2017). Pretreatment methods can be classified as physical, chemical, biological, and combined pretreatments, respectively (X. Wang et al., 2020; Zhou et al., 2017; Ferdes et al., 2020; H. Yi et al., 2013). Pretreatment methods to improve the AD process are shown in the Figure 2.10.

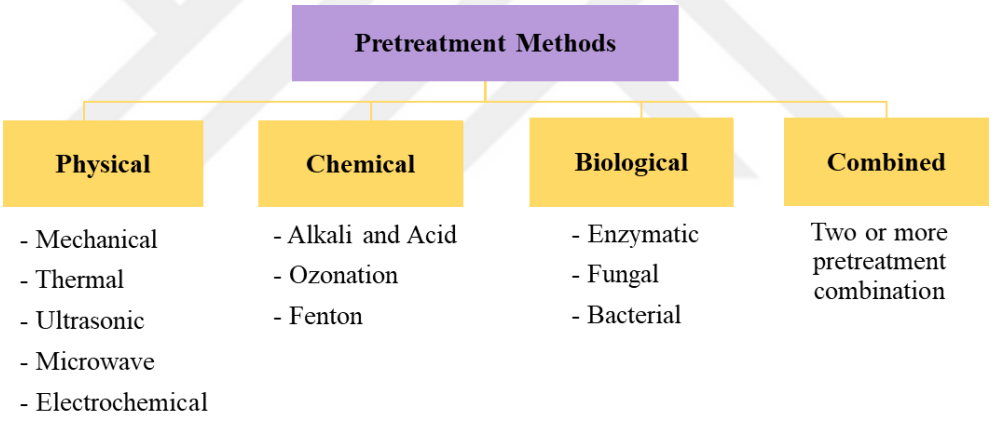

Figure 2.10 Pretreatment methods to improve the AD process (Atelge et al., 2020)

2.3.1 Physical pretreatment

The basic process in physical pretreatment is to change the structure of biomass and reduce the size of the particles by applying physical forces. This application leads to an increase in the surface area of the particles, as a result polymer in organic materials become susceptible to microbial and enzymatic attacks, which enhances methane production (Zheng et al., 2014). Although physical pretreatment also has a

positive effect on biogas production, the main disadvantage of this method is its high energy consumption. The physical pretreatment can be classified as mechanical, thermal, ultrasonic, microwave irritation, and electrochemical pretreatment (Neumann et al., 2016).

Mechanical pretreatment: Mechanical pretreatment, also known as milling, is usually applied especially for lignocellulose and algae biomass for substrate size reduction (Motte et al., 2015). It also increases the surface area, bulk density, and porosity of the substrate. Mechanical pretreatment has a positive effect on biogas production by improving the interaction between substrate and microorganisms (Montgomery & Bochmann, 2014).

Thermal pretreatment: Thermal pretreatment, also known as liquid hot water pretreatment, is a well-known method and depends on temperature, pressure, and time. In this pretreatment, heat is applied to system to provide more soluble substrate (Sayara & Antoni, 2019). This process aims to increase biodegradability of the substrate to make it more accessible to bacteria. The heat applied for maximum biogas production depends on the type of substrate and generally ranges between 150 °C – 180 °C for different sludge characteristics (Hendriks & Zeeman, 2009).

Ultrasonic pretreatment: Ultrasonic pretreatment is seen as an environmentally friendly process to increase the biodegradability of organic matter in AD (Zeynali et al., 2017). This method is generally used for sewage sludge treatment in WWTPs. In the study conducted by Zeynali et al. (2017), ultrasonic pretreatment was applied to AD process and results showed that biogas and methane production increased significantly. However, energy consumed during this pretreatment method is a challenge, especially for biogas plant where energy generation is the main objective (Zeynali et al., 2017).

Microwave pretreatment: Microwave pretreatment, one of the thermal pretreatment methods, aims to accelerate biological, chemical, and physical

processes in AD process by using electromagnetic radiation. Studies in the literature show that microwave pretreatment increases methane yield (Amin et al., 2017).

Electrochemical pretreatment: Electrochemical pretreatment is widely used in AD process. It is considered as an effective method for improving sludge biodegradability (Ye et al., 2016). In this pretreatment, high voltage is applied to sludge to dissolve organics by breaking the bonds between polymers. At the end, sludge cells become degraded and proteins and polysaccharides in the sludge become soluble available for the microorganisms (Panigrahi & Dubey, 2019).

2.3.2 Chemical pretreatment

In chemical pretreatment, powerful reagents are used to hydrolyze the cell wall and membrane and increase solubility of organics (Appels et al., 2008). Chemical pretreatment is used more frequently than either biological or physical pretreatment. Since it increases biological degradation of complex materials, and give more effective results in terms of biogas production and methane yield (Amin et al., 2017). Besides, chemical pretreatment is also effective in reducing the HRT of anaerobic reactors. Various chemical applications have been developed considering different operating principles. Chemical pretreatment methods can be classified as alkali and acidic pretreatment, ozonation pretreatment, and Fenton pretreatment (Tyagi & Lo, 2011; Zhen et al., 2017) .

Acid pretreatment: Acid pretreatment is an effective method in breaking down the van der Waals forces, hydrogen, and covalent bonds that hold the biomass components together (Amin et al., 2017). This causes sludge disintegration and cell lysis in anaerobic processes and makes the sludge more bioavailable to increase the rate and efficiency of the digestive process (Karuppiah & Ebenezer Azariah, 2019). However, acids such as CH_3COOH, HCl and H_2SO_4 used in chemical pretreatments are dangerous, corrosive, and toxic. Also, acid recovery after hydrolysis requires a secondary treatment process, which increase the cost of treatment. In the case of

H_2SO_4 or HNO_3 used as chemical agents, H_2S and N_2 formation is observed in the reactors and this causes a decrease in methane production (Amin et al., 2017).

Alkali pretreatment: Alkaline pretreatment is carried out by adding bases to system. This causes an increase in the inner surface area, a decrease in polymerization, and the destruction of bonds between polymers. The feedstock becomes easily available for hydrolytic enzymes (Patinvoh et al., 2017). For alkali pretreatment, solution which consists of different portion of CaO, NaOH, and KOH is prepared with pH ranges 10–13. Due to this high pH values, after the hydrolysis stage pH adjustment is required for the coming AD process. This situation causes an increase in operating cost and also environmental concerns (Atelge et al., 2020).

Ozone pretreatment: Ozone is a powerful oxidant widely used in the water disinfection and pathogens destruction. By reacting with polysaccharides, proteins, lipids and other recalcitrant compounds, ozone converts them into biodegradable molecules (Neumann et al., 2016). Thus, it leads to improvement in the biogas and methane formation during AD process (Ariunbaatar et al., 2014). Ozonation does not require additional chemicals other than ozone and with this feature it differs from other chemical pretreatments methods (Tyagi & Lo, 2011). Besides increasing methane formation and destroying pathogens, ozone pretreatment does not cause odor formation in AD system (Atelge et al., 2020).

Fenton pretreatment: Fenton pretreatment is a combination of H_2O_2 and ferrous iron addition. Ferrous iron reacts with H_2O_2 to form highly reactive hydroxyl radicals. These radicals destroy organic material during reactions without any toxic or inhibitory byproducts. The Fenton pretreatment is often applied for sludge treatment and increases the solubility and biodegradability of the sludge to increase biogas production (Erden & Filibeli, 2010).

2.3.3 Biological pretreatment

Biological pretreatment is considered as an environmentally friendly approach, in which organisms are directly or indirectly involved. The main purpose of using this method is to degrade the insoluble materials in the substrates (Karuppiah & Ebenezer Azariah, 2019). For this purpose, enzymes and fungal pretreatments are applied to the systems as a biological pretreatment method (Keskin et al., 2019). Enzyme addition increases solubility of sludge by degrading polymers in the substrate. Depending on the composition of organic substrates, different enzymes such as lipase, cellulase, amylase and protease can be added to AD reactor (Ferdes et al., 2020). Increased cell lysis has many positive effects on sludge treatment such as improving filterability and increasing methane production (Atay & Akbal, 2016).

2.3.4 Combined pretreatment

The application of combined pretreatment has increased in recent years. With this application, it is aimed to overcome the limitations of the single methods stated in Table 2.5 (Neumann et al., 2016). Combined pretreatment methods appear to be an attractive for sludge treatment due to higher efficiency in methane and biogas production, and the most common combinations are physical/chemical and thermo/chemical pretreatments (Jaai Kim et al., 2013; Tian et al., 2015).

As described above, each pretreatment application used in AD, have positive effects on increasing process rates, biodegradability of substrates, pathogen removal and high biogas and methane yield. Besides these advantages, each method has some drawbacks. Table 2.5 shows the advantages and disadvantages of pretreatment methods mentioned.

Table 2.4 Advantages and disadvantages of pretreatment methods (Atelge et al., 2020)

Pretreatment method		Advantages	Disadvantages
Physical pretreatment	Thermal pretreatment	- Increase enzyme accessibility - High AD process rate - Reduce lignocellulose - Reduce dewatering time	- High energy demand - Increased temperature
	Ultrasonic pretreatment	- Increase components solubility - Increase process rate - Reduce pathogens	- Require High-tech system and regular maintenance - High energy demand - Limitation of operating conditions
	Microwave pretreatment	- Size reduction - Pathogen reduction - Reduce dewatering time	- High energy demand - Narrow influence area (scalability)
Chemical pretreatment	Acid & Alkali pretreatment	- Low capital cost - Reduction of lignin and hemicellulose - Improved hydrolysis phase - Short retention time	- High chemical content in reactor - Inhibitor effects on AD - High cost of chemicals - Corrosion of equipment
	Ozonation	- No addition of chemicals - Decrease pathogens - Short retention time - Reduces lignin content - No toxic compound formation is observed	- High capital cost - Large O_3 amount
	Fenton pretreatment	- Hemicellulose degradation - Improve removal rate of VS, TS, and COD	- High cost of chemicals
Biological pretreatment		- Low energy - No additional chemicals - Environmentally friendly	- Slow process - High enzyme price - Enzyme addition - Sterile conditions need - Further development required
Combined pretreatment		- High biodegradability - Increase solubility - HRT reduction	- High capital cost - Generation of non-biodegradable components - High energy demand

Pre-treatment methods include thermal, chemical, mechanical, and biological methods have been studied in the literature to assets biogas and methane production. In previous studies, alkaline pretreatment is evaluated as prominent application

among other pretreatment methods, whereas, in recent years ozonation has gained great attention in the scientific research as it provides higher biodegradability of and toxic compound removal (Torres & Lloréns, 2008).

2.3.5 Ozone pretreatment

As mentioned in the previous section, many pretreatment methods are used to improve the biogas and methane production in AD process. Ozonation is one of the most widely used chemical pretreatment methods. Ozone is widely applied in water and wastewater treatment with the aim of disinfection and increasing the biodegradability of organic matter (Chiavola et al., 2019). During the oxidation process since no oxidant residues remain and no additional chemical is required, ozonation gains attention among other pretreatment methods (Atelge et al., 2020).

As a powerful oxidant, ozonation is one of the processes used in anaerobic processes to enhance the solubility of the sludge (Bougrier et al., 2007). By combining AD and ozonation, organic solids in sewage sludge become more easily degradable and more accessible for the microorganisms. In this way, the hydrolysis phase is accelerated, and methane production is improved (Bougrier et al., 2007). In this pretreatment method, the most important factor is applied ozone dose (Silvestre et al., 2015). There is a correct relationship between applied ozone dose and sludge solubility. High dose ozone application causes a decrease in methane yield due to oxidative effect of ozone. Many studies have been conducted in the literature by applying various ozone doses (Bougrier et al., 2007).

As a result of the study conducted by Weemaes et al. (2000), 100% increase in methane production was observed at the 0.083 g O_3/g TSS dose applied to anaerobic batch digester. In another study, 4 different ozone doses 0.043 g O_3/g TSS, 0.06 g O_3/g TSS, 0.08 g O_3/g TSS, and 0.1 g O_3/g TSS were applied to anaerobic digesters (Silvestre et al., 2015). In this study, the optimum ozone dose was determined as 0.06 g O_3/g TSS in which two-fold higher methane production was achieved

compared to untreated one. On the other hand, Chacana et al. (2017) found that 0.08 g O_3/g TSS ozone dose increased methane production by 52%. Chiavola et al. (2019) investigated the effects of ozonation on anaerobic digestion. For this purpose, ozone doses of 3.5 to 53.6 mg O_3/g TS were applied to the activated sludge alone and mixture with primary sludge. 30% more methane was achieved at the lowest ozone dosage of 3.5 mg O_3/g TS applied to activated sludge. In another study, Yeom et al. (2002) achieved a 110% increase in methane production in the anaerobic process using an ozone dose of 0.1 g O_3/g TSS. In the study conducted by Bougrier et al. (2007), 145% increase in methane production was achieved by applying 0.15 g O_3/g TS ozone dose. In these studies, ozone pretreatment is applied to SADs and optimum ozone doses were determined for these digesters. In the study completed by Yue et al. (2020), TSADs pretreated with ozone dose of 0.02 g O_3 /g TVS were operated to treat food waste led to the highest methane yield. However, excessive amount of ozone doses decreased methane production.

As can be seen, various ozone doses have been studied in the literature. The joint result of these studies is that the success of ozonation in increasing biogas production requires optimum dose determination to avoid exceeding ozone dose to worsen anaerobic biodegradability (Silvestre et al., 2015).

2.4 Microbiology of AD

AD process has been successfully utilized in the treatment of solid waste, urban and industrial wastewater and in energy production for many years (Cabezas et al., 2015). Recently, one of the most researched areas related to this technology is the microbiology of AD process. Studies are carried out to understand microbial community structure using culture-dependent and culture-independent molecular approaches. The biology and biochemistry of AD has been extensively studied to avoid process errors and optimize methane production (Achinas et al., 2020).

The AD process is the complete decomposition of organic compounds into CH_4 and CO_2 by complex microorganisms belonging to *Bacteria* and *Archaea* domains. As shown in Figure 2.3, methane formation is a complex process. This formation takes place in 4 steps and they are hydrolysis, acidogenesis (fermentation), acetogenesis, methanogenesis (Cabezas et al., 2015). Each degradation steps are proceeded by different consortia of microorganisms. These microorganisms are partially syntrophic interrelated and have different environmental requirements (Weiland, 2010). Complex microbial communities in AD systems consist of hydrolytic, acidogenic and acetogenic bacteria and archaea (methanogens). Centrophic relationships between microorganisms significantly affect metabolism in AD (Achinas et al., 2020).

AD process starts with the action of hydrolytic bacteria and these organisms are facultative. They catalyze the hydrolysis reaction with the help of extracellular enzymes. In hydrolysis step, proteins and polysaccharides are converted into monomeric sugars, fatty acids, amino acids and alcohols (Achinas et al., 2020). This step is carried out by hydrolytic microorganisms such as *Clostridium, Bacillus, Bacteroides, Hydrogenoanaerobacterium,* and *Eubacterium* (Saha et al., 2020).

Hydrolysis stage is followed by acidogenesis. At this stage, acidogenic bacteria form fermentation products such as VFAs, alcohols, CO_2 and H_2 by using the end products of hydrolysis stage. Acidogenic bacteria consist of facultative and obligatory microorganisms. The former can survive in both aerobic and anaerobic conditions, whereas the latter is strictly anaerobic (L. N. Nguyen et al., 2019). Acidogenic bacteria in AD can be classified as *Clostridium, Flavobacterium, Peptococcus, Eubacterium, Bifidobacterium and Pseudomonas* (L. N. Nguyen et al., 2019; Saha et al., 2020).

In the third stage of methane formation, acetogenic bacteria convert VFAs and alcohols into acetate, H_2, and CO_2, which are used by methanogens in following stage. Acetogenesis is an intermediate stage in methane production and includes bacterial genus such as *Syntrophomonas* and *Syntrophobacter* (Shah et al., 2014).

The most acetogenic bacteria at this stage are as follows *Clostridium*, *Syntrophobacter*, *Syntrophomonas*, *Pelotomaculum* and *Smithllela propionica* (Amani et al., 2010).

The last stage of the AD process is called methanogenesis. At this stage, methane is produced by methanogenic microorganisms. Methanogens are strictly anaerobic *Archaea*, and they have slow growth rates and are sensitive to environmental changes (pH, temperature and ammonia). Numerous studies show that the most common methanogenic species found in anaerobic digesters are *Methanobacterium*, *Methanothermobacter*, *Methanobrevibacter*, *Methanosarcina*, and *Methanosaeta* (Achinas et al., 2020).

The stages of methane formation and the microorganisms involved in these stages are shown in Figure 2.11.

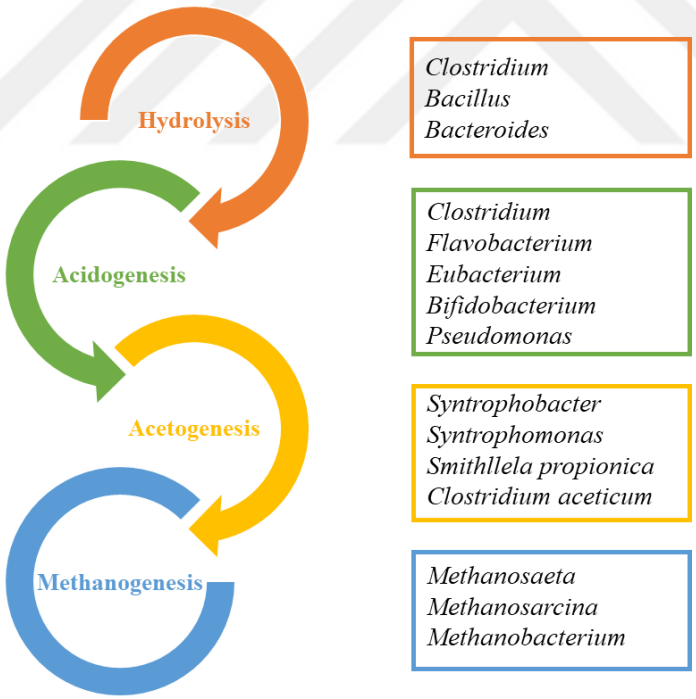

Figure 2.11 Simplified schematic representation of AD process and microorganisms adapted from Nielsen et al., 2020

Apart from mentioned microorganisms, AD system also includes different types of microorganisms such as sulfate reducers and denitrifies and these microorganisms compete with those involved in methane production and adversely affect methane production (Scholten et al., 2002).

Sulfate is one of the most common components found in most wastewater. During AD process, sulfate reducers use sulfate as an electron acceptor and produce hydrogen sulfide (H_2S). In the AD process, there is competition for acetate and hydrogen between sulfate reducers and methane producers (Z. hua Liu et al., 2018). Sulfate reducers reduce sulfate to H_2S using hydrogen, whereas methane-forming bacteria use hydrogen to produce methane. In case of sulfate presence in AD, methane-producing bacteria use less substrate (Gerardi et al., 2016). In this way, sulfate reducing bacteria inhibit methanogens and cause negative effect on methane formation. It has been reported that the sulfate-reducing bacteria species in Mesophilic anaerobic digesters are *Desulfovibrio*, *Desulfobacter*, *Desulfobacterium*, *Desulfobulbus*, *Desulfomicrobium*, *Desulfomonas*, *Desulfococcus*, *Desulfosarcina*, and *Desulfomonas* (Z. hua Liu et al., 2018).

Besides sulphate, nitrate is known to suppress the methane formation (Scholten et al., 2002). In denitrification process, denitrifiers reduce nitrate to nitrous oxide and molecular nitrogen. Denitrifiers use acetate and hydrogen to complete nitrate reduction by competing with methanogens (Carlini et al., 2020). In addition to acetate competition, production of toxic intermediates (NO_2, NO, N_2O) of denitrification cause inhibition of methanogenesis. The three genera that include the most denitrifying bacterial species are *Alcaligenes*, *Bacillus* and *Pseudomonas* (Gerardi et al., 2016).

2.4.1 Microbial analyses

As mentioned in the previous sections, AD is a complex bioprocess in which microorganisms belonging to different functional groups are involved. It is believed

that a better understanding of the underlying microbiology facilitates the optimization of biological processes and consequently improve methane production efficiency by improving treatment in the AD process (Kirkegaard et al., 2017). However, our understanding of the function and metabolic capabilities of microbial communities in AD is limited (Zamanzadeh et al., 2016). For this purpose, culture-dependent and culture-independent methods have been used to define the microbial community of AD for many years. These methods are illustrated in Figure 2.12.

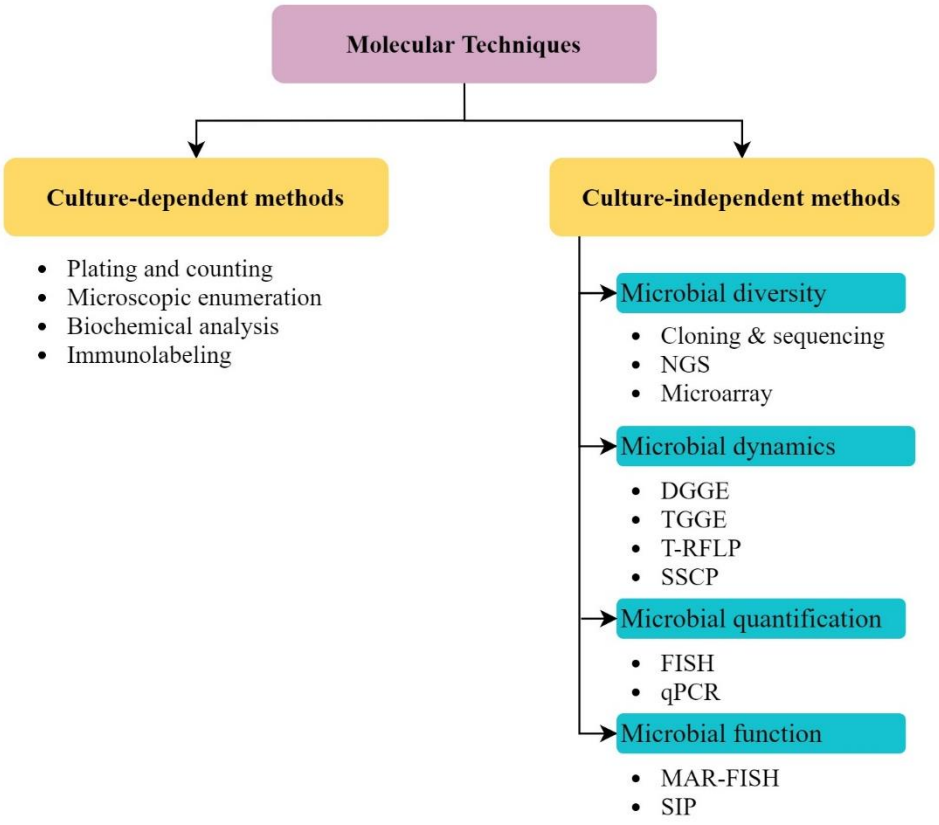

Figure 2.12 Microbial analyses used for microbial communities adapted from Cabezas et al., 2015

NGS, next generation sequencing; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature 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; MAR-FISH, micro-autoradiography FISH; SIP, stable isotope probing.

As seen in Figure 2.12, traditional culture-depended molecular techniques are also used in microbial analysis of anaerobic systems. However, these techniques cannot

provide detailed information about the structure, function, dynamics, and diversity of microbial communities. Culture-independent molecular techniques can measure cell DNA and RNA and provide more robust information (Khanal, 2009). By using these molecular techniques, genes, proteins (enzymes) and metabolic products associated with biogas production can be determined without the difficulties in culturing. Techniques used to understand the diversity, dynamics, quantities, and functions of microbial communities are shown in Figure 2.12. Advantages and disadvantages of each method is summarized in the Table 2.5.

Table 2.5 Advantages and disadvantages of molecular techniques (Khanal, 2009; Rincon-Florez et al., 2013)

Methods	Advantages	Disadvantages
Cloning & sequencing	- Provides more accurate identity - Contains larger sequences	- Large number of clones must be sequenced - Bias from PCR - Laborious and time consuming
NGS	- Rapid method - Information about functions of microbial communities	- Expensive method - Require replication and statistical analysis - Challenging data analysis
Microarray	- Analysis of multiple genes - Diversity/pattern analysis - Identification at group/ species level - High sensitivity - Rapid method - Not laborious	- Expensive method - Long time required
DGGE & TGGE	- Pattern and diversity analysis - Does not require further sequencing - Bands can be cloned and sequenced for identification	- Multiple bands for a single species - Difficult to reproduce - PCR bias problems - Further sequencing requirement
T-RFLP	- Analyses of a wide microbes - Suitable for pattern analysis - One band per species - Highly reproducible - Provide data storage and comparison between different samples	- Restriction enzyme selection problem - PCR bias problems - Some species show the same length of fragments

Table 2.5 cont'd

Methods	Advantages	Disadvantages
SSCP	- Identify community members - Identify new mutations	- Short fragments - Lack of reproducibility
FISH	- Direct analysis - Quantification - No heterogeneity problem - Suitable for targeting specific group/species	- Requires genes/RNA with high number of copies - Limit for total diversity mapping - Highly dependent on specificity of probe
qPCR	- Quantification - Rapid, accurate and highly sensitive method - Relatively cheap and easy	- Used only for targeting of known sequences - DNA impurities can create false-positive results
SIP	- High sensitivity - Provides information on the active microorganisms	- Incubation and cycling of the stable isotope may cause bias in the microbial community analysis.

2.4.1.1 Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization (FISH) is an important method that has been used for more than 30 years to detect microorganisms in environmental samples (Lukumbuzya et al., 2019). FISH is recognized as one of the most powerful techniques in modern microbial ecology, since it has a clear advantage of direct cell counting in their natural environment without cultivation (Kumar et al., 2011). Besides quantification, FISH technique allows identification of target organisms at genus or species level. Identification by this method is based on hybridization of probes with specific target genes. Probes are single-stranded DNA or RNA which are short usually consist of 18 to 25 base pairs, fluorescently labeled and complementary to the target gene (Zamanzadeh et al., 2016). Numerous types of probes are stored in the database and new probes can be designed for better analysis of the composition and dynamics of microbial communities (Kumar et al., 2011).

FISH method is successfully applied in many areas for many years. The FISH protocol differs depending on the type of sample, the target sequences and the probe

used. However, all tests usually follow a few common steps, and these are (Rodriguez-Mateos et al., 2020):

- Sampling
- Cell preparation: Cells are immobilized on a glass slide.
- Enzymatic pretreatments: Enzymes are used to increase cell wall permeability and thus facilitate probe penetration.
- Fixation and dehydration: Paraformaldehyde and / or ethanol treatments are performed in a series. The aim is to avoid any metabolic activity and to protect cellular structure.
- Hybridization: In this step hybridization occurs between target gene and fluorescent probe.
- Washing: After hybridization step, unbound fluorescent probes should be thoroughly washed to avoid false positive results.
- Visualization: Cells are visualized by fluorescence microscope.

AD has been comprehensively described in terms of the process, but information on microbial communities is still insufficient (Khan et al., 2018). Different approaches are carried out in this direction. One of these approaches is use of FISH. The FISH method is frequently used in studies because it allows the localization and identification of certain groups of microorganisms. In addition, it allows to examine the structure of the syntrophic and synergistic consortium in AD (Kumar et al., 2011). In this context, FISH method was firstly used by Raskin et al., 1994 to identify methanogenic microorganisms in AD process. For this purpose, they designed probes for methanogens and as a result of the study they found that *Methanosarcina* and *Methanosaeta* species were abundant in the AD (Raskin et al., 1994). In the study carried out by Stabnikova et al., 2006, FISH method was used to monitor methanogenic *Archaea* during AD process and 16S rRNA targeted oligonucleotide probe (Arc915) was chosen as probe. The same probe was used in another study conducted by Dinova et al., 2018 for the determination and localization of methanogenic *Archaea* in anaerobic digesters employed in a WWTP. Crocetti et al.,

2006 phylogenetically analyzed 3000 *Euryarchaeota* 16S rRNA gene sequences and evaluated previously published oligonucleotides. The updated probe set has been optimized in the study for use in FISH to get more accurate detection of methanogenic *Archaea* (Crocetti et al., 2006). In the research by Khan et al. (2018), the FISH technique was used to detect and quantify methanogenic *Archaea* in sludge samples. As a result of this study, it was found that acetoclastic organisms (*Methanosarcinaceae* & *Methanosaetaceae*) were more common than hydrogenotrophic organisms (*Methanobacteriales, Methanomicrobiales, Methanobacteriaceae* and *Methanococcales*). Apart from these studies, Santegoeds et al. (1999) used the FISH method to identify sulfate reducers along with methanogens in AD. Reyes et al. (2015) studied microbial populations such as sulfate reducers, denitrifiers and methanogens for identification and quantification by FISH method. In the study 34 probes including *Methanosarcinales, Methanomicrobiales, Desulfobulbus, Desulfobacteraceae,* and *Nitrobacter* spp. were used (Reyes et al., 2015). In these studies, microbial analyzes were carried out for SADs. Microbial analyzes for TSADs have been performed in only a few studies in the literature. Cirne et al. (2007) analyzed microbial community in TSAD of energy crops. In the study, FISH technique was performed with probes available and considered to be relevant in the context of AD: EUBMIX (for all *Bacteria*) ARC915 (for *Archaea*); HGC69a (for *Actinobacteria*); CF319a (for *Flavobacterium*) (Cirne et al., 2007). In the research conducted by Giuliano et al. (2014), microbial analysis was performed in long-term pilot scale two-stage anaerobic digesters by using FISH method. Microbial analysis showed that when significant methane production was detected in digesters, *Methanobacteriales* and *Methanosarcinales* were the dominant species (Giuliano et al., 2014).

As seen from the mentioned studies and results, the FISH method is used for microbial analysis in the AD process. However, a comprehensive research investigating all microbial communities in two-stage anaerobic digesters is a gap in the literature. Similarly, the effect of ozone pretreatment is still unknown on these microbial communities. Therefore, in this study, microbial communities as well as

biogas and methane production in two-stage anaerobic digesters and their reaction to ozone pretreatment were examined by FISH method.

CHAPTER 3

MATERIALS AND METHODS

Within the scope of the study, the effect of ozone pretreatment on TSAD was evaluated in terms of biogas production and microbial population dynamics. Various experiments were planned and applied for this purpose. The main steps of the experiments are summarized in the Figure 3.1.

Figure 3.1 Main experimental steps followed in the study

3.1 Sludge sampling

In this study, lab-scale two-stage digesters were operated with two types of sludge: seed and feed sludge. These sludge samples were collected from Ankara Central WWTP. The WWTP is designed as activated sludge process with 765,000 m^3/day capacity. In the facility, mesophilic anaerobic digesters are operated at 35.5 °C and 14 days SRT and 60000 m^3 of biogas with 60-70% methane is produced by digesters. At about 80-85% of the energy requirement of the facility is met by obtained biogas (ASKİ, 2019). Process configuration and a picture of the Ankara Central WWTP are given in the Figure 3.2.

The seed sludge was taken once from the anaerobic digester unit. The feed sludge was taken from inlet of anaerobic digesters which was thickened raw sludge line weekly. After samples were taken, sludges were brought directly to the laboratory and characterization experiments were carried out. The seed sludge was used directly at setup as inoculum whereas the feed sludge was stored at +4 °C for later use. The

sludge lines from which the seed and feed sludges collected are specified in Figure 3.2.

a)

b)

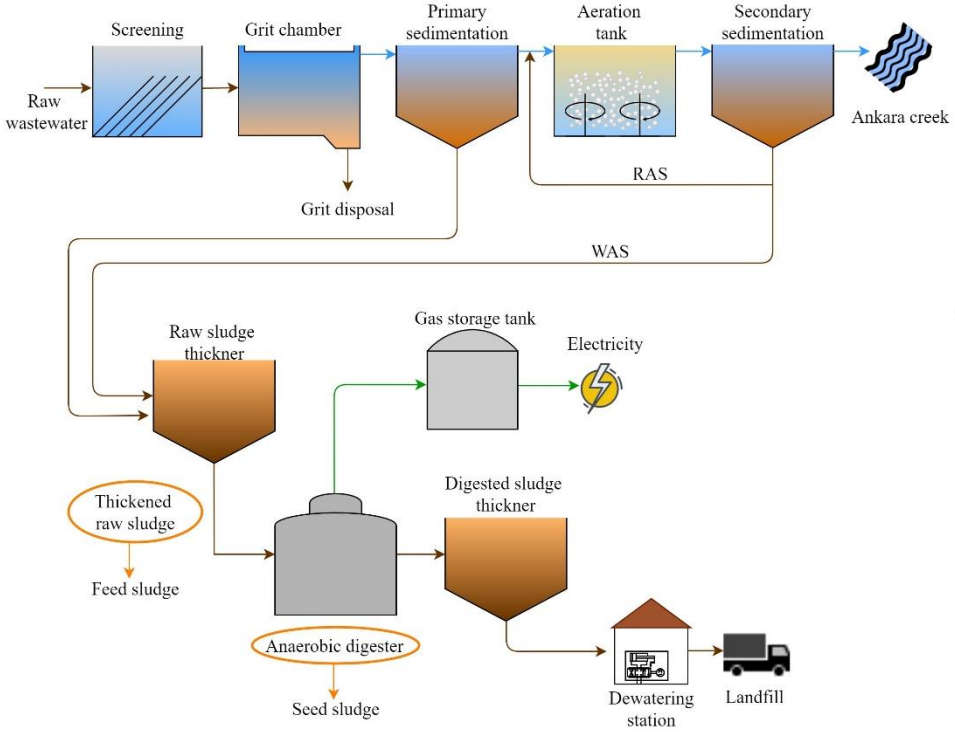

Figure 3.2 Picture (a) and process configuration of Ankara Central WWTP (b) (ASKİ, 2019)

3.2 Start-up and operation of two-stage anaerobic digesters

Within the research framework, two-stage anaerobic digesters were established. For the installation, a lab-scale digester system was ordered from Germany under the

distributorship of Çalışkan Laboratuvar Ürünleri Tic. Ltd. Şti. The AD System was made of Schott brand borosilicate glass digesters and 304 stainless steel pipes and included 5 L digesters, 2 L graduated cylinders, valves, pipes, hose adapters, clamps, and rubber O-rings. In addition to these materials, 2 Masterflex L/S Cartridge Pump System 115/230 VAC (77919-35) pumps were ordered from Sümer Analitik and Medikal Teknolojiler to provide sludge transfer between digesters.

For start-up initially, pumps were placed between digesters to obtain two-stage anaerobic digesters. Magnetic mixers are used to provide homogeneity. System components and graduated cylinders were connected, and the cylinders were filled with saturated salt (NaCl) solution to minimize CO_2 absorption. In order to provide a suitable anaerobic environment for digesters, the system was installed in hot room with 35.5 °C and purged with N_2 gas. Schematic diagram and picture of installed two-stage anerobic digesters given in Figure 3.3 and Figure 3.4, respectively.

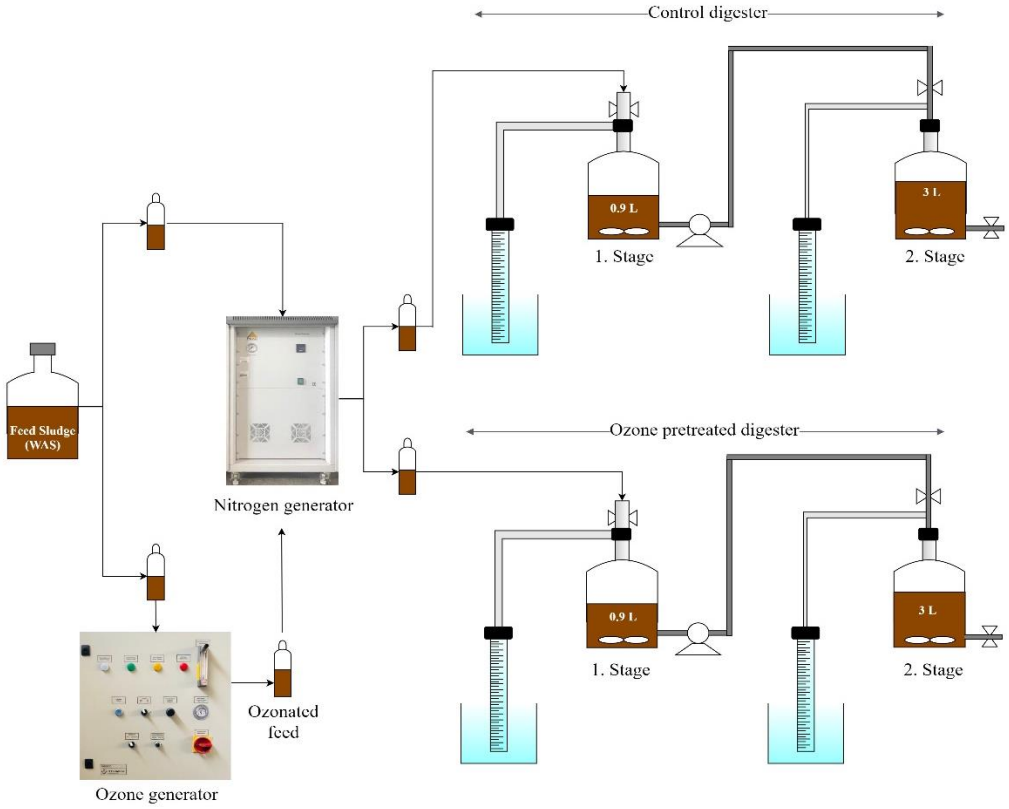

Figure 3.3 Schematic diagram of two-stage anerobic digesters used in the study

Figure 3.4 Picture of installed two-stage anerobic digesters

Two-stage anaerobic digesters were operated under mesophilic conditions. Volume of the first stage was determined as 0.9 L whereas the second stage volume was determined as 3 L. In this context, two-stage digesters were operated as indicated in Table 3.1 with a total volume of 3.9 L.

Table 3.1 Operating conditions of two-stage anaerobic digesters used in the study

	Control digester		Ozone pretreated digester	
Parameters	**1. stage**	**2. stage**	**1. stage**	**2. stage**
Temperature (°C)	35.5	35.5	35.5	35.5
pH	5.5-6.5	7.5-8.5	5.5-6.5	7.5-8.5
Total volume (L)	5.0	5.0	5.0	5.0
Effective volume (L)	0.9	3.0	0.9	3.0
Feeding (mL/d)	150	-	150	-
SRT (d)	6	20	6	20
Ozone dose (g O_3/g TSS)	-	-	0.06	-

At the beginning of the operation, the specified sludge volumes were added to the digesters after purging with N_2. The first stages of the TSAD setups were fed daily with 150 mL of feed sludge purged with N_2 and the same volume of digested sludge was withdrawn from the second stages for characterization and microbial analyses during unsteady-state period. After reaching steady-state, one of the TSAD setup was daily fed with sludge pretreated with 0.06 g O_3/g TSS ozone and then purged with N_2. The volume of sludge added to the first stage of digester was pumped to the second stage daily.

3.3 Ozone pretreatment

In the study, 0.06 g O_3/g TSS ozone dose was used for pretreatment of TSAD. This optimum ozone dose was previously determined for SAD process in our lab. Ozonation applied to two-stage anaerobic digester by employing WEDECO OCS Modular 4 HC ozone generator (WEDECO, Germany) shown in Figure 3.5.

Figure 3.5 Ozone generator used in the study

Before ozonation, the generator was calibrated. Required ozone amount to obtain 0.06 g O_3/g TSS ozone dose was calculated by using Equation 3.1 based on the TSS concentration of the feed sludge which was taken from WWTP weekly. Ozone concentration was calculated by using Equation 3.2 with constant flow rate of 50 L/h. The potentiometer adjustment of the ozone generator was obtained from the performance curve shown in Figure 3.6. Details and results of these calculations are given in Table 3.2.

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

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

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

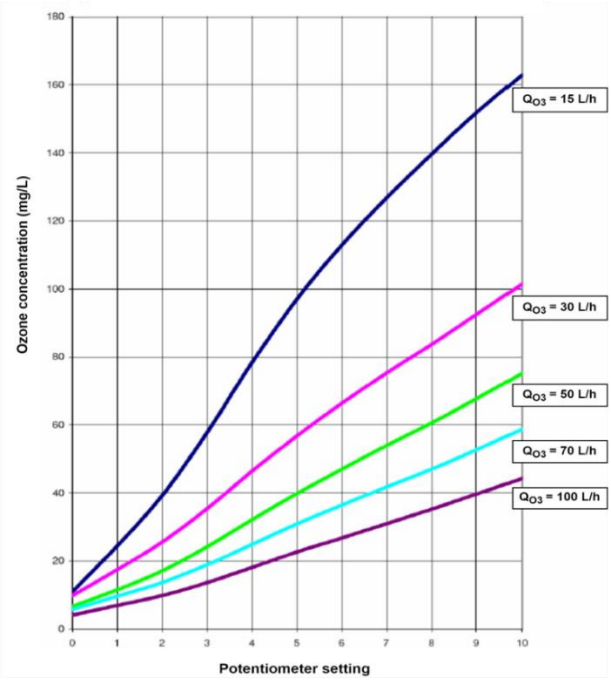

Figure 3.6 Performance curve of the ozone generator used in the study

Table 3.2 Ozone calculations

Week	D_{O3} (g O_3/g TSS)	V_{feed} (mL)	$[TSS]_{feed}$ (g/L)	TSS amount in feed sludge (mg)	m_{O3} (mg)	t (min)	Ozone mass flow (mg/h)	C_{O3} (mg/L)	Q_{O3} (L/h)	Potentiometer setting*
1	0.06	150	20.810	3121.5	187290	2	5618.7	112.37	50	5.6
						5	2247.5	44.95	50	
						10	1123.7	22.475	50	
						15	749.16	14.983	50	
2	0.06	150	27.750	4162.5	249750	2	7492.5	149.85	50	7.9
						5	2997.0	59.94	50	
						10	1498.5	29.97	50	
						15	999.0	19.98	50	
3	0.06	150	22.110	3316.5	198990	2	5969.7	119.39	50	6.2
						5	2387.9	47.758	50	
						10	1193.9	23.879	50	
						15	795.96	15.919	50	
4	0.06	150	16.720	2508.0	150480	2	4514.4	90.288	50	4.4
						5	1805.8	36.115	50	
						10	902.88	18.058	50	
						15	601.92	12.038	50	

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

As seen in Table 3.2, the two-stage anaerobic digester was pretreated with 0.06 g O_3/g TSS ozone dose for 4 weeks. Feed sludges with a volume of 150 ml were subjected to ozone during 5 min at 5.6, 7.9, 6.2 and 4.4 potentiometer settings, respectively. Before feeding the digesters, ozone pretreated, and non-pretreated sludges were purged with N_2.

In this study, N_2 was used to remove O_2 in the seed and feed sludges to assure anaerobic environment. For this, Peak Scientific NM30LA-MS nitrogen generator (Peak Scientific, United Kingdom) was used to provide the necessary nitrogen gas to the sludge (Figure 3.7). In the study, considering the volume of seed sludges, 0.9 L and 3 L, the first and second stages of TSAD were purged with N_2 during 45 and 150 min, respectively. Also feed sludges were purged during 7.5 min daily.

Figure 3.7 Nitrogen generator used in the study

3.4 Analytical analyses

Within the scope of the study, the following parameters were measured for sludge samples; pH, temperature, COD, TN, solids content and VFA. For measurements, 150 mL of sludge samples were taken daily from the second stage of the two-stage digesters. The amount of 500 µL biogas samples were also taken from the system for methane content analysis.

3.4.1 pH and temperature

After calibrating pH meter with pH 4, pH 7 and pH 10 buffer solutions, pH and temperature of sludge samples were measured by using HACH sensION 2 waterproof pH/ISE/mV meter (HACH, USA). Measurements were carried out daily according to Standard Method 4500H (APHA-AWWA-WEF, 2017).

3.4.2 COD

In the study, COD was measured by HACH LCK 514 COD kits in the range of 100-2000 mg/L and for evaluation HACH DR 2800 spectrophotometer was used (HACH, USA). Before analysis, the sludge samples were diluted 1/50 with ultrapure water (ddH2O). COD measurements were performed as duplicate and removal efficiencies were calculated weekly.

3.4.3 TN

TN concentrations was measured by HACH LCK 338 Laton TN kits in the range of 20-100 mg/L and for evaluation HACH DR 2800 spectrophotometer was used (HACH, USA). Before analysis, the sludge samples were diluted 1/50 or 1/100 with ddH2O. TN measurements were performed as duplicate.

3.4.4 Solids content

By using Standard Methods 2540B, 2540D, 2540E and 2540G; TS, VS, TSS and VSS concentrations were determined for sludge samples, respectively (APHA-AWWA-WEF, 2017). In TSS and VSS analyses, the sludge samples were diluted in the ratio of 1/10 to filter easily. All measurements were performed as duplicate and removal efficiencies were calculated weekly.

3.4.5 Total VFA

Total VFA measurements were performed by using the simple titration method suggested by Anderson & Yang, 1992 through the following steps: Firstly, pH of the samples was measured, then 50 mL samples were titrated with 0.1 N sulfuric acid (H_2SO_4) in two steps (first to pH 5.1, then to pH 3.5). Finally, total VFA concentrations were calculated using equations 3.3 and 3.4.

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

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

A1: Molar equivalent of H2SO4 consumed to reach pH 5.1 (first end point)

A2: Molar equivalent of H2SO4 consumed to reach pH 3.5 (second end point)

[HCO3-]: Bicarbonate (HCO3) concentration

[VA]: VFA 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: HCO3 dissociation constant, 6.6×10^{-7}

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

These equations were solved in excel program and VFA concentrations were calculated using the readings obtained from titration experiments.

3.4.6 Biogas and methane production

In the study, water displacement method was used to measure total biogas production in two-stage anaerobic digesters. For this purpose, graduated cylinders were connected to the digesters and filled with saturated salt solution (Figure 3.4). After

that, valves between the digesters and the cylinders were opened and biogas was collected in the inverted graduated cylinders by displacing salt solution. Amount of biogas produced was calculated in mL from level decrease in the liquid (Selvankumar et al., 2017).

In order to determine composition of biogas, gas samples were withdrawn from the digesters by using sterilized 500 µL Hamilton SampleLock syringe (Hamilton Company, USA). Biogas samples were injected into Agilent Technologies 6890N Network gas chromatograph (GC) (Agilent Technologies, USA) which was equipped with a HP-Plot Q capillary column and a thermal conductivity detector using helium (He) and N_2 as carrier gases (Figure 3.8). Methane contents in biogas samples were determined as percentage by the help of GC and these percentages were multiplied with measured biogas volumes to get methane volume. Methane yields and biogas yields were calculated as mL CH_4/g VS and mL Biogas/g VS, respectively. Biogas and methane measurements were performed on daily basis.

Before injections, GC calibration was performed with two standard gas mixtures and pure CH_4. The first mixture consisted of 25% CH_4, 55% CO_2 and 20% N_2 whereas the second one consisted of 65% CH_4, 25% CO_2 and 10% N_2.

Figure 3.8 GC used in the study

3.5 Microbial analyses by FISH method

After the characterization analyses, the remaining sludge samples were fixed and stored for microbial analyses through FISH method. In the study, all experiments were carried out in triplicate. The FISH method was carried out by following the modifications designed by Nielsen et al. (2009) for activated sludge in biological wastewater treatment and the procedure was summarized in Figure 3.9.

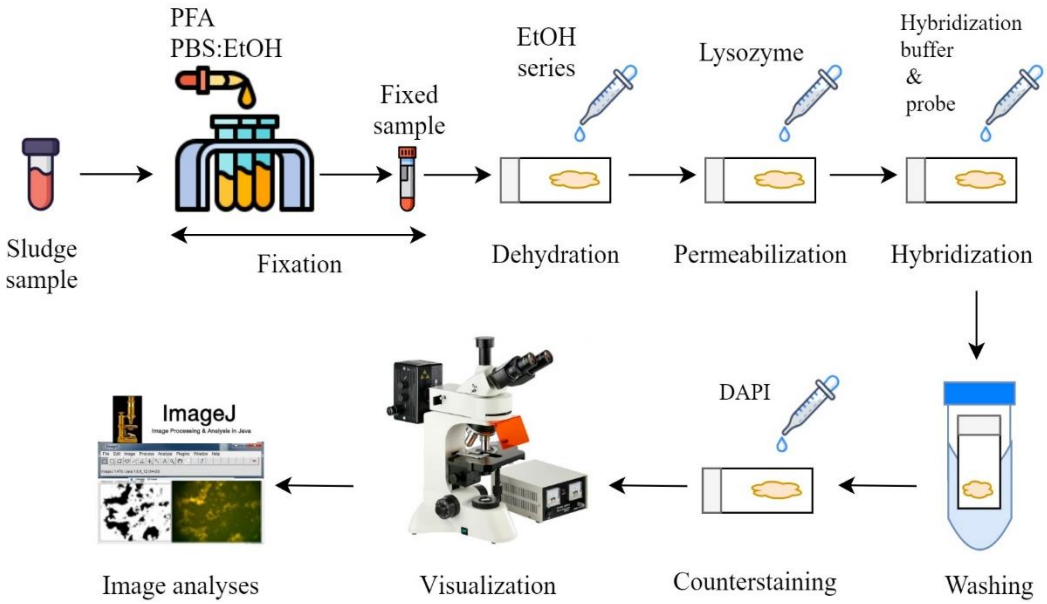

Figure 3.9 Schematic diagram of FISH procedure followed in the study

3.5.1 Probes and optimization of hybridization conditions

In the study, specific probes of microorganisms were selected from the literature for investigation of the microbial consortium in two-stage anaerobic digesters. Before FISH analyses, optimization experiments were carried out with probes and relevant pure cultures, and stringency conditions were determined to obtain the highest hybridization efficiency. Pure cultures used in optimization analysis were provided from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany) in lyophilized form. The lyophilized pure cultures were revived in the anaerobic chamber at Gülhane Military Medical

Academy Microbiology Laboratory (GATA, Turkey) and fixed for later use according to procedure of supplier. Probes and their target organisims used in the study were selected from probeBase and given in Table 3.3 (Greuter et al., 2016). 16S rRNA targeted oligonucleotide probes, labeled with fluorescein isothiocyanate (FITC) at 5' end, were supplied by Alpha DNA (Alpha DNA, Canada). Stock and working solutions of the probes were prepared by resuspending in Tris-EDTA (TE) buffer and diluting the probes by following procedure of the supplier.

As seen in Table 3.3, probes belonging to acidogens, acetogens, sulfate reducers denitrifiers, methanogens, *Archaea* and total *Bacteria* groups were used in the study. For total *Bacteria* detection, EUBmix which was prepared by mixing of EUB338, EUB338 II and EUB338 III probes were used. NONEUB probe was used as negative control in the analyses.

For the success of FISH technique, probes were empirically tested to optimize the stringency conditions (Hristova et al., 2000). For this purpose, target and non-target microorganisms for each FITC-labeled probe were used. In FISH experiments, pure cultures of probes were used as positive control and *Escherichia coli* used as negative control. For probe optimization, hybridization was performed by changing formamide (FA) and NaCl concentrations. The percentage of FA used in the hybridization buffer was selected from the literature, and FISH experiments were performed at three different percentages using the given percentage and ±%5 differences of this value. The NaCl concentrations in the wash buffers were adjusted according to Table 3.4 depending on the FA concentrations (P. H. Nielsen et al., 2009). Subsequently, the FISH procedure was performed in triplicate for optimization experiments as described in sections 3.5.3 to 3.5.6 and the stringency conditions of the probes were determined.

Table 3.3 Overview of 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	-	(Greuter et al., 2016)

*EUB338, EUB338 II and EUB338 III probes were mixed and called as EUB_{mix} 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

The first step of FISH method, fixation is a critical step to inactivate microbial cells and enzymatic activities and to prevent growth after sampling for more accurate results (Rocha et al., 2018). In this step, 6 mL sludge samples were transferred to falcon tubes and centrifuged for 5 min at 10000 rpm, after centrifugation the supernatant was removed. 3 mL of cold 4% paraformaldehyde (PFA) was added to the pellet and mixed through vortexing. The falcon tubes were kept on ice for 4 h, then centrifuged at 10000 rpm for 5 min to separate the PFA. In the last step of fixation, 3 mL of cold 1:1 phosphate-buffered saline and ethanol solution (PBS: EtOH) was added onto the pellet and mixed (Nielsen et al., 2009). The fixed samples were stored at -20 °C in 1.5 mL eppendorf tubes as triplicates for later use.

3.5.3 Dehydration and permeabilization

After the fixed samples reached room temperature, 10 µL of the sample was placed on the microscope slide and gently spread with a pipette and left for air-drying. After that, 50, 80 and 96% ethanol were applied to slides for 3 min. The slides were again left for air-drying and then 10 µL of cold lysozyme was applied. The samples on slides were covered with glass covers for cell wall permeabilization. The slides were incubated in a humid environment, at 37 °C for 30 min. Then cover glasses were removed by washing with ddH20 and the slide was air dried finally.

3.5.4 Hybridization and washing

In this step of FISH method, firstly, 2 mL of hybridization buffer depending on the optimum FA concentrations of each probe and 50 mL of washing buffer depending on this FA concentration were prepared according to Table 3.3. After that, 8 µL of hybridization buffer and 1 µL of probe working solution were applied to the samples.

Cover glass was placed, and the slides were incubated at 46 ° C for 2 hours in a dark and humid environment.

After hybridization, washing buffers were placed in 50 mL falcon tubes and heated in 48 °C water bath. Cover glasses placed in the hybridization step were removed with washing buffer. Slides were transferred to heated falcon tubes and incubated in water bath at 48 °C for 15 min. Then, slides were removed and immersed in cold ddH2O. Lastly, slides were left for air-dry (Nielsen et al., 2009).

After hybridization completed, samples were counterstained with a 4, 6-Diamidino-2-phenylindole dihydrochloride (DAPI) solution (1 µg / mL). After 10 min waiting, slides were rinsed with ddH20 and left for air-drying. Until visualization, slides were stored at -20 ° C in the dark.

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; ddH_2O, ultrapure water; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; EDTA, Ethylenediaminetetraacetic acid.

3.5.5 Visualization

Slides stained with DAPI were brought to room temperature for microscopic visualization. Visualization was performed by BEL Photonics FLUO3 fluorescence microscope (BEL Engineering, Italy) equipped with an HBO 100W mercury lamp, and had objective lenses of 4x/0.1, 10x/0.25, 40x/0.65 and 100x/1.25 and ultraviolet (UV), violet (V), green (G) and blue (B) filters.

In the microscope, all microorganisms stained with DAPI were monitored under a 330-400 nm spectrum UV filter as blue, while target microorganisms hybridized with FITC labeled probes were monitored under 420-485 nm spectrum B filter as yellow. From the slides, 10 representative areas for each microorganism were selected and images were captured with the help of CCD camera and microscope software under both UV and B filters.

3.5.6 Image analyses

The captured digital images were analyzed by ImageJ software which is an image processing program designed for scientific multidimensional images (Schneider et al., 2012). In the study, ImageJ analyses were performed according to the procedure provided by Bankhead, 2014 as follows:

Firstly, the image to be analyzed is opened with the *File → Open* command and then copied by *Image →Duplicate*. The opened image was converted to 8-bit color by *Image → Type → 8-bit*. After that, with Image → Adjust → Threshold command, threshold value of the 8-bit image was adjusted to include stained microorganisms with reference to the original image. After adjusting the threshold value of the image, *Analyze → Analyze Particles* command is used for pixel analysis and the analysis was obtained in the *Summary* window. ImageJ analysis process is summarized in Figure 3.10.

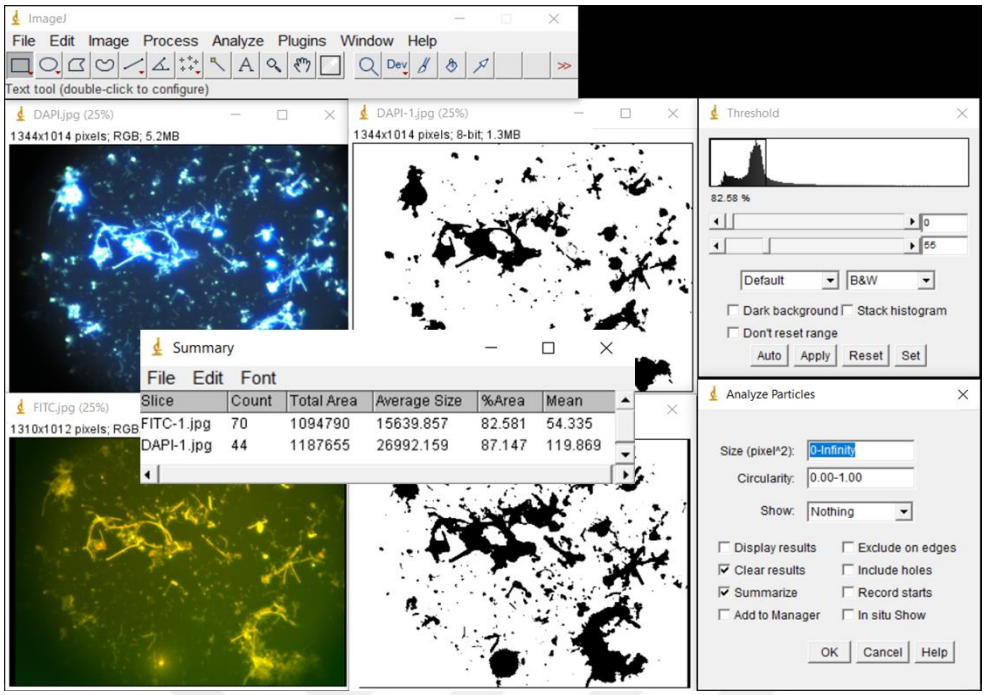

Figure 3.10 Screenshot of ImageJ analysis used in the study

In the fluorescence microscope, the UV filter gave the image of all microorganisms stained with DAPI, while the BLUE filter gave the image of only microorganisms that hybridize with the specific probe labeled with FITC. Based on this, after the ImageJ analysis, the Total Area data obtained in the Summary window were transferred to the excel program. By using Equation 3.5, target microorganisms in microbial communities were quantified (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 calculations, pixel areas obtained with the NONEUB negative control probe were subtracted from the results to eliminate measurement errors caused by contamination or non-specific binding. EUBmix and ARC915 probes were also used to determine the total *Bacteria* and *Archaea* populations in the samples. The relative abundances of target microorganisms were calculated for all microorganisms as a percentage by scaling to 100%.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization analysis for seed and feed sludge

Seed and feed sludges were used in the operation of two-stage anaerobic digesters as mentioned in section 3.1. The sludge samples were characterized in terms of pH, temperature, COD, and solids content for 16 weeks. The properties of the seed and feed sludges were given in Table 4.1. Two-stage anaerobic digesters were operated at unsteady-state condition (without ozonation) up to week 12. Steady-state conditions were assumed to be reached when daily data changes were within ± 10% fluctuation. After that, one of anaerobic digester was pretreated with ozone dose of 0.06 g O_3/g TSS while the control digester without treatment continued to be operated for 28 days. The required ozone amounts were calculated according to TSS values of feed sludge (Table 4.1), and the results were given in Table 3.2. Moreover, VS/TS ratios were also given in the table to show organic amount of feed sludge, and values varied between 0.49 and 0.74. Additionally, OLR values of feed sludge were calculated according to VS concentration of feed sludge and given in the same table. OLR values changed between 1.68 – 4.45. It was observed that values for the parameters varied close to each other, except for the 3rd week. Due to technical failures in WWTP, the feed sludge characteristic was not observed as expected for week 3.

Table 4.1 Characteristics of seed and feed sludge and OLR values used in the study

Parameters	pH	T (°C)	COD (g/L)	TN (g/L)	TS (g/L)	VS (g/L)	TSS (g/L)	VSS (g/L)	VS/TS	OLR (gVS/L.day)
Seed sludge	7.58±0.04	29.60±0.10	14.75±0.12	2.03±0.07	20.16±0.17	8.91±0.14	17.13±0.04	6.52±0.18	0.44	-
Feed sludge										
1st week	6.20±0.01	25.00±30	38.00±0.07	2.57±0.02	35.00±0.09	20.33±0.22	31.13±0.11	15.90±0.17	0.58	3.39±0.22
2nd week	6.55±0.03	27.00±0.10	32.75±0.08	2.02±0.06	31.62±0.25	21.22±0.01	29.92±0.05	19.38±0.14	0.67	3.54±0.01
3rd week	7.44±0.28	28.10±0.00	4.87±0.18	0.84±0.15	2.16±0.34	1.29±0.10	1.90±0.02	1.270±0.31	0.60	0.22±0.10
4th week	6.52±0.14	24.10±0.20	37.30±0.09	2.32±0.03	28.02±0.17	17.95±0.08	25.70±0.18	13.50±0.29	0.64	2.99±0.08
5th week	6.07±0.13	27.20±0.20	48.25±0.27	2.24±0.04	42.84±0.51	26.71±0.06	38.45±0.12	23.80±0.08	0.62	4.45±0.06
6th week	6.02±0.02	26.00±0.20	18.20±0.17	2.79±0.03	14.69±0.18	10.10±0.09	12.95±0.03	8.30±0.01	0.69	1.68±0.09
7th week	6.09±0.15	28.60±0.30	27.15±0.04	2.04±0.04	21.15±0.08	14.15±0.25	19.80±0.17	13.05±0.13	0.67	2.36±0.25
8th week	6.23±0.09	22.90±0.10	43.65±0.11	2.11±0.05	43.26±0.15	21.20±0.11	40.75±0.04	19.20±0.15	0.49	3.53±0.11
9th week	6.88±0.06	28.60±0.10	38.00±0.05	2.08±0.11	30.66±0.23	17.54±0.07	28.95±0.08	15.90±0.07	0.57	2.92±0.07
10th week	6.13±0.15	22.40±0.50	36.25±0.02	3.71±0.07	30.41±0.23	20.61±0.13	27.95±0.17	17.85±0.20	0.68	3.43±0.13
11th week	6.03±0.07	25.60±0.10	28.00±0.13	3.02±0.01	30.64±0.04	16.77±0.05	19.04±0.07	12.99±0.14	0.55	2.80±0.05
12th week	6.68±0.18	21.50±0.30	30.15±0.06	2.28±0.01	27.16±0.11	17.58±0.01	22.40±0.03	14.80±0.14	0.65	2.93±0.01
13th week	6.31±0.08	20.70±0.20	38.55±0.01	2.19±0.05	28.12±0.17	20.22±0.20	20.81±0.19	13.95±0.06	0.72	3.37±0.20
14th week	6.34±0.01	21.70±0.10	37.65±0.04	2.39±0.14	32.36±0.02	23.81±0.03	27.75±0.27	15.65±0.18	0.74	3.97±0.03
15th week	6.21±0.11	26.10±0.10	36.00±0.15	2.17±0.03	28.15±0.06	18.49±0.07	22.11±0.11	10.97±0.09	0.66	3.08±0.07
16th week	6.03±0.22	25.40±0.20	32.74±0.07	1.76±0.01	29.43±0.04	17.45±0.15	16.72±0.06	12.10±0.03	0.59	2.91±0.15

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

4.2 Analytical analyses

150 mL of sludge and 500 µL biogas samples were taken daily from two-stage anaerobic digesters for analytical analyses. As mentioned, two-stage anaerobic digesters were operated at unsteady-state condition for 12 weeks. During unsteady-state period, pH and temperature were measured daily, whereas COD, TN, solid contents, total VFA, biogas, and methane measurements were performed weekly as duplicates. Steady-state conditions were assumed to be reached when daily data changes were within ± 10% fluctuation. All measurements were performed daily at steady-state conditions. Results of each parameter for unsteady- and steady-state conditions were discussed below for control and ozonated digester.

4.2.1 pH

pH measurements of two-stage anaerobic digesters were carried out daily. pH values of digesters before ozone pretreatment are shown in Figure 4.1. Fluctuations in pH values were observed until the 65th day due to the adaptation process and the changing pH of the feed sludge. After the day 65, pH become more stable. The pH values of control and ozonated digesters were measured during the ozone pretreatment period (Figure 4.2).

The results given in Figures 4.1 and 4.2 belong to the second stage of the digesters in which methanogenesis phase takes place. In two-stage anaerobic digesters, the methanogenesis digester is usually operated with a pH range of 6-8 to facilitate methane formation (Cremonez et al., 2021; Yan et al., 2021). In the current study, pH values were measured between 7.5 and 8.0. Additionally, no significant changes were observed in the pH values of the digesters after ozonation, and daily change was smaller than ± 0.3. These findings were in parallel with Kosowski et al. (2020) who pointed out that ozone pretreatment did not cause any pH change.

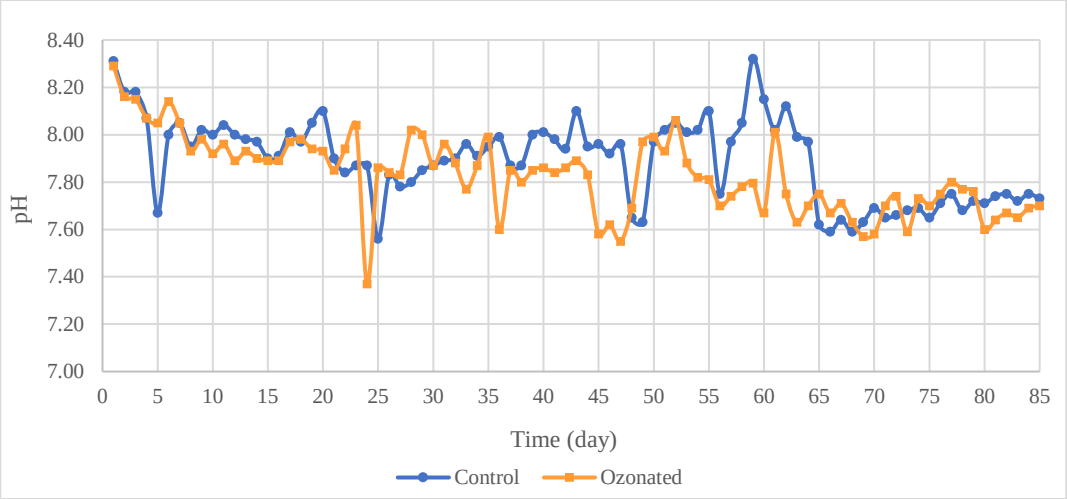

Figure 4.1 pH changes during unsteady-state operation

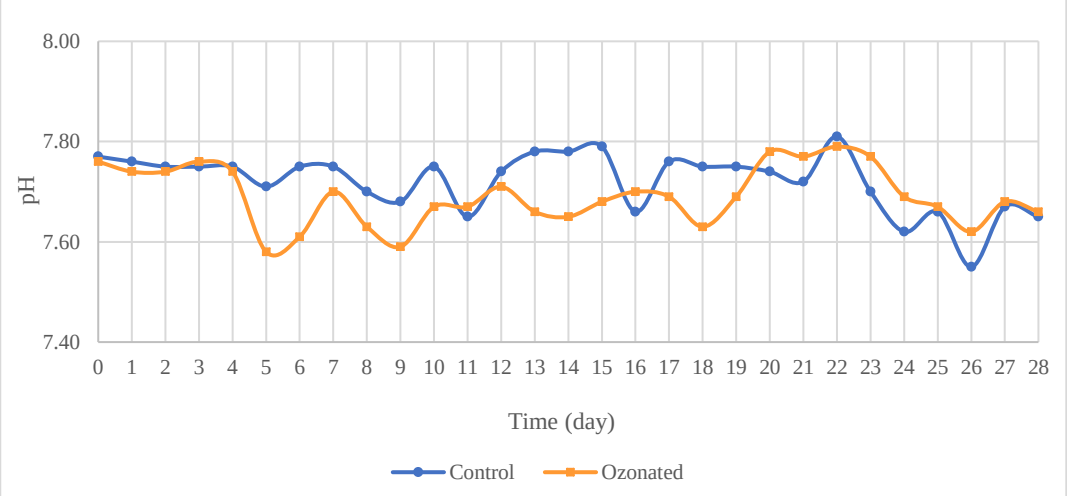

Figure 4.2 pH changes during steady-state operation

In previous studies, it was underlined that the hydrolysis / acidogenesis stages usually operated in the low pH range of 5.0-6.5 (Srisowmeya et al., 2020; Cremonez et al., 2021). Therefore, throughout the study pH of the first stages were also measured weekly to control the system. The result of pH measurements was given in Figure 4.3. In the first weeks, high values between 7.0 and 8.1, and fluctuations in pH were observed due to the adaptation process. In addition, the high pH of the feed sludge in the 3rd week led to pH increase in the digesters. On the other hand, digesters

were fed with low pH sludge except 3rd week as shown in Table 4.1. Low pH sludge acted as buffer in the system and led to decrease in pH. The pH values were observed between 6.32 – 5.78, therefore no pH control was required.

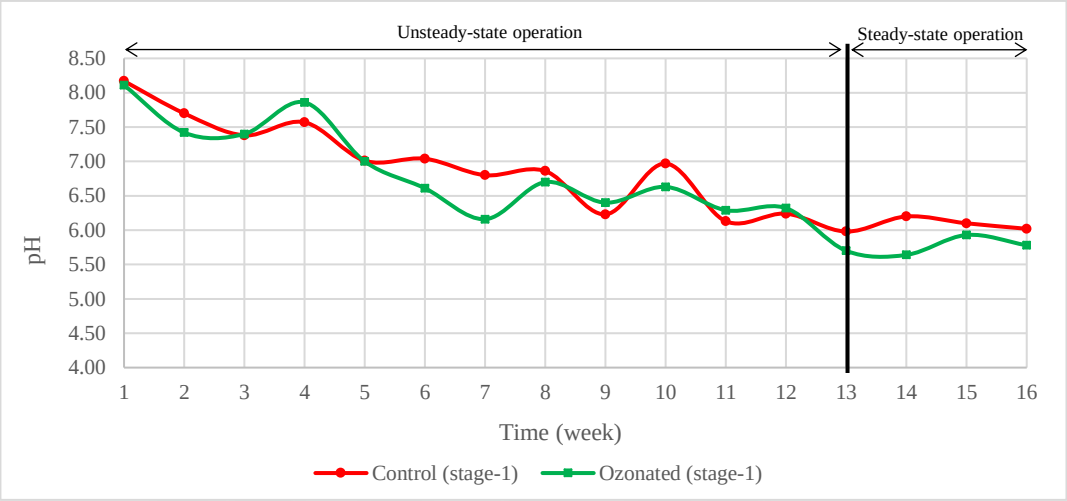

Figure 4.3 pH changes in the first stages of control and ozonated digesters

4.2.2 Temperature

Two-stage anaerobic digesters were operated under mesophilic conditions and temperature measurements were carried out daily throughout the study. The results were given in Figure 4.4 and 4.5 for unsteady- and steady state conditions, respectively. In previous studies, the temperature was kept between 30 – 40 °C in mesophilic anaerobic digesters for optimum biogas and methane production (C. Zhang et al., 2014; Rusín et al., 2021). Likewise, as seen in Figures 4.4 and 4.5, temperature values in the current study varied between 34 - 37 °C. Although same fluctuations were observed, these were thought to be caused by the temperature difference between the hot room at where the reactors were operated and the laboratory environment, and the time elapsed until the analyses were completed.

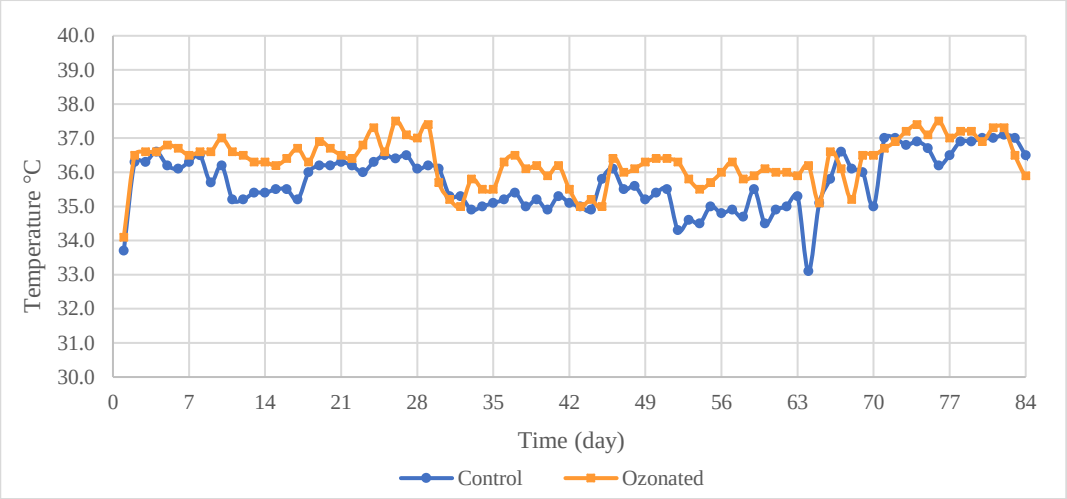

Figure 4.4 Temperature changes during unsteady-state operation

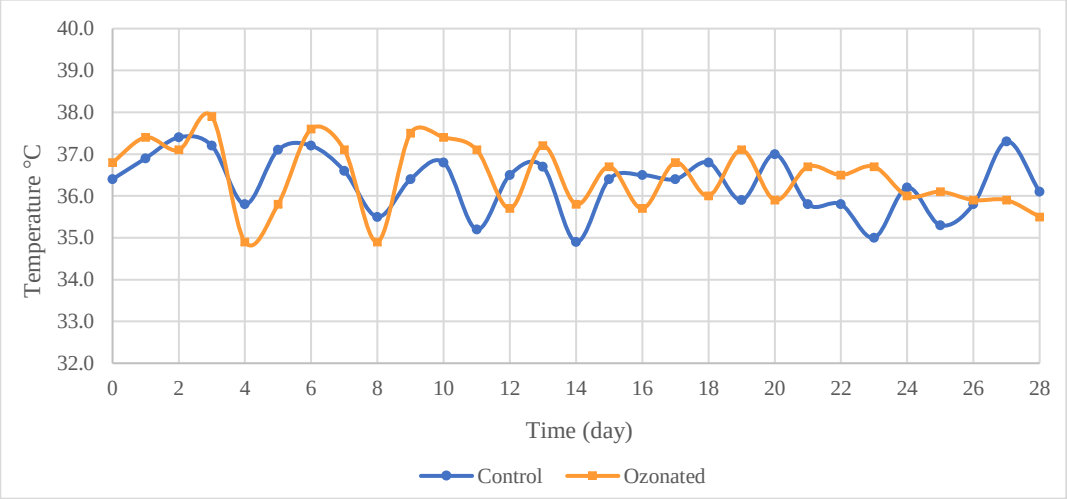

Figure 4.5 Temperature changes during steady-state

4.2.3 COD

COD concentrations of control and ozonated digesters were monitored weekly during unsteady-state operation and the results were given in Figure 4.6. The COD values were measured over 12 weeks and varied between 17.9 – 38.0 g/L.

After digesters reached steady-state conditions, COD concentrations were measured daily to monitor the effect of 0.06 g O_3/g TSS ozone dose and given in Figure 4.7. During ozone pretreatment operation, COD removal efficiencies for control and ozonated digesters were calculated weekly by regarding COD values of feed sludge. The COD values of feed sludge and removal efficiencies were also shown in the figure. In the first week, feed sludge with a COD value of 38.55 g/L was introduced into the digesters. At the end of this week, COD removal efficiencies for control and ozonated digesters were found to be close to each other (44% and 43% respectively). For the control digester, the highest COD removal was observed in the 3rd week and 51% removal was achieved by reducing the COD value from 36.0 g/L to 17.65 g/L. On the other hand, for the ozonated digester, the highest COD removal was observed in the 2nd week, and 63% removal was achieved by reducing the COD value from 37.65 g/L to 13.74 g/L. After the week 1, it was observed that higher COD removal was achieved in ozonated digester than control digester with the effect of ozone pretreatment.

In previous studies, no information was given about the effect of ozone pretreatment on COD removal in two-stage anaerobic digesters. In single-stage anaerobic digesters, ozone pretreatment resulted in 64% COD removal and provided 25% more removal than the control reactor (Le et al., 2019). On the other hand, in the study conducted by Carballa et al. (2007), 65% COD removal was observed in the single-stage digester while ozone pretreatment with 0.02 g O_3/g TSS ozone dose did not show any effect on removal. In the study conducted by Ambrose et al. (2020), two-stage anaerobic digesters were subjected to hybrid microwave-oxidative pretreatment and COD removal was calculated as 68% and 82% for untreated and pretreated digesters, respectively. In the study conducted by Pascual et al. (2017), wastewater sludge was used as feed stock and an average of 58% COD removal was obtained in two-stage anaerobic digesters. Different COD removal efficiencies were reported in studies. Although ozone pretreatment lagged behind microwave-oxidative pre-treatment, COD removal achieved in the current study was similar to the results obtained in the literature.

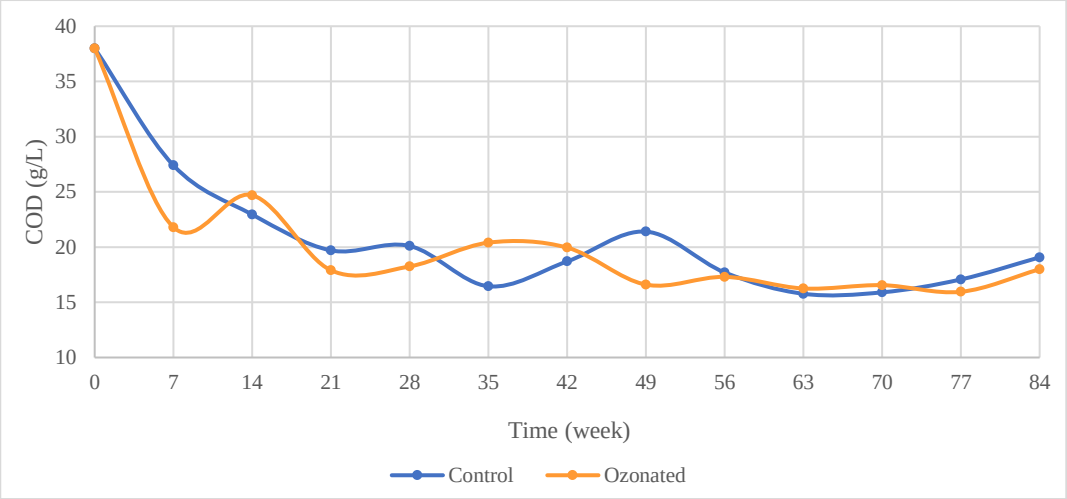

Figure 4.6 COD changes during unsteady-state operation

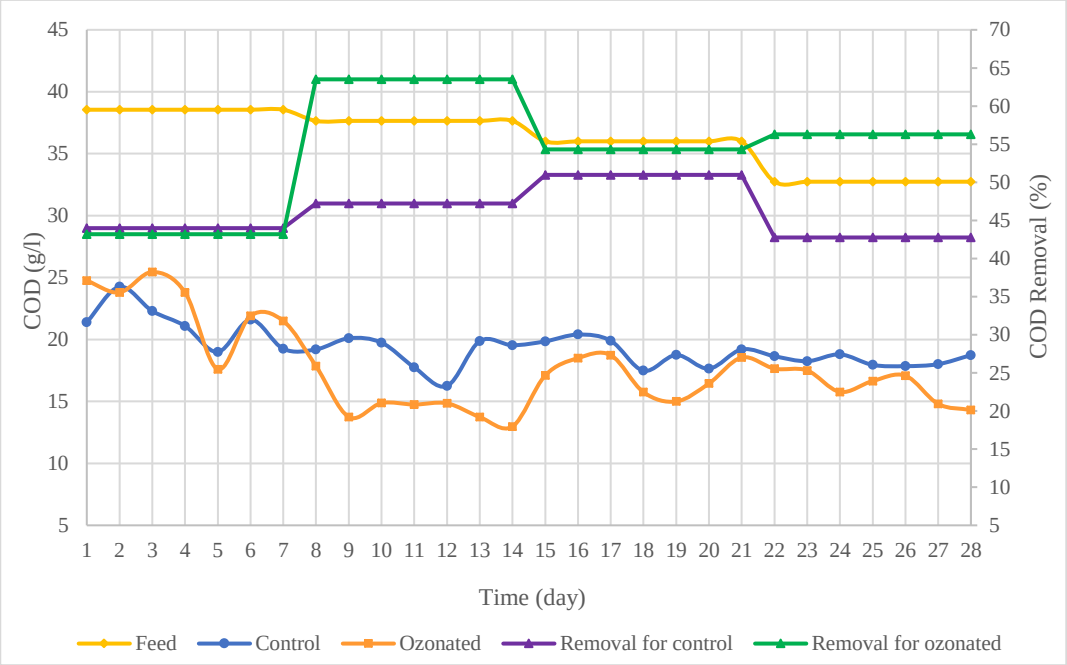

Figure 4.7 COD changes during steady-state operation

4.2.4 TN

TN concentrations were monitored weekly at unsteady-state and daily at steady-state operations and the results were given in Figure 4.8 and 4.9, respectively. TN values for digesters were varied between 2.03 – 3.92 g/L before ozone pretreatment and

2.19 – 3.73 after ozonation. As Harrison & Ndegwa (2020) stated, TN concentrations were not changed in the study during operations. Additionally, it was observed that the TN concentration was not affected by ozone pretreatment.

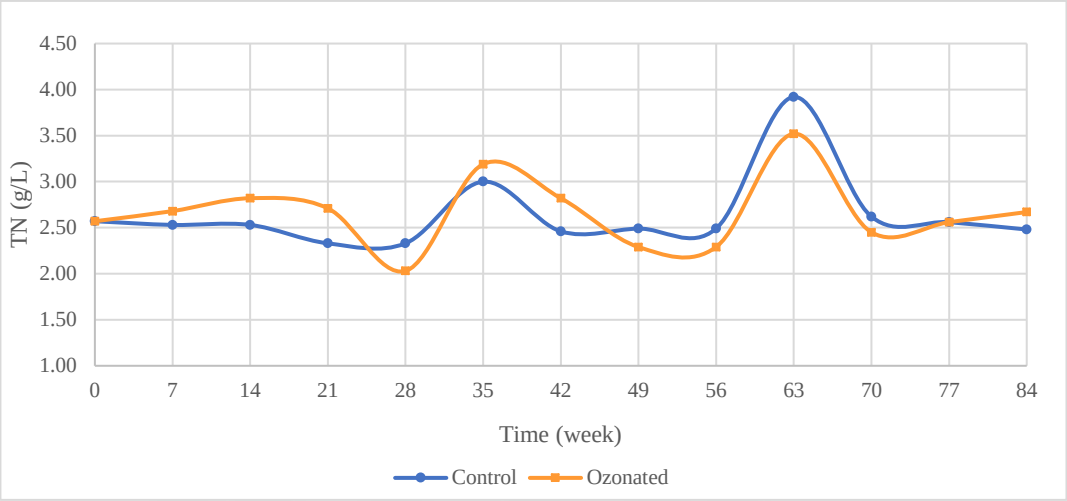

Figure 4.8 TN changes during unsteady-state operation

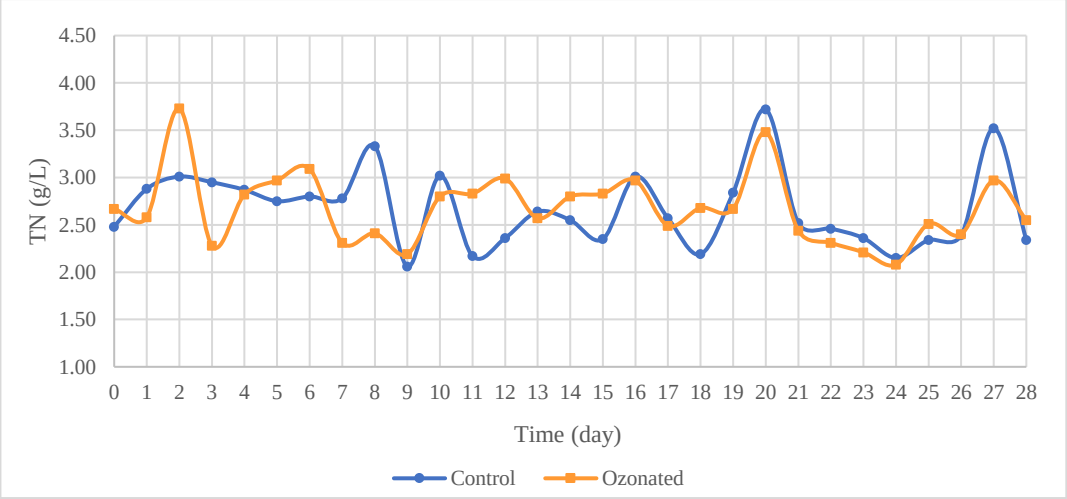

Figure 4.9 TN changes during steady-state operation

4.2.5 Solids content

TS, VS, TSS and VSS concentrations were monitored for two-stage anaerobic digesters weekly at unsteady-state and daily at steady-state operations.

4.2.5.1 TS

TS concentrations of control and ozonated digesters were measured weekly during unsteady-state operation. The results were given in Figure 4.10 and values were varied between 15.6 – 35.0 g/L for 12 weeks.

TS concentrations were measured daily after digesters reached steady-state condition and given in Figure 4.11. During ozone pretreatment, TS removal efficiencies for control and ozonated digesters were calculated weekly. Removal efficiencies and TS concentration of feed sludge were also illustrated in the figure. The highest TS removal for both digesters was observed in the 2nd week. TS value in the control digester decreased from 32.36 g/L to 17.08 g/L and 47% removal was achieved while for ozonated digester, TS value decreased from 32.36 g/L to 15.77 g/L and 51% removal was achieved. Although higher TS removal was recorded in ozonated digestion in other weeks, no difference exceeding 6% was observed between them. Considering the removal efficiencies in both cases, it was concluded that ozone pretreatment did not make a remarkable difference in TS removal.

In previous studies, effect of ozone treatment on TS removal was not mentioned for two-stage anaerobic digesters. On the other hand, in a study conducted by Bougrier et al., (2007), it was reported that ozone pretreatment did not affect TS for ozone doses below 0.15 g O_3/g TS, which was also supported by the results of the current study. Apart from this study, C. Li et al. (2015) evaluated effect of thermo-chemical pretreatment in two-stage anaerobic digesters and employed co-digestion of sludge and FOG (fat, oil and grease), 65.7% and 68.2% TS removals were reported for untreated and pretreated digesters, respectively. In the study conducted by Ambrose et al. (2020), TS removal for untreated and microwave-oxidative pretreated two-

stage anaerobic digesters were %53 and %58, respectively. Although different pretreatment methods and different feedstocks were used in these studies, the results were similar to ozone pretreatment case and noticeable change was not observed in TS removal efficiencies.

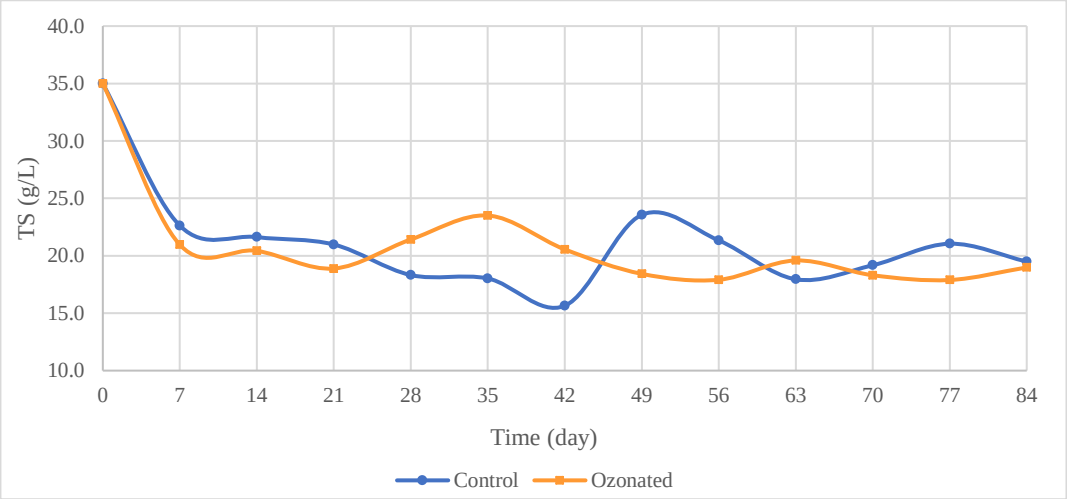

Figure 4.10 TS changes during unsteady-state operation

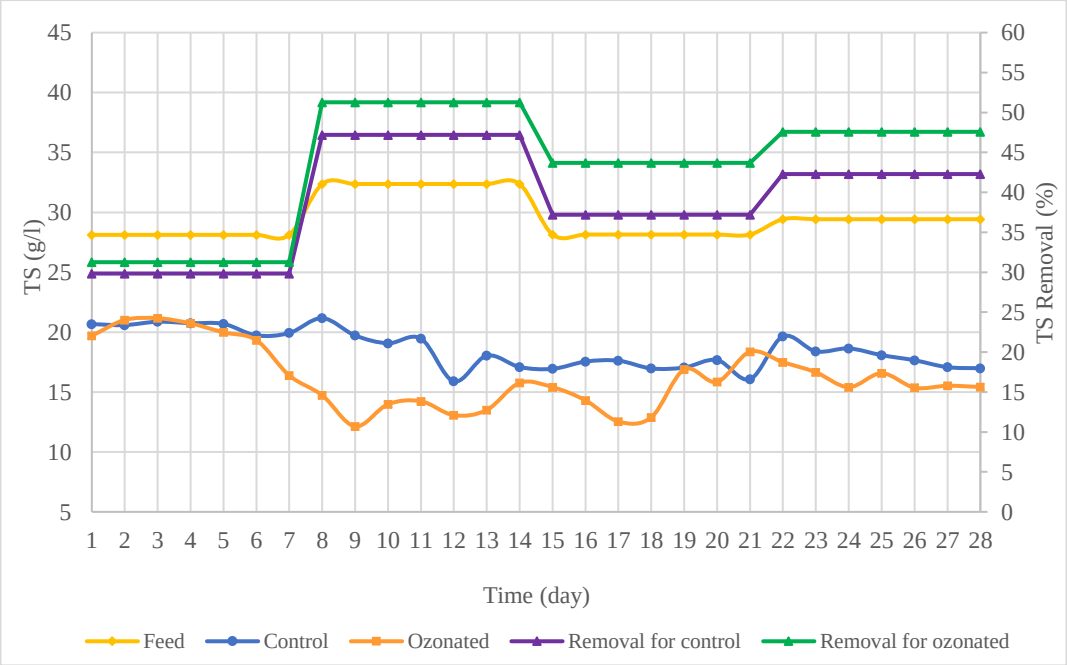

Figure 4.11 TS changes during steady-state operation

4.2.5.2 VS

VS concentrations of control and ozonated digesters were given in Figure 4.12 for unsteady-state operation. In VS concentrations, no major changes observed for 12 weeks and the values varied between 15.6 – 23.5 g/L.

After the digesters became stable, VS concentrations were measured daily and given in Figure 4.13. During ozone pretreatment, VS removal efficiencies for control and ozonated digesters were calculated weekly. Removal efficiencies and VS concentration of feed sludge were also indicated in the figure. The highest VS removal for both digesters was observed in the 2nd week. In control digester 51% VS removal was achieved by reducing TS from 23.81 g/L to 17.74 g/L. For ozonated digester, VS value decreased from 23.81 g/L to 9.27 g/L and 61% removal was achieved. Higher VS removals were also recorded in ozonated digestion in other weeks. Considering the average VS removal efficiencies observed over 4 weeks in both digesters, the values for the control and ozonated digesters varied between 47±3.21% and 53±5.36%. It was concluded that higher VS removal was achieved in ozonated digester than control digester with the effect of ozone pretreatment.

The effect of ozone pretreatment on VS removal for two-stage anaerobic digesters was not studied in the literature. However, for single-stage digesters, 30%, 38% and 60% VS removals were reported in control digester and pretreated digesters with ozone doses of 0.015 and 0.05 g O_3/TS, respectively, by Goel et al. (2003). On the other hand, in the study conducted by Braguglia et al. (2012), VS removals were reported as 27%, 29% and 34%, respectively, for control and pretreated digesters with 0.05 and 0.07 g O_3/TS. When the current study results were compared to mentioned studies, it was concluded that two-stage anaerobic digesters provided advantages in terms of VS removal.

Different pretreatment methods have been applied in the literature for two-stage anaerobic digesters and VS removals were reported as follows: In the study conducted by Monlau et al. (2015), two-stage anaerobic digesters were subjected to

alkali pretreatment and 41% and 50% VS removals were found, respectively, in the untreated and pretreated digesters. 73% and 62% VS removals were reported by Ambrose et al. (2020) for two-stage digesters with and without hybrid microwave-H_2O_2 pretreatment. Moreover, C. Li et al. (2015) evaluated effect of thermo-chemical pretreatment with co-digestion of sludge and FOG (fat, oil and grease), 75% and 77% TS removals were reported for untreated and pretreated digesters, respectively. As a result, while ozone pretreatment provided an advantage over alkaline pretreatment in two-stage anaerobic digesters, it lagged behind microwave-H_2O_2 and thermo-chemical pretreatment for %VS removal.

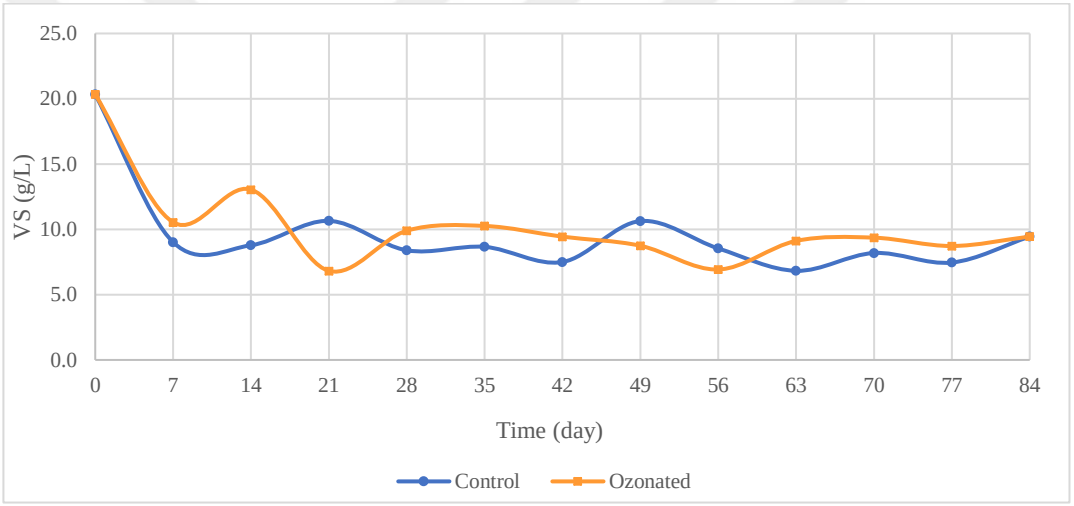

Figure 4.12 VS changes during unsteady-state operation

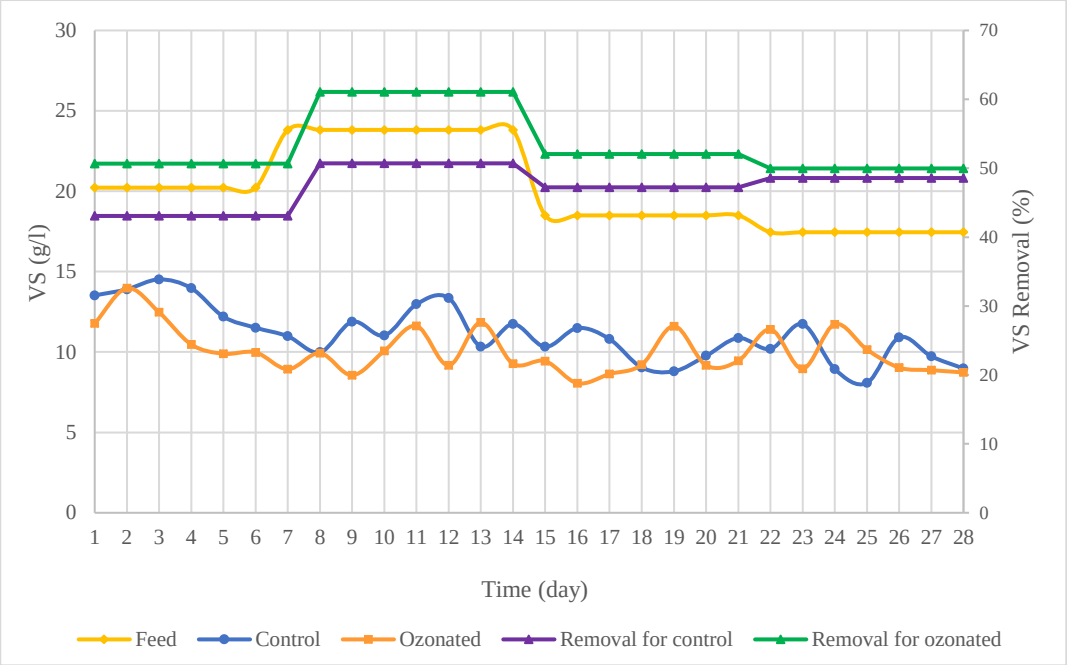

Figure 4.13 VS changes during steady-state operation

4.2.5.3 TSS

TSS values of unsteady-state condition were given in Figure 4.14 for two-stage anaerobic digesters. Fluctuations in TSS concentrations were observed, depending on the changing characteristics of the feed sludge and removal efficiencies.

TSS concentrations of control and ozonated digesters were measured weekly after the digesters reached steady-state condition. TSS concentrations observed in digesters and feed sludge were given in Figure 4.15 with corresponding removal efficiencies. In second week, 50% TSS reduction was obtained in control digester whereas TSS removal reached to 56% in ozonated digester with the effect of ozone pretreatment. Although higher TSS removals were found in the ozonated digester at week 2 and 3, more TSS removals were observed in the control digester at week 1 and 4. As in TS removal, no noticeable effect of ozone pretreatment was observed in TSS removal in two-stage anaerobic digesters. On the contrary, in a study conducted by Salsabil et al. (2010), 78% TSS removal was observed in anaerobic digester subjected to ozone pretreatment, while 66% TSS removal was observed in the

untreated digester. Elbeshbishy & Nakhla (2011) evaluated the effect of ultrasonication in two-stage anaerobic digesters and reported TSS removals of 44% and 55% for untreated and pretreated digesters, respectively.

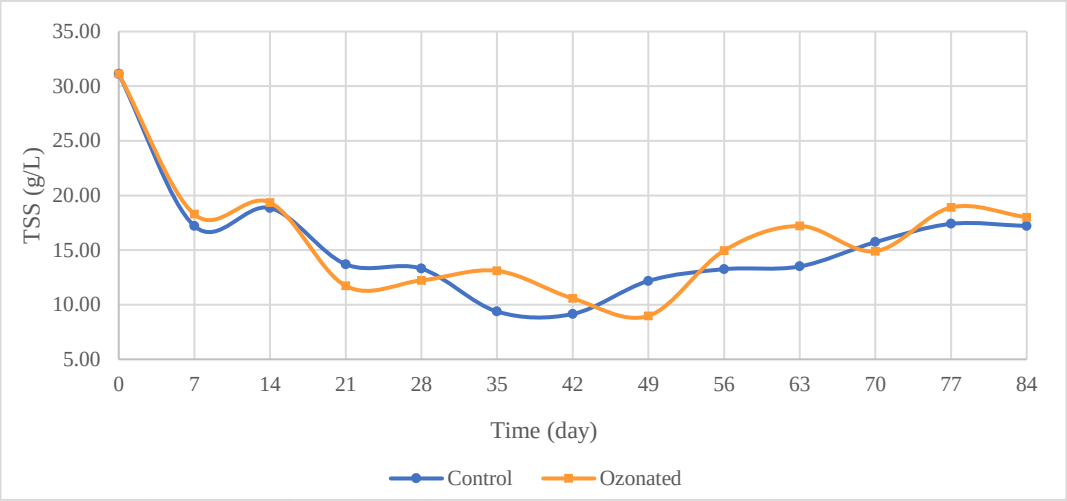

Figure 4.14 TSS changes during unsteady-state operation

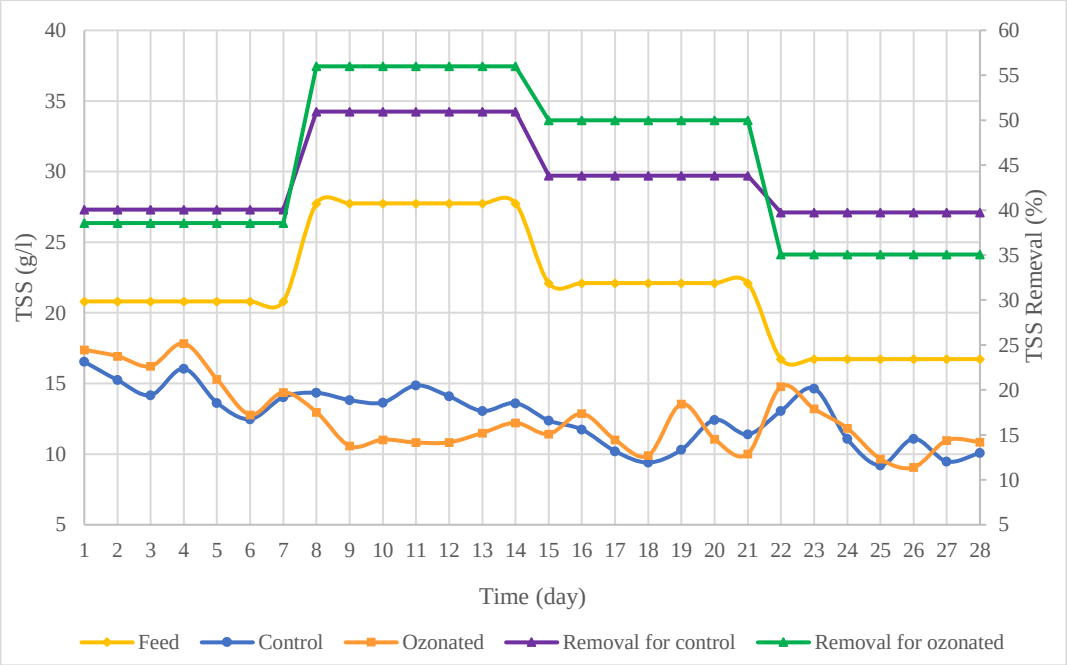

Figure 4.15 TSS changes during steady-state operation

4.2.5.4 VSS

VSS concentrations of control and ozonated were given in Figure 4.16 for unsteady-state operation. In VSS concentrations, no major changes observed for12 weeks similar to VS concentration and values varied between 6.82 and 10.65 g/L.

After the digesters became stable, VSS measurements were carried out daily for control and ozonated digesters. VSS concentrations of digesters and feed sludge were given in Figure 4.17 with corresponding removal efficiencies. Maximum VSS removal was observed in 2nd week for control and ozonated digesters with 46% and 58% removals, respectively. As indicated in the figure, more VSS removal was recorded in the ozonated digester than in the control digester and it was concluded that ozone pretreatment increased VSS removal in the current study. The effect of ozone pretreatment on VSS removal has not been studied in the literature for two-stage anaerobic digesters. Weemaes et al. (2000) stated that VSS removals were 41%, 55% and 73% for digesters pretreated with 0.05, 0.1 and 0.2 g O_3/g COD ozone doses. While 55% VSS removal was achieved with 0.1 g O_3/g COD by Weemaes et al. (2000), 58% VSS removal was achieved with the ozone dose of 0.06 g O_3/g TSS in a two-stage anaerobic digester in the current study. Thus, two-stage anaerobic digester provided greater VSS removal with lower ozone dose.

Elbeshbishy & Nakhla (2011) evaluated the effect of ultrasonication in two-stage anaerobic digesters and reported VSS removals of 51% and 59% for untreated and pretreated digesters, respectively. In a study conducted by Rivero et al. (2005), two-stage anaerobic digesters were subjected to H_2O_2 pretreatment, and 50% and 61% VSS removals were reported for untreated and pretreated digesters. Thus, application of different pretreatment methods resulted in similar VSS removal efficiencies.

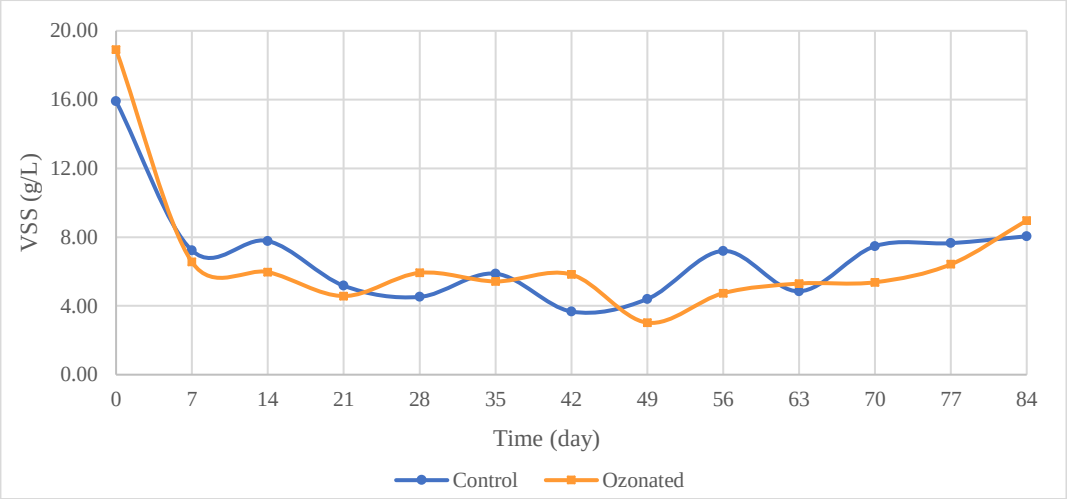

Figure 4.16 VSS changes during unsteady-state operation

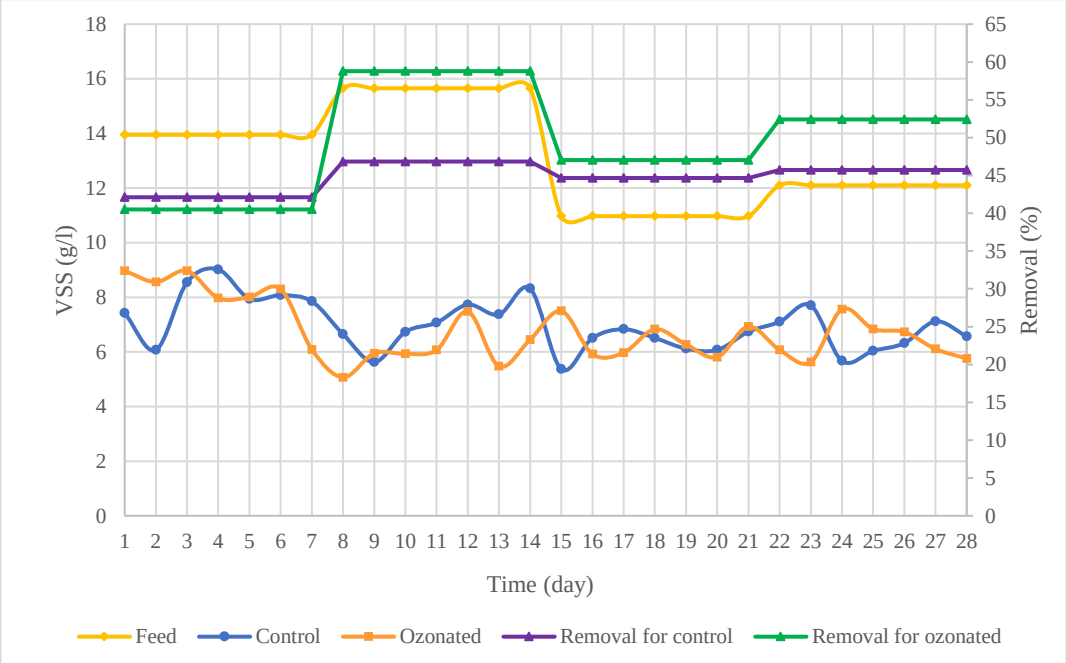

Figure 4.17 VSS changes during steady-state operation

For control and ozonated digester, removal efficiencies observed during ozone pretreatment period were given in the Table 4.2 in terms of COD, TS, TSS, VS, and VSS.

Table 4.2 %Removal efficiencies for control and ozonated digester

	Weekly removals (%)							
	Control digester				Ozonated digester			
Parameters \ Weeks	1	2	3	4	1	2	3	4
COD	44.0	47.2	51.0	42.8	43.2	63.5	54.3	56.3
TS	29.8	47.2	37.2	42.3	31.3	51.3	43.7	47.6
TSS	40.1	51.0	43.8	39.7	38.5	56.0	50.0	35.1
VS	43.1	50.7	47.2	48.6	50.7	61.1	50.4	50.0
VSS	42.1	46.8	44.7	45.7	40.5	58.8	47.0	52.4

4.2.6 Total VFA

Total VFA concentrations of control and ozonated digesters were monitored weekly at unsteady-state operation and the results were given in Figure 4.18. Generally, in anaerobic digesters, total VFA values vary inversely to pH (Hori et al., 2006; K. Wang et al., 2014). However, total VFA concentrations in the digesters increased in the first weeks despite increasing pH values. This situation was thought to be originated from activities of acidogens and methanogens. Acidogens are fast-growing microorganisms and they adapt changing environmental conditions easily (S. Li & Yang, 2016). On the other hand, methanogens are sensitive and slow-growing microorganisms and cannot adequately utilize the produced VFA due to changing environmental conditions (Anukam et al., 2019). Although fluctuations were observed in the total VFA values at the beginning, after day 35, the total VFA changed inversely with the pH values and varied between 10.69 and 14.31 mM.

Total VFA concentrations for steady-state operation were given in Figure 4.19 for control and ozonated digesters. After the reactors reached steady-state, total VFA concentrations changed depending on the pH values and varied between 10.21 and 14.79. Throughout the study, total VFA concentrations of the first stages were also measured weekly to control the system. The result of total VFA measurements was given in Figure 4.20. Total VFA increased in parallel with decrease in pH as shown in the Figure 4.3. Thus, with ozone pretreatment the total VFA values observed in

the first stage and the second stage of two-stage anaerobic digesters were varied between 40-47 mM and 10-12 mM, respectively. No inhibitory values of VFA were observed in the digesters and ozonation did not show a significant impact on VFA profile.

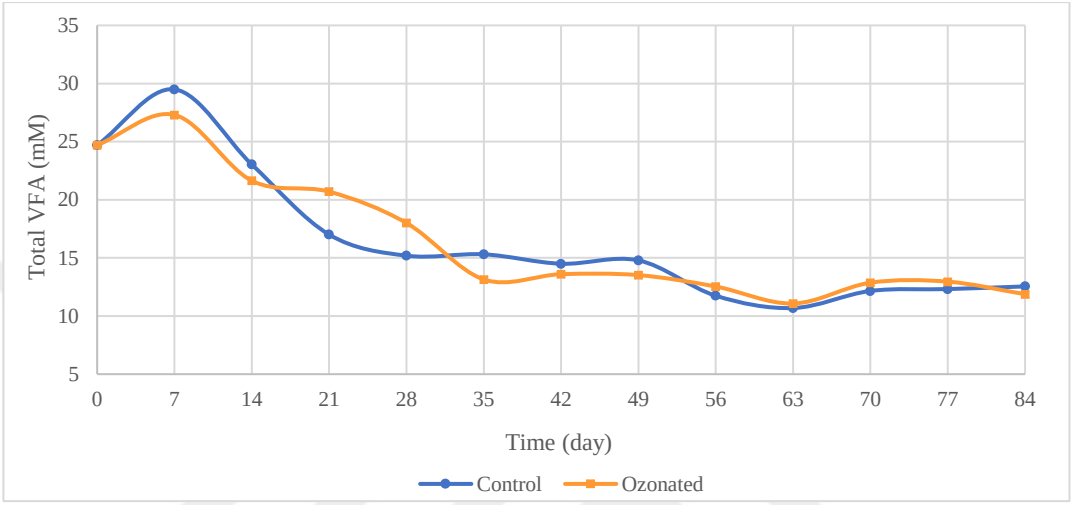

Figure 4.18 Total VFA changes during unsteady-state operation

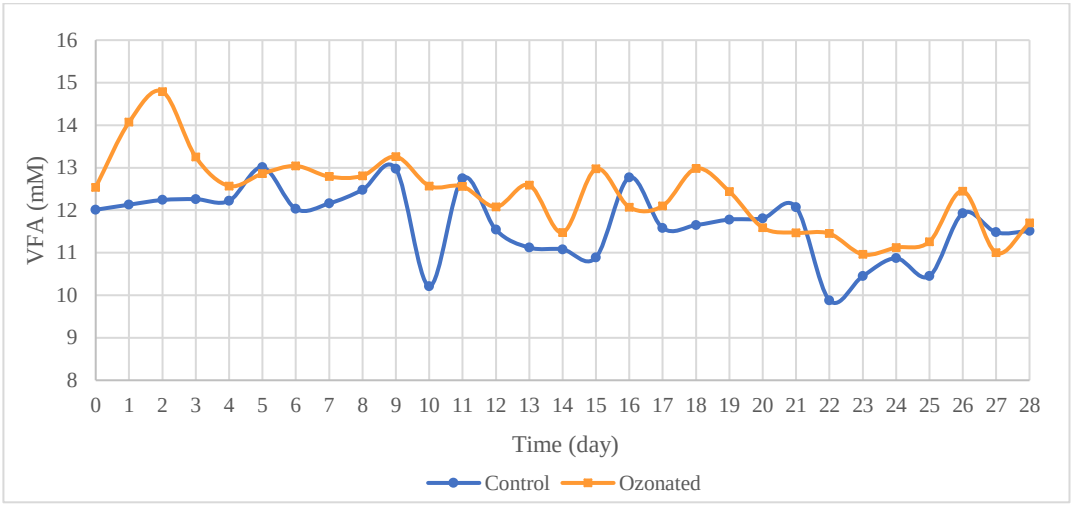

Figure 4.19 Total VFA changes during steady-state operation

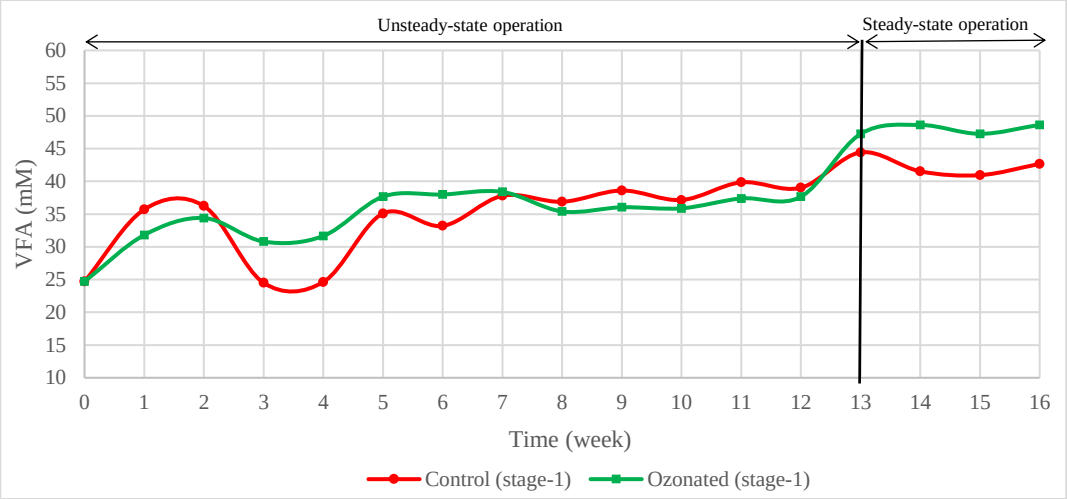

Figure 4.20 Total VFA changes in the first stages

4.2.7 Biogas production

The amount of biogas produced in two-stage anaerobic digesters was followed throughout the operation. The biogas values measured until the digesters reach steady-state condition and were shown in Figure 4.21. During this time, average daily biogas production observed in digesters was 463 and 483 mL for control and ozonated, respectively. After the steady-state achieved, biogas amount for control and ozonated digesters was measured daily to monitor the effect of ozonation, and the results were given in Figure 4.22 in terms of mL in Figure 4.23 in terms of mL Biogas/g VS. Ozone pretreatment initially caused a decrease in biogas production in the ozonated digester. The reason for this situation was thought to be the adaptation of microorganisms to ozonated feed sludge. This case was also stated by Le et al. (2019). This response was replaced by increase in biogas after the 4th day. From day 4 to day 28, daily biogas production was higher in ozonated digester compared to control digester. During ozone pretreatment period, daily average amount of biogas produced in control and ozonated digesters was 176 and 242 mL Biogas/g VS, respectively. Thus, ozone pretreatment with 0.06 g O_3/g TSS increased average daily biogas production by 38% in two-stage anaerobic digesters.

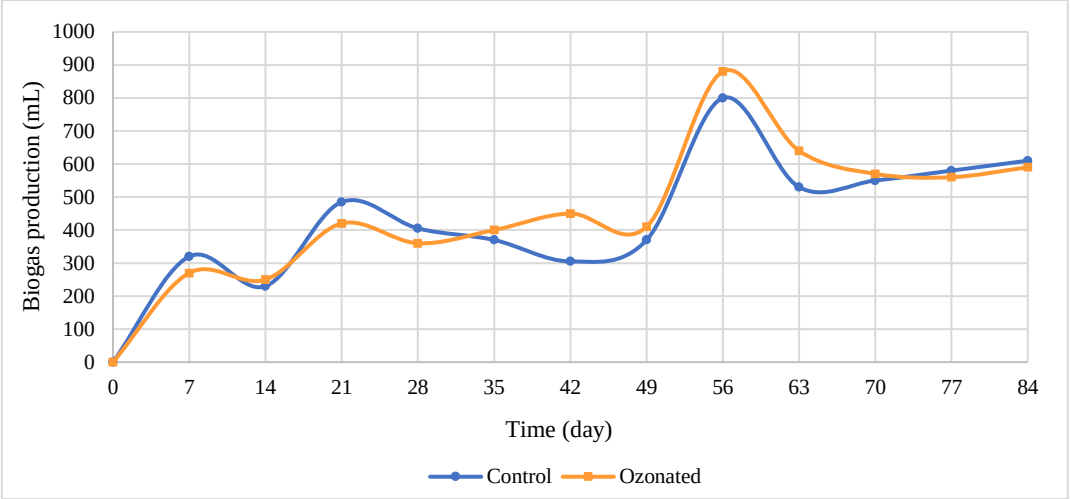

Figure 4.21 Daily biogas production during unsteady-state operation

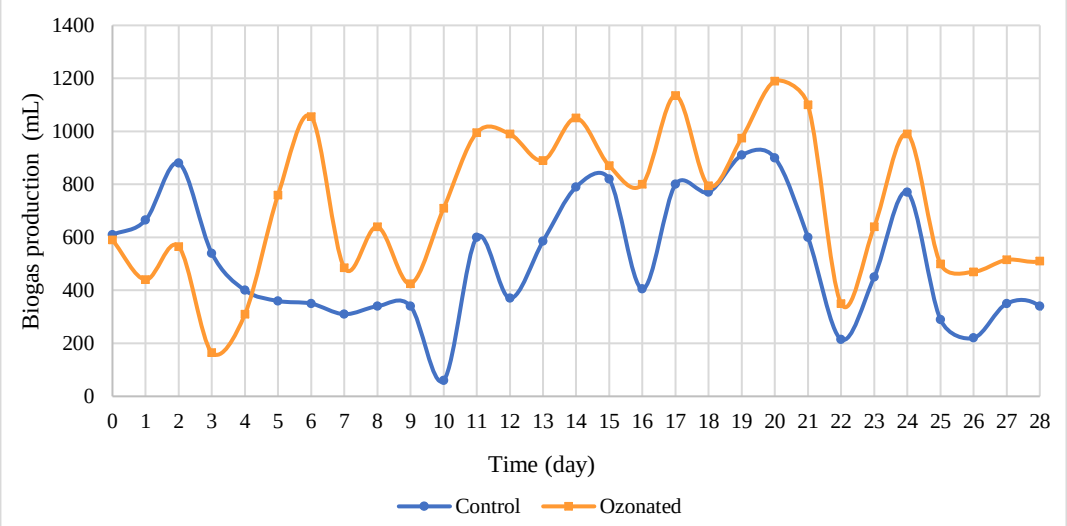

Figure 4.22 Daily biogas production during steady-state operation

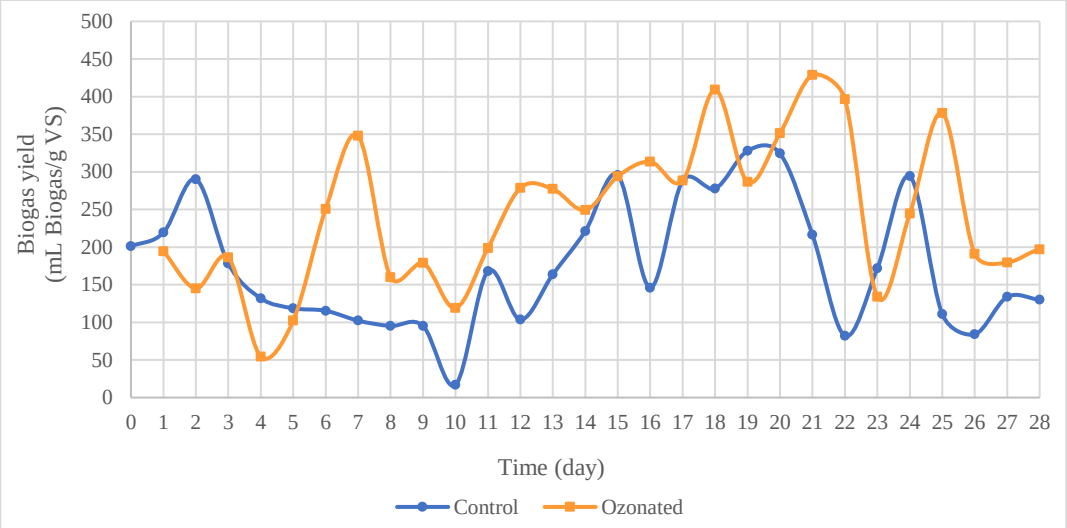

Figure 4.23 Daily biogas yield during steady-state operation

In Figures 4.24 and 4.25, cumulative biogas productions were given for control and ozonated digesters before and during steady-state in terms of mL, respectively. For the digesters operated without treatment up to 12 weeks, the cumulative biogas amount was 5555 and 5800 ml. During ozone pretreatment, the cumulative biogas yields obtained in control and ozonated digesters were given as mL Biogas/g VS in the Figure 4.26. The values were 5103 and 7029 mL Biogas/g VS for control and ozonated digesters. Thus, at the end of 28 days, 38% increase was achieved in the two-stage anaerobic digester with ozone pretreatment.

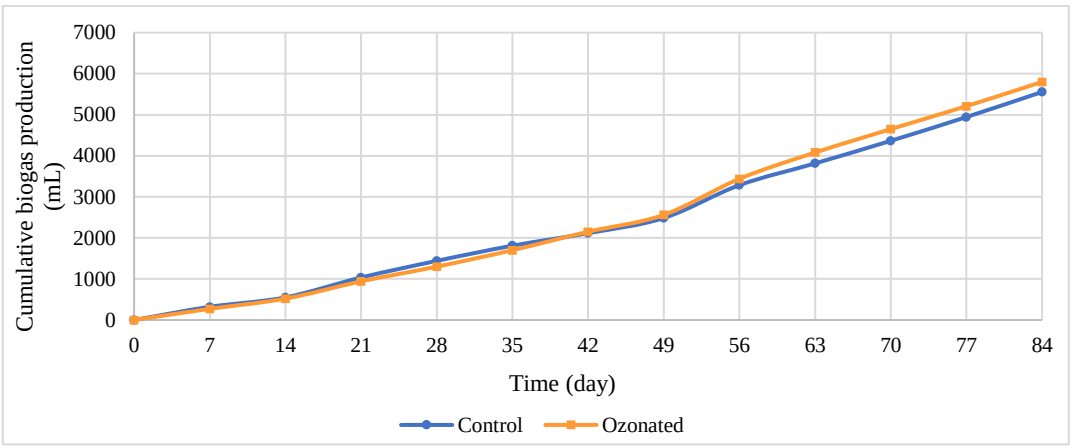

Figure 4.24 Cumulative biogas production during unsteady-state operation

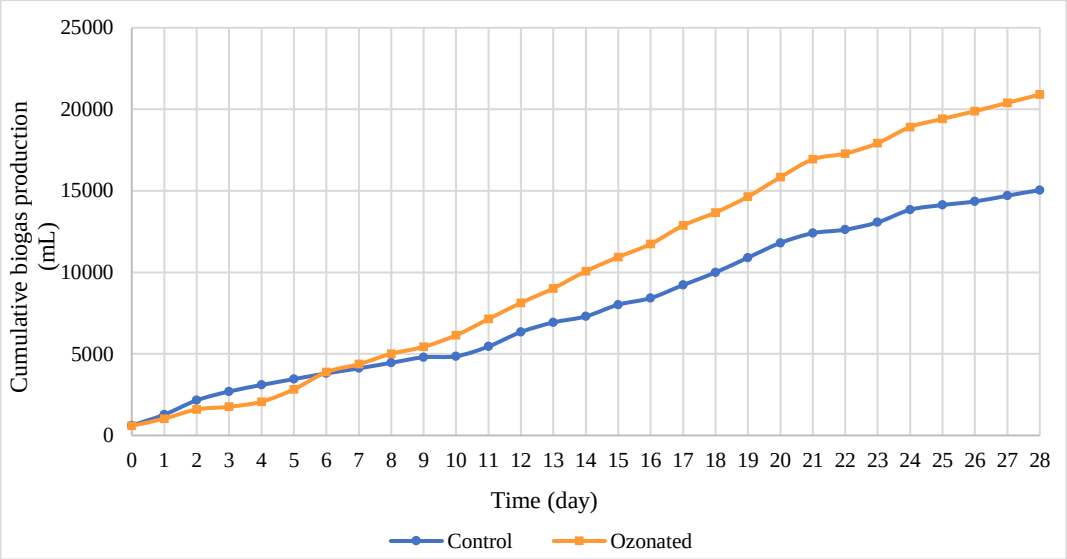

Figure 4.25 Cumulative biogas production during steady-state operation

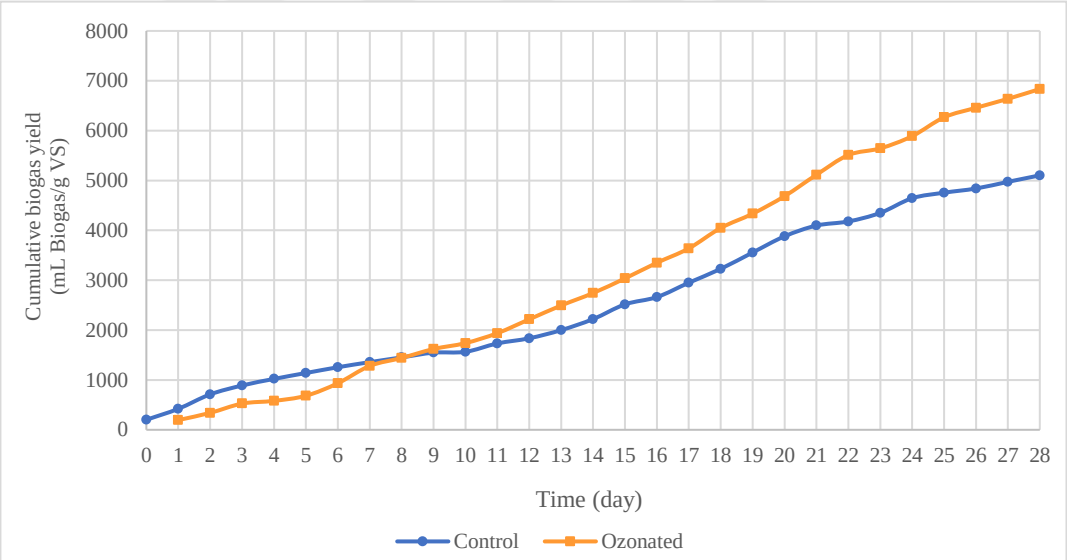

Figure 4.26 Cumulative biogas yield during steady-state operation

Although there are many studies in the literature where ozone pretreatment was applied to single-stage anaerobic digesters, there exists only two ozone pretreatment studies for two-stage anaerobic digesters. In the study conducted Yue et al. (2020), food wastes and glycerol trioleate were used as feedstock and subjected to ozone

pretreatment and methane production in two-stage anaerobic digesters. Although it was stated in the study that ozone pretreatment increased biogas production in two-stage digesters, numerical data were not given. In another study by conducted by Santos et al. (2018), coffee husks were used as substrate and subjected to ozone pretreatment. This attempt resulted in a 50% increase in biogas production. Besides, ozone pretreatment provides an increase in biogas has been concluded by many studies in the literature. For example, Le et al. (2019) obtained 52.9% increase in biogas production by using ozone dose of 0.02 g O_3/g TS. On the other hand, Silvestre et al. (2015) stated that ozone doses of 0.043 and 0.063 g O_3 / g TSS increased biogas by 5% and 21%, respectively. In another study, ozone doses of 0.04, 0.06 and 0.09 g O_3/g TS increased biogas production by 14%, 24% and 58%, respectively, (Bougrier et al., 2007). As mentioned in many studies, using different ozone doses and different configurations, increase in biogas production has been achieved in the AD process.

4.2.8 Methane Production

Methane productions in two-stage anaerobic digesters were followed throughout the operation. Methane content of biogas was determined by GC, both until the digesters became stable and during ozone pretreatment. For control and ozonated digesters, methane contents before ozone pretreatment were given in Figure 4.27. After the day 56, methane contents became more stable and were around 44±4.3% and 47±1.3% for control and ozonated digesters, respectively. Results of steady-state operation with ozone pretreatment were given in Figure 4.28. A sudden decrease was reported in methane content of ozonated digester with ozone pretreatment between days 0 and 2. As stated in biogas production, the decrease in methane content was due to adaptation process and initial reactions of microorganisms to ozone (Le et al., 2019). It has been observed that the methane content sharply increased starting from the 3rd day of ozone pretreatment. This increase lasted until day 14 and maximum methane contents were achieved for control and ozonated digesters 76% and 93%,

respectively. In other words, maximum methane content for two-stage anaerobic digesters in the current study was 77% and this value reached 93% with ozone pretreatment. A decrease in methane contents was observed for both digesters between day 14 and day 20. Methane contents changed steadily from day 20 to the end of operation. The average daily methane contents during 28 days for control and ozonated digesters were 55% and 64%, respectively.

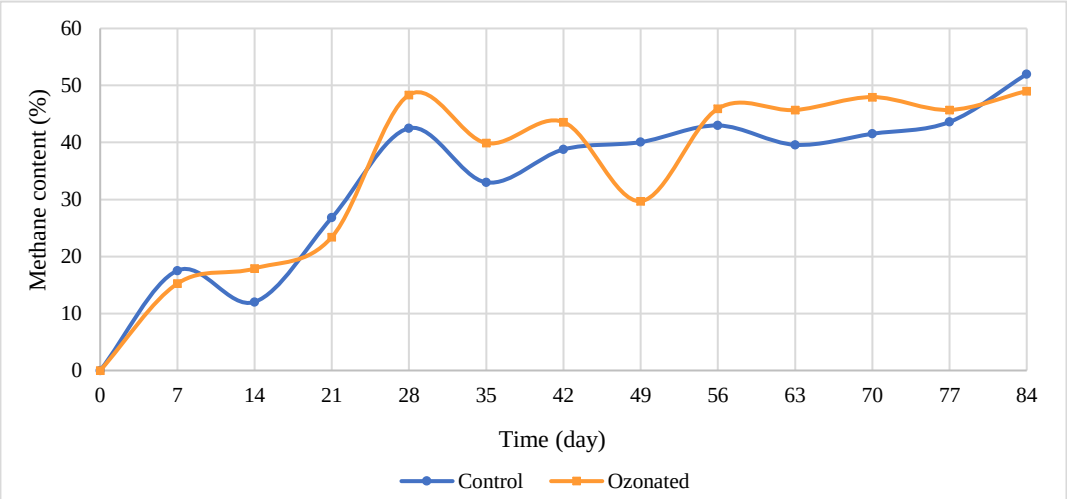

Figure 4.27 Methane content of biogas during unsteady-state operation

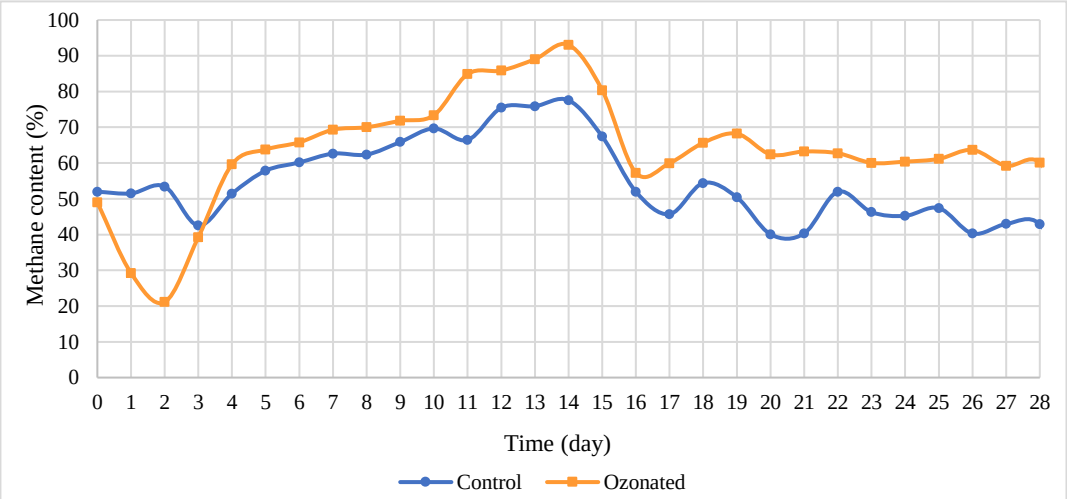

Figure 4.28 Methane content of biogas during steady-state operation

Daily methane productions were calculated with methane contents and biogas productions and given in Figure 4.29 and Figure 4.30. Also, for the steady-state operation period daily methane yield were calculated as mL CH_4/g VS and given in Figure 4.31. Daily methane productions showed similar trend with biogas productions. After the steady-state achieved, methane production of digesters was measured daily to monitor the effect of ozonation. As shown in Figure 4.30, daily methane production started to rise after the 3rd day similar to methane content. From day 4 to 28, more methane production was observed in ozonated digester compared to control digester on a daily basis. During ozone pretreatment period, the average daily methane yield was calculated as 94 and 159 mL CH_4/g VS for control and ozonated digesters, respectively. Thus, ozone pretreatment with 0.06 g O_3/g TSS increased average daily methane production by 69% in two-stage anaerobic digesters.

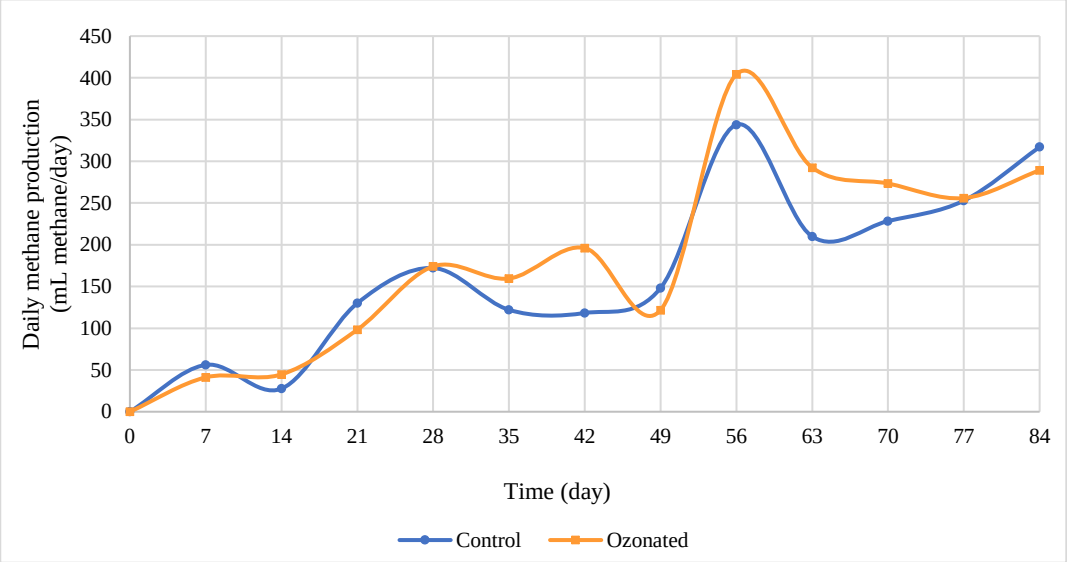

Figure 4.29 Daily methane production during unsteady-state operation

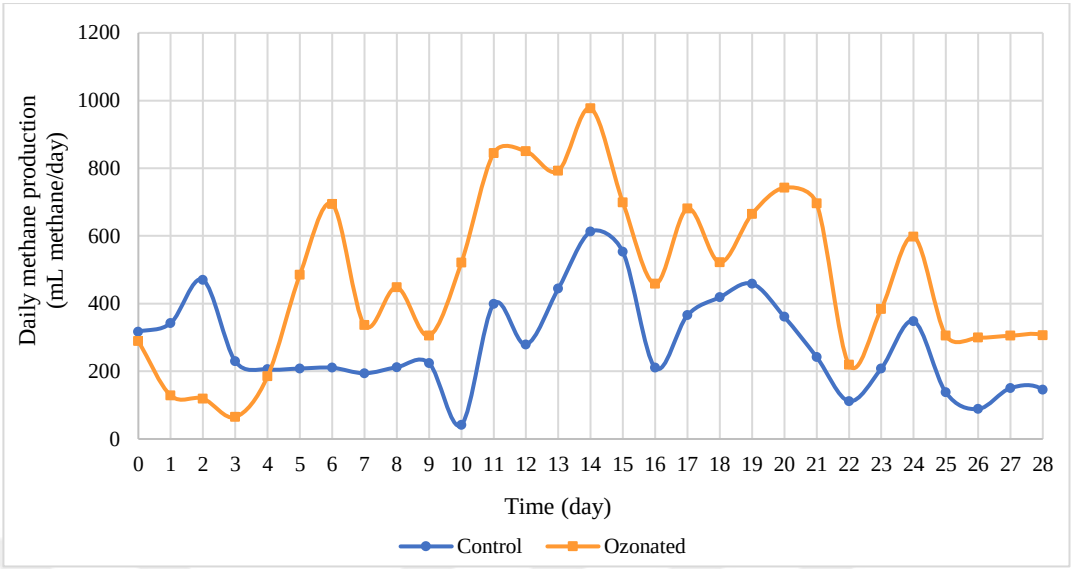

Figure 4.30 Daily methane production during steady-state operation

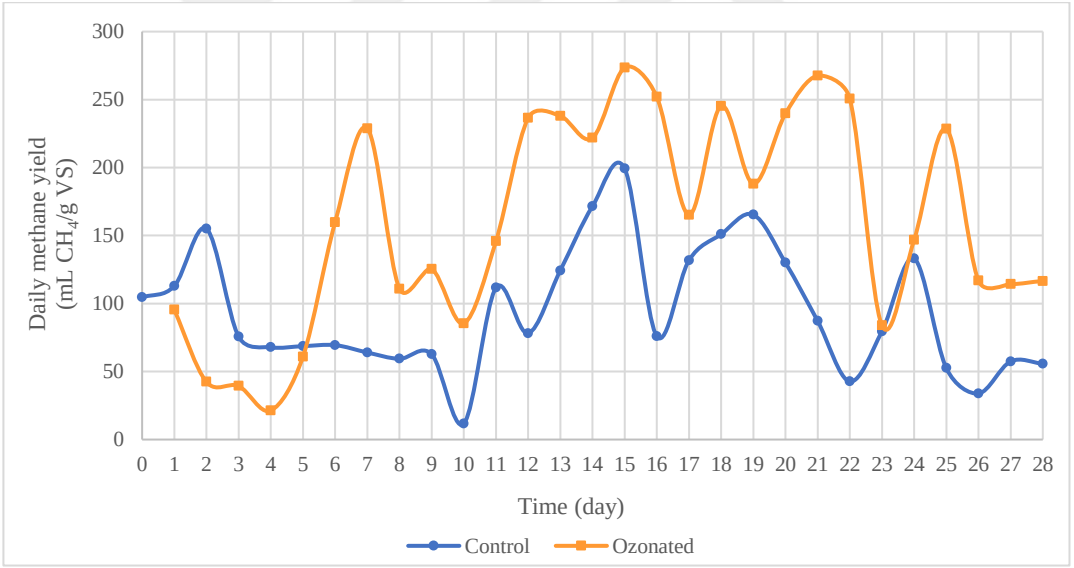

Figure 4.31 Daily methane yield during steady-state operation

In Figures 4.32 and Figure 4.33, the cumulative methane productions were given for control and ozonated digesters before and during steady-state conditions in terms of mL, respectively. Cumulative methane yields were given in Figure 4.34 for control and ozonated digester in terms of mL CH_4/g VS. During ozone pretreatment,

obtained cumulative methane yields were 2732 mL for control and 4618 mL CH_4/g VS for ozonated digester. Thus, at the end of 28 days, 69% increase was achieved in the two-stage anaerobic digester with ozone pretreatment.

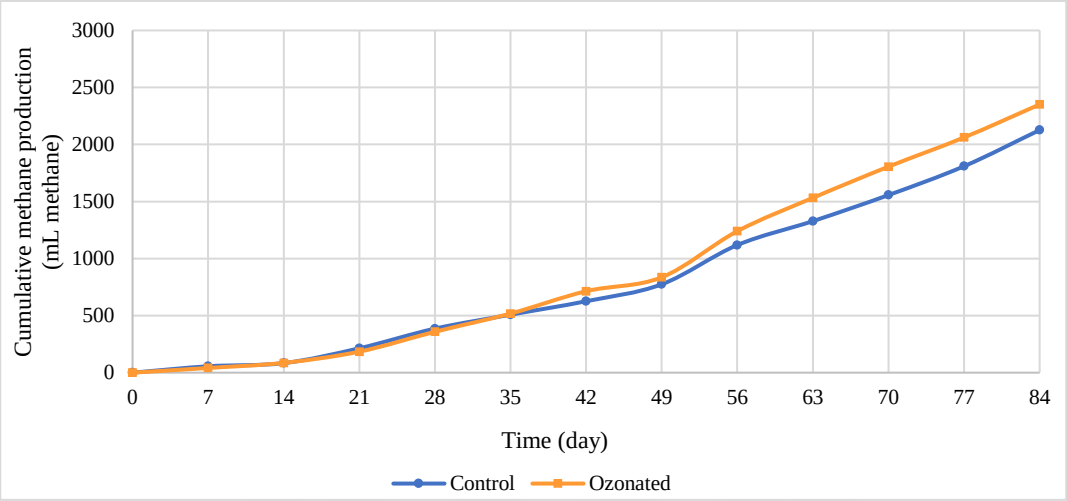

Figure 4.32 Cumulative methane production during unsteady-state

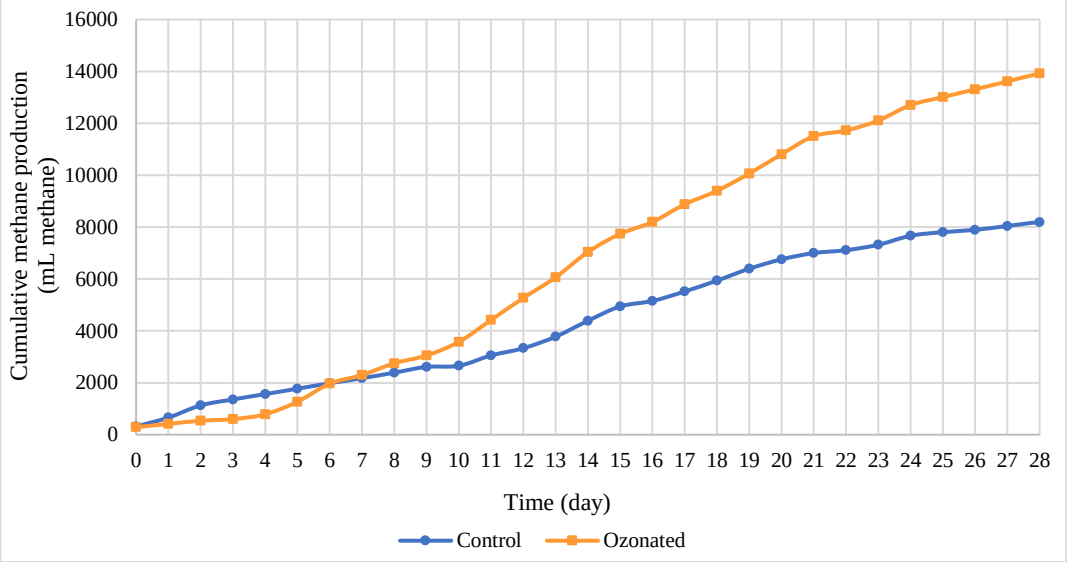

Figure 4.33 Cumulative methane production during steady-state operation

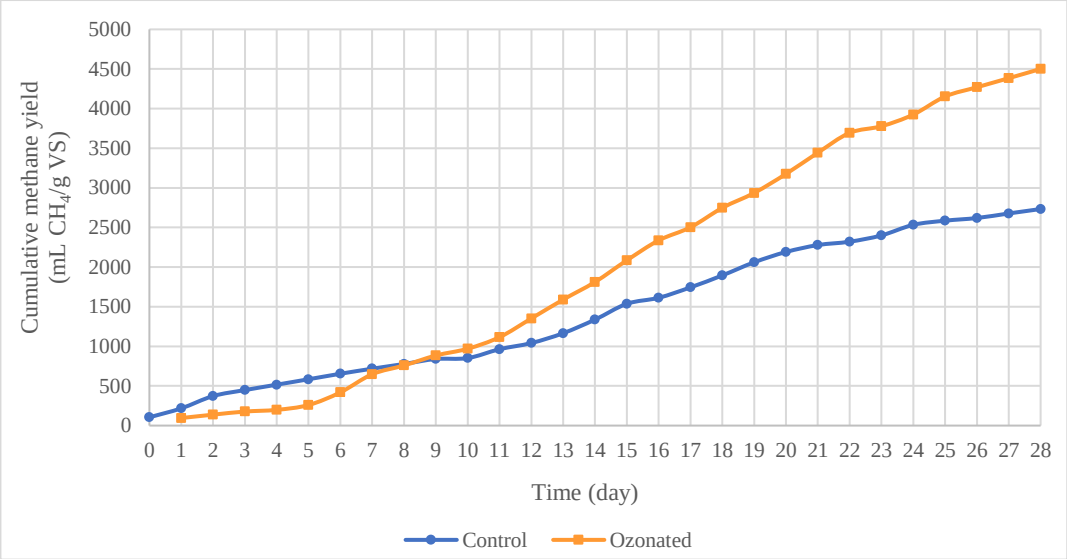

Figure 4.34 Cumulative methane yield during steady-state operation

As stated in the biogas production section, in the literature, studies including two-stage anaerobic digesters operated with ozone pretreatment are limited. In the study conducted by Yue et al. (2020), food waste and glycerol trioleate were used as feedstock for two-stage anaerobic digesters, and the effect of ozone pretreatment on methane production was observed. In the study, co-digestion of food waste and ozone pretreatment with 0.2 g O_3/TVS resulted in an 82% increase in methane production. In another study conducted by Santos et al. (2018), co-digestion of coffee husks and ozone pretreatment resulted in a 53% increase in methane production. In the current study, two-stage anaerobic reactors were fed with sludge subjected to 0.06 g O_3/g TSS ozone dose and resulted in 69% increase in methane production. Therefore, the effect of ozone pretreatment on methane production in two-stage anaerobic digesters was thought to depend on the feedstock and ozone dose.

Apart from mentioned studies, change in methane content and methane production were examined by using different pretreatment application and feed stocks in two-stage anaerobic digesters. Baldi et al. (2019) evaluated the combined digestion of food waste and activated sludge in two-stage anaerobic digesters and observed an average of 70% methane content. Kumanowska et al. (2017) proved that TSAD

helped achieving methane content of up to 80%. In another study, Ambrose et al. (2020) investigated the effects of hybrid microwave oxidative (MW-H_2O_2) pretreatment in two-stage semi-continuous anaerobic digesters. In that process, the highest methane percentage was %71 in methanogenesis stage and with pretreatment methane production was increased by %75. On the other hand, in the study conducted by Monlau et al. (2015), alkaline pretreatment, applied to two-stage anaerobic digesters, resulted in only 4% and 31% increase for methane content and methane production, respectively. Also, Ding et al. (2017) applied hydrothermal pretreatment to two-stage anaerobic digesters and was observed 32% increase in methane production. In the current study, ozone pretreatment was applied to sewage sludge and the highest methane percentage was obtained as 93%, while the average methane content was observed around 64±15.4%. Also, 69% increase was achieved in methane production with ozone pretreatment.

4.3 Microbial community structure of two- stage anaerobic digesters

After the analytical analyses, remaining sludge samples were fixed and stored for microbial analyses through FISH method during ozone pretreatment period. FISH experiments were carried out in triplicate as mentioned in Section 3.5. The results of the probe optimization experiments were given below. Also, results of FISH experiments performed under optimum conditions were discussed following sections.

4.3.1 Optimization of probes

At the beginning of study, FISH procedure was performed to determine the stringency conditions of the probes as mentioned in section 3.5.1. For this purpose, experiments were carried out using varying concentrations of FA and NaCl. In this section, results for MS1414 probe were given as representative. In the experiment, MS1414 probe was hybridized with *Methanosarcina* spp. (DSM2256) as positive

and *E. coli* (DH5α) as negative control. Optimization images taken under UV and B filters were given in Figure 4.35 for MS1414 probe. FA and NaCl concentrations, and corresponding signal intensity measurements were given in Table 4.3. Consequently, the optimum stringency conditions for MS1414 probe depending on the highest signal intensities were determined as 55% FA and 0.020 M NaCl concentration. The optimum hybridization stringency conditions were determined as indicated for each probe separately and the results were given in Table 4.4.

Table 4.3 Optimization for MS1414

		Positive control	**Negative control**
[FA] (%)	**[NaCl] (M)**	***Methanosarcina spp.* (DSM17206) (% signal intensity)**	***E. coli* (DH5α) (% signal intensity)**
45	0.040	73.87±2.06	3.24±1.34
50	0.028	69.92±0.45	3.48±0.63
55	0.020	86.03±1.51	2.67±1.42

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

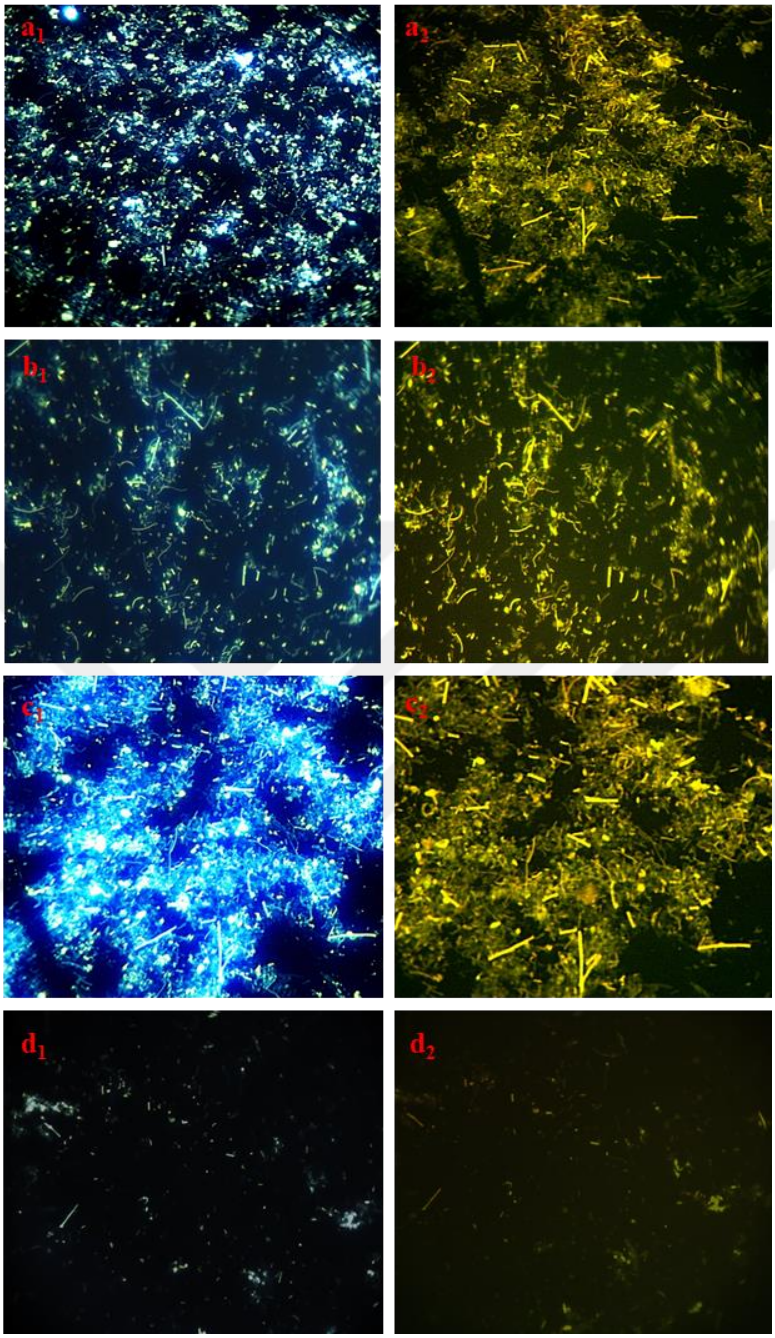

Figure 4.35 Representative optimization images for MS1414 probe hybridized with pure culture of *Methanosarcina* spp. (DSM2256) as positive control at 45% FA and 0.040 M NaCl (a_1-a_2), 50% FA and 0.028 M NaCl (b_1-b_2), 55% FA and 0.020 M NaCl (c_1-c_2); and with pure culture of *E. coli* (DH5α) as 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 hybridized MS1414 probe (a_2, b_2, c_2, d_2)

Table 4.4 Optimum stringency conditions of 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

Sludge samples were taken from control and ozonated digesters on days 1, 7, 14, and 21, after steady-state conditions achieved. Of these days, 14th day is the time when the highest methane content was reached for both digesters. After the 21st day, the production of methane and biogas in digesters became more stable and no major changes were observed in the values. In other words, according to the methane and biogas production conditions in the digesters the days were chosen to determine microbial changes. FISH experiments were performed as described in Section 3.5 by using the optimum stringency conditions specified in Table 4.3. Representative hybridization images of probes were given in Figure 4.36.

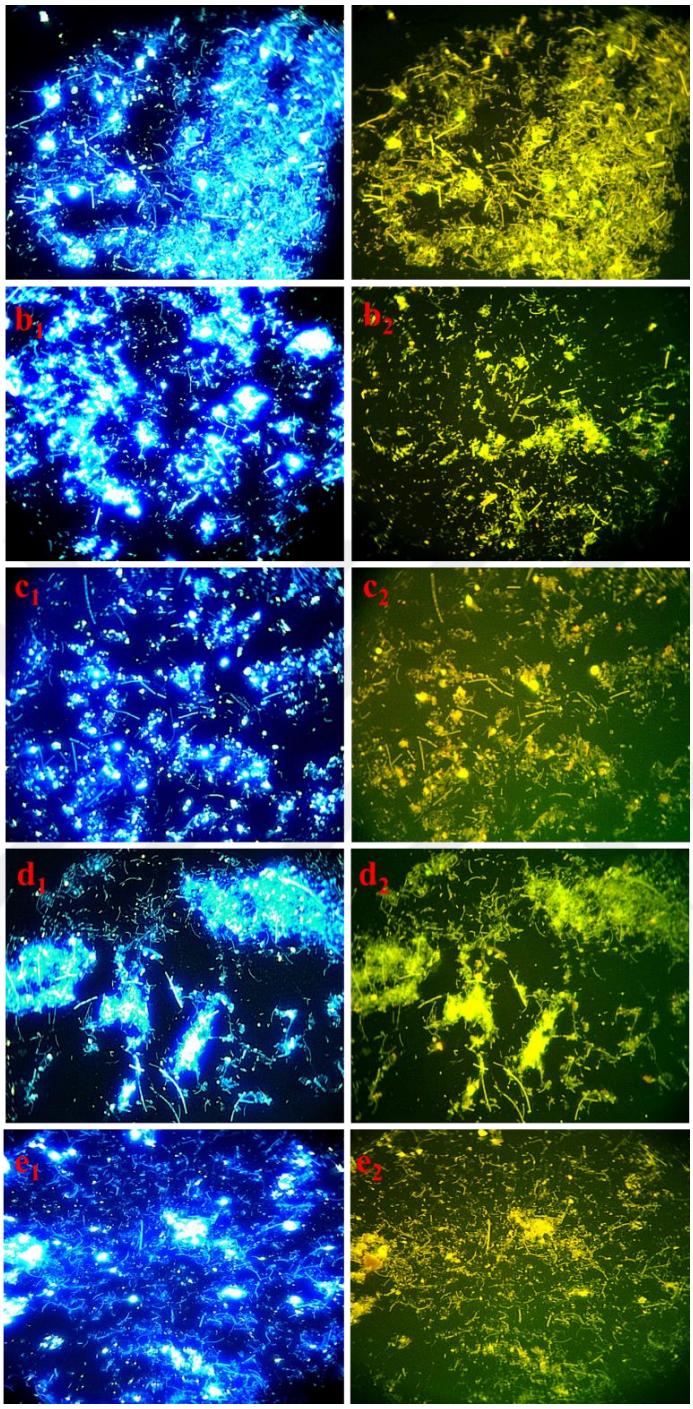

Figure 4.36 Representative hybridization images for methanogenic probes of MX825 (a_1-a_2), MS1414 (b_1-b_2), MG1200 (c_1-c_2), MB1174 (d_1-d_2) and ARC915 (e_1-e_2) at optimal stringency conditions. Total microorganisms stained with DAPI (a_1, b_1, c_1, d_1, e_1) and target microorganisms hybridized with FITC-labeled probes for corresponding areas (a_2, b_2, c_2, d_2, e_2)

The microscope images taken in the visualization step were analyzed with the ImageJ program as mentioned earlier. Relative abundancies of each microorganisms were calculated with the help of excel and only mean values of replicate experimental results were given in this section. Raw ImageJ data and detailed relative abundancies of images were provided in Appendix C. The results summarizing the microbial changes in two-stage anaerobic digesters were illustrated in Figures 4.37 and 4.38 for domain and microbial communities, respectively.

As indicated in Figure 4.37, until 21st day, bacterial population increased whereas archaeal population decreased in control digester. On the other hand, with the effect of ozone pretreatment, *Archaea* population increased from 43.1% to 61.2% on the 7th day and was dominated until the end of the operation in ozonated digester. Ozone pretreatment resulted in the highest methane production on the 14th day.

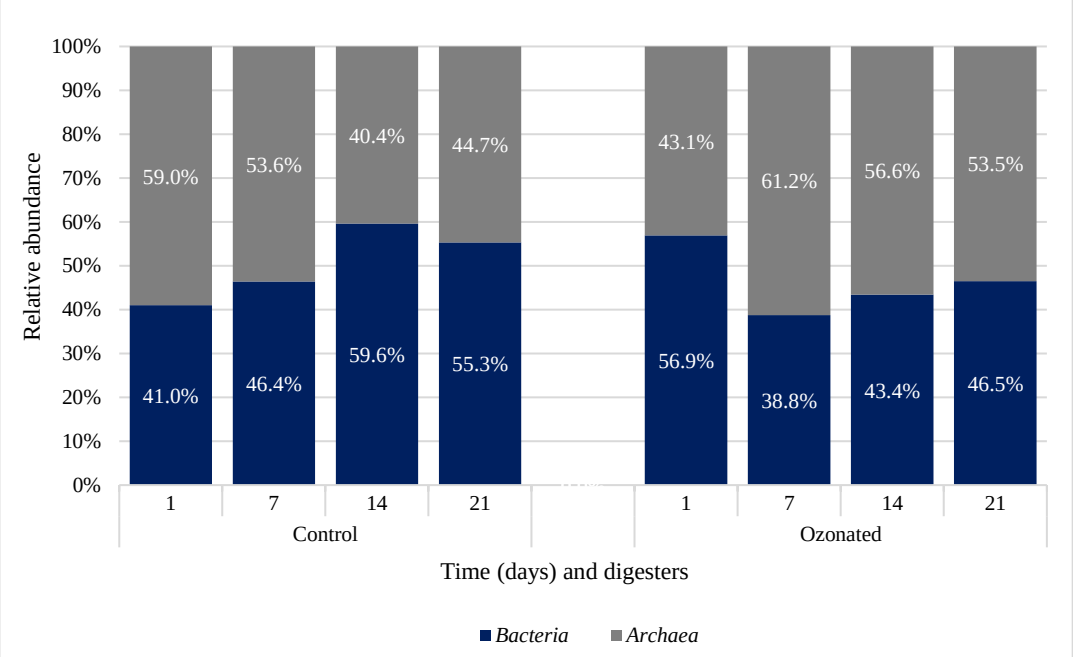

Figure 4.37 Microbial changes in two-stage anaerobic digesters on domain basis

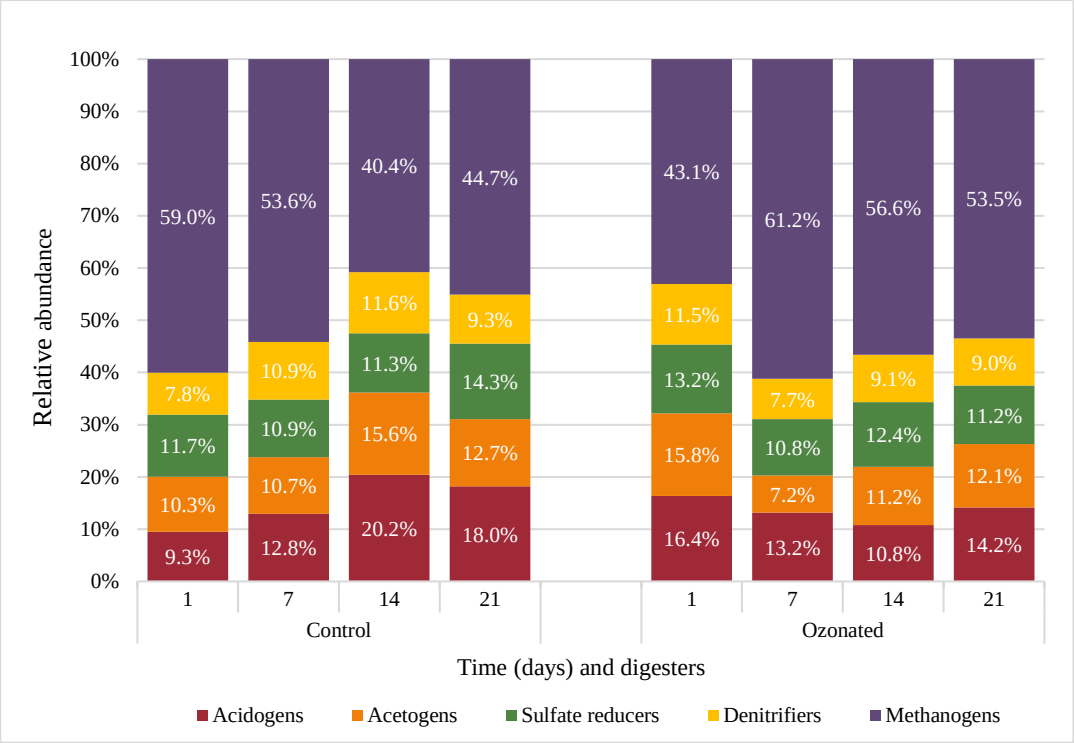

Figure 4.38 Microbial changes in two-stage anaerobic digesters on microbial community basis

In Figure 4.38, relative abundancies of microbial communities were indicated individually. An unexpected increase in the acidogens was observed on day 14 in control digester (from 12.8% to 20.2%). However, this situation did not cause an increase in total VFA and methane production reached the highest level in the digester. In ozonated digester, a decrease in acidogens was observed in day 7 and 14 with application of ozone treatment, followed by decreased VFA concentration. Overall, low acidogens populations were monitored in both digesters depending on the concept of the two-stages. In control digester, while acetogens increased from 10.7% to 15.6% on 14th day, no significant change was reported on the other days. In ozonated digester, acetogens decreased significantly on the 7th day (from 15.8% to 7.2%), increased to 11.2% on the 14th day, and remained stable afterwards.

In control digester, during the highest methane production, population of the sulfate reducers was lower than the day 1. It was observed that the percentage of sulfate

reducers increased from 11.3% to 14.3% between day 14 and 21 when methane production decreased. These findings were in parallel with Liu et al. (2018) who stated that sulfate reducers and methanogens compete for acetate and hydrogen and this negatively affects methane production. In ozonated digester, with the effect of ozone pretreatment, the population of sulfate reducers decreased from 13.2% to 10.8% between the day 1 and 7. Although the population of sulfate reducers increased from 10.8% to 12.4% between the 7^{th} and 14^{th} days, the methane production increased significantly. In denitrifiers population, an increase was observed in the control digester between day 1 and 14 and their population decreased from 11.6% to 9.3% on 21^{st} day. In ozonated digester, it was observed that denitrifiers population was decreased by ozone pretreatment on day 7 and day 14 when high methane production was observed in the digester. These findings were in parallel with Carlini et al. (2020) who stated that denitrifiers use acetate and hydrogen to complete nitrate reduction by competing with methanogens and this negatively affects methane production.

Lastly, methanogens were dominant community in both digesters entirely, it also provided evidence that the maximum methane production observed in digesters was between the 7^{th} and 14^{th} days. Moreover, an increase in the methanogen population with ozone pretreatment was achieved in ozonated digester compared to control digester. 0.06 g O_3/g TSS ozone dose increased the methanogenic activity, and this result was reflected in methane content and methane amount. Considering the distribution of microbial communities, two-stage concept was successfully implemented in the current study.

For both two-stage anaerobic digesters, microbial changes on genus/species basis were detailed in the sections below:

4.3.3 Microbial changes in control digester

Microbial changes of control digester were summarized in Figure 4.39.

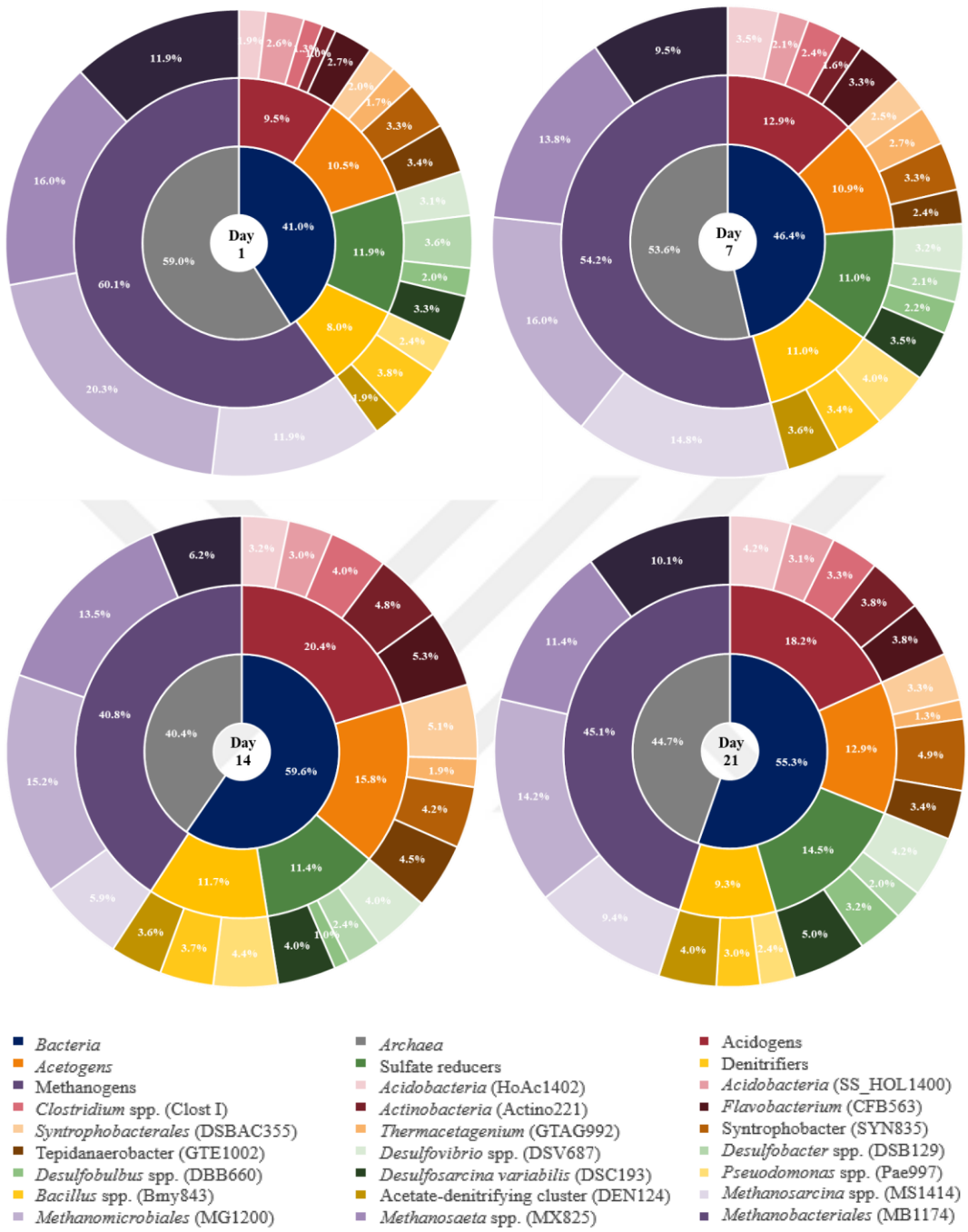

Figure 4.39 Microbial changes with respect to relative abundancies in control digester in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

During operation, on 14th day, an unexpected increase (from 9.5% to 20.84%) was observed in acidogens and they decreased slightly after the following days. Among the acidogens, *Flavobacterium* (CFB563) with 5.3% had the highest increase and followed by *Actinobacteria* (Actino221) with 4.8% and *Clostridium* spp. (Clost I) with 4.0%. This increase in acidogens did not cause VFA accumulation in the digesters, and maximum methane production was observed on day 14. In the digester, acetogens remained same on 7th day but increased on 14th day. On day 14, among acetogens, *Syntrophobacterales* (DSBAC355), *Syntrophobacter* (SYN835) and *Tepidanaerobacter* (GTE1002) increased, while *Thermacetagenium* (GTAG992) decreased in numbers.

No significant change was noticed in the population of sulfate reducing bacteria between day 1 and day 14. On the other hand, it was observed that sulfate reducers increased from 11.4% to 14.5% rapidly on the 21st day. By comparing the results of 14th and 21st day, population increase, among sulfate reducers, was mostly observed in *Desulfosarcina variabilis* (DSC193) (from 4.0% to 5.0%) and *Desulfobulbus* spp. (DBB660) (from 1.0% to 3.2%). This was interpreted as *Desulfosarcina variabilis* and *Desulfobulbus* spp. were responsible for the decrease in methane production in the control digester after day 14. Among denitrifiers, population of *Pseuodomonas* spp. (Pae997) increased on 7th and 14th days and decreased on 21st days. *Bacillus* spp. (Bmy843) with 3.4±0.33% population remained almost same during operation, while the Acetate-denitrifying cluster (DEN124) population increased from 1.9% to 4.0%.

In the control digester, a decrease in the methanogen population was noted between day 1 and day 14. Between 0 and 7 day, the highest decrease was seen in the population of *Methanomicrobiales*. Considering the daily methane production graph, the decrease, observed in methane production after the 2nd day, was originated from this situation. Although the daily methane production and methane content increased between the 7th and 14th days, the result was not reflected in the methanogen population. During this time, methane content reached its maximum value (76%) in the digester. *Methanomicrobiales* and *Methanosaeta* spp. (MX825), the

methanogens with the highest populations (15.2% and 13.5%), was found to be responsible for this result on 14th day. On the 21st day, *Methanosarcina* spp. (MS1414) and *Methanobacteriales* (MB1174) populations increased, while *Methanomicrobiales* and *Methanosaeta* spp. populations decreased. Throughout the operation, order of *Methanomicrobiales* was reported as the predominant methanogen in digestion with average 16.2% population.

According to the literature, *Methanosaeta* spp. and *Methanosarcina* spp. are the most dominant methanogens reported in two-stage anaerobic digesters (Gonzalez-Martinez et al., 2016; Botello Suárez et al., 2018). However, in the current study, *Methanomicrobiales* and *Methanosaeta* spp. were found as dominant methanogens.

4.3.4 Microbial changes in ozonated digester

Microbial changes of digester pretreated with 0.06 g O_3/g TSS ozone dose were summarized in Figure 4.40. During operation, there was an overall decrease in the acidogens population following by ozone pretreatment. *Flavobacterium* (CFB563), *Acidobacteria* (HoAc1402), and *Clostridium* spp. (Clost I) were affected by ozone pretreatment mostly, with a sharp decline in populations compared to other acidogens. The lowest acidogens population with 10.8% was reported on day 14 when the maximum methane production was reached. On the 21st day, population of acidogens was increased to 14.2% in ozonated digester and this was accounted for pH decrease and fast growth rate of acidogens as indicated by S. Li & Yang (2016). By comparing day 1 and 7, it was found that acetogens were negatively affected by ozone pretreatment and decreased dramatically. Among acetogens, *Syntrophobacterales* (DSBAC355) was impacted by ozone pretreatment and almost disappeared, with 0.9% population percentage.

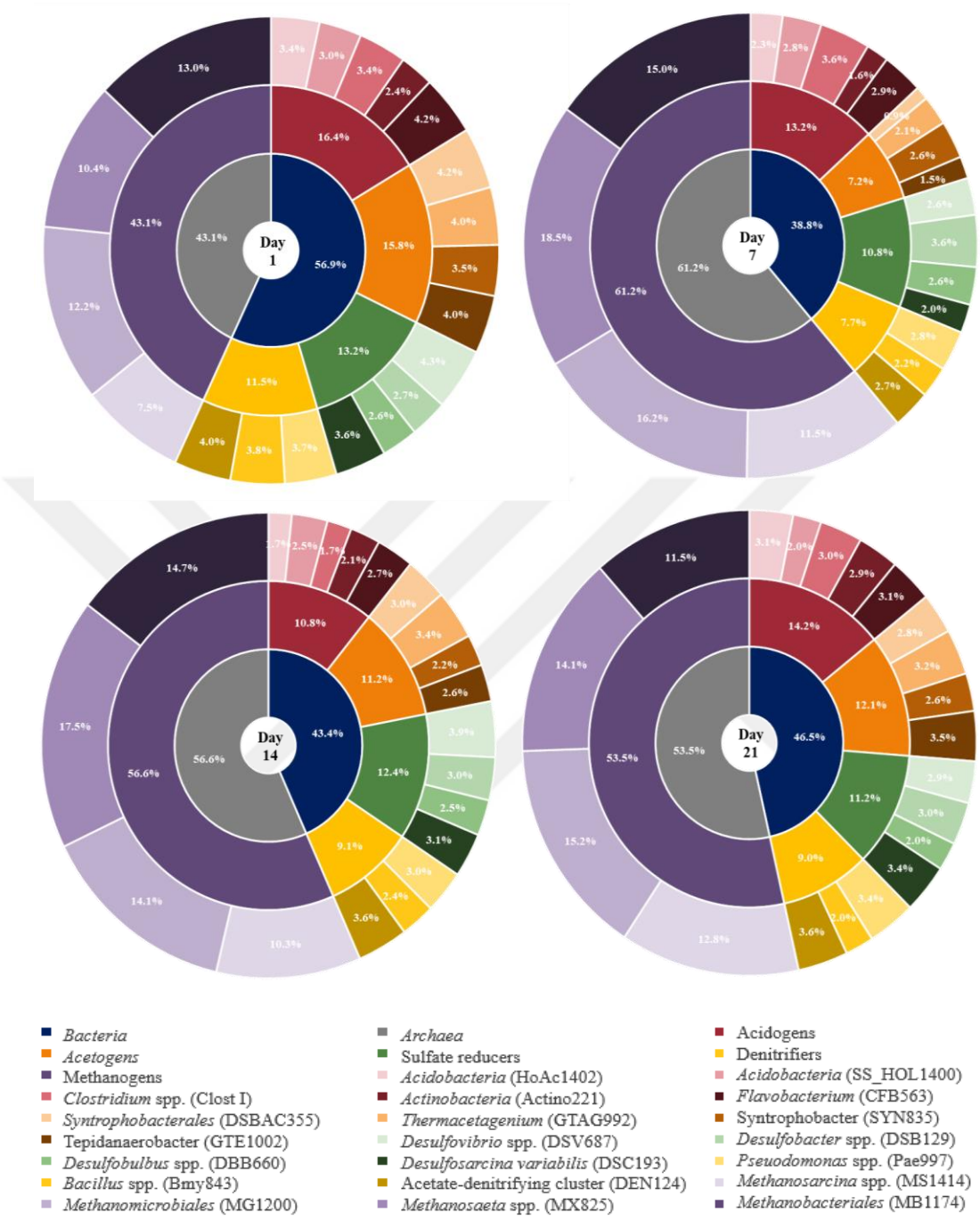

Figure 4.40 Microbial changes with respect to relative abundancies in the digester pretreated with 0.06 g O3/g TSS in terms of domain (inner circle), microbial community (middle circle) and genus/species (outer circle) levels

Population of sulfate reducing bacteria was decreased suddenly from 13.2% to 10.8% on the 7th day by ozone application. On day 7, ozone pretreatment caused a decrease in the population of *Desulfovibrio* spp. (DSV687) (from 4.3% to 2.6%) whereas *Desulfobacter* spp. (DSB129) population increased (from 2.7% to 3.6%) with the ozone pretreatment. Considering the inhibitory effect of sulfate reducers on methane production, it was concluded that the increase in *Desulfobacter* spp. did not hinder methane production.

Population of denitrifiers declined from 11.5% to 7.7% on day 7 and were around 9.1% on days 14 and 21. Acetate-denitrifying cluster population increased from 2.7% to 3.6% between 7th and 14th day when high methane production was observed. *Pseuodomonas* spp. and *Bacillus* spp. populations increased only 0.2% during this period. Considering the competitive relationship between methanogens and denitrifiers, it was concluded that Acetate-denitrifying cluster did not inhibit methane production.

Methane production increased rapidly up to day 14 and maximum methane content was achieved (93%) in the digester. Although methane production decreased after 14th day, stable changes were detected, and methane content varied between 64 – 69%. Depending on these changes, increases in the methanogen population were observed on the 7th and 14th days with 61.2% and 56.6%. Although methanogens experienced a small decrease with 53.5% on day 21, the values were always higher than day 1 with 43.1%. On 7th and 14th days, it was observed that *Methanosaeta* spp. (MX825) with 18.5% and 17.5%, respectively, were predominant among the methanogens with the highest percentage. *Methanosaeta* spp. were followed by *Methanobacteriales* (MB1174) with 14.7% and *Methanomicrobiales* (MG1200) with 14.1%. Similarly, in the study conducted by Botello Suárez et al. (2018), *Methanosaeta* spp. was found as a dominant methanogen in a two-stage upstream anaerobic sludge blanket reactor. Gonzalez-Martinez et al. (2016) also found *Methanosaeta* as predominant methanogen in a bench-scale two-stage anaerobic digester.

4.3.5 Comparison of methanogens

In order to understand the effect of ozone pretreatment on methanogens in two-stage anaerobic digesters, the methanogenic populations were illustrated in Figure 4.41 for control and ozonated digesters with corresponding daily methane productions. As illustrated in the figure the methane productions and methane contents of the digesters on day 1 were almost same, however, differences in methanogen distributions were observed in digesters. On day 1, *Methanomicrobiales* had the highest population with 19.9% in control digester, whereas *Methanobacteriales* had the highest population with 13.0% in ozonated digester. In the control digester, *Methanomicrobiales* were the dominant methanogen throughout the operation. On the other hand, in ozonated digester, the dominant methanogen has shifted from *Methanobacteriales* to *Methanosaeta* spp., with ozone pretreatment. This shift resulted in 38% and 69% increase in biogas and methane production, respectively.

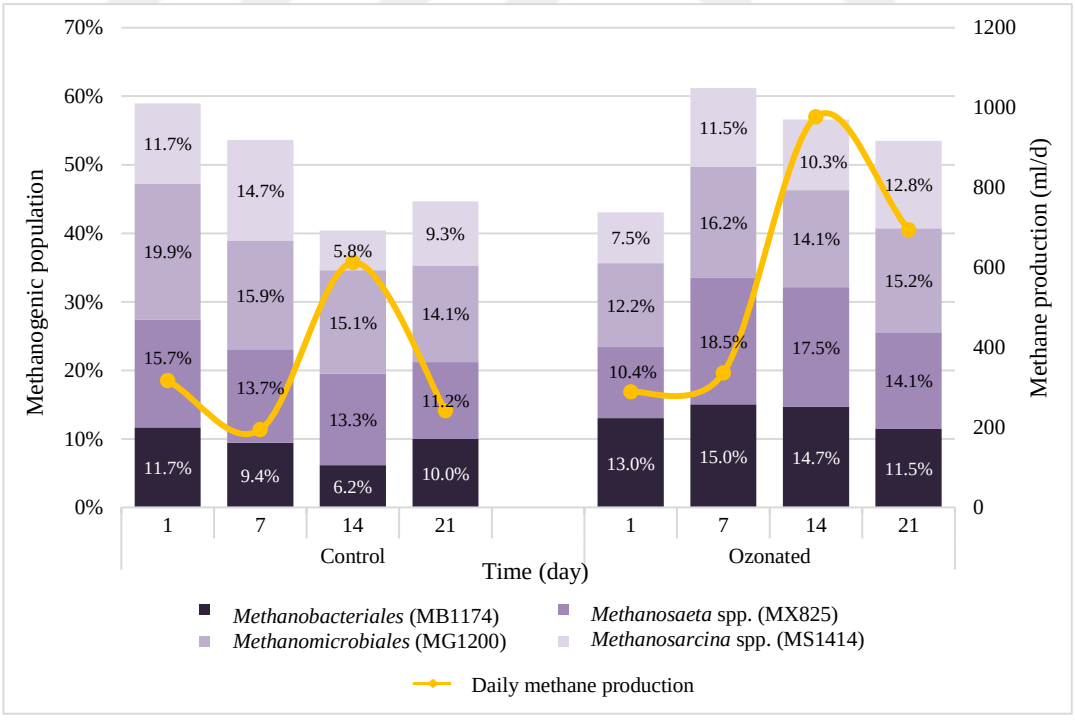

Figure 4.41 Changes in methanogenic population and daily methane production in anaerobic digesters

Methanosarcina spp. showed a similar trend in control and ozonated digesters. Population of *Methanosarcina* spp. increased on 7^{th} and 21^{st} days and decreased on 14^{th} day. *Methanosarcina* spp. were noted as methanogen with the lowest population throughout the study. This situation contradicts with some microbial analysis studies conducted in two-stage anaerobic digesters in the literature. In a study done by Shin et al. (2010), two-stage anaerobic digesters were operated with food waste and in microbial analysis, *Methanosarcina* spp. were reported as dominant methanogen in digesters. Similarly, cattle manure was digested in two-stage anaerobic digesters and it was concluded that *Methanosarcina* spp. were the dominant methanogen as a result of microbial analysis (Akyol et al., 2016).

In control and ozonated digesters, order of *Methanomicrobiales* and *Methanosaeta* spp. were reported as dominant methanogens in the study, respectively. Although 0.06 g O_3/g TSS ozone dose generally caused population increase in all methanogens, *Methanosaeta* spp. was found as the most affected methanogen. In two-stage digester with ozone pretreatment, dominant methanogen shifted from *Methanobacteriales* to *Methanosaeta* spp.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The aim of this study is to indicate the effect of ozone pretreatment on biogas/methane production and microbial community structure in mesophilic two-stage anaerobic digesters. Within the scope of the study, two-stage digesters were operated in series. One of the digesters was operated as a control reactor without any treatment, while the other was pretreated with 0.06 g O_3/g TSS ozone dose. Digesters were tracked throughout the operation in terms of pH, temperature, COD, TN, TS, VS, TSS, VSS and total VFA. Biogas and methane productions were monitored by water displacement method and GC. Microbial community structure was investigated by FISH. The results of the study pointed out that:

- The highest methane contents were achieved for untreated and ozonated digesters as %76 and %93, respectively.
- Two-stage anaerobic digester ozonated with 0.06 g O_3/g TSS ozone dose produced higher methane (69%) and biogas (38%) than untreated one.
- Ozonated two-stage anaerobic digester showed higher removal efficiencies of COD (63%), TS (49%), VS (62%), TSS (56%) and VSS (58%) than untreated one.
- Ozonation in two-stage anaerobic digestion led to the dominance of *Methanosaeta* spp. over *Methanomicrobiales*.
- Ozonation of two-stage anaerobic digester suppressed the dominant sulfate reducer *Desulfovibrio* spp.

5.2 Recommendations for future studies

- Two-stage anaerobic digester should be investigated by using other ozone doses.
- Feasibility studies should be carried out for pilot- and full-scale application of and ozone pretreatment in TSAD,
- Due to high cost of ozone and two-stage installation, cost/benefit analyses should be performed.
- Different pretreatment methods should be applied to two-stage anaerobic digesters for the comparison of ozone pretreatment.
- Combination of ozone pretreatment with other pretreatment methods should be investigated.
- Metagenomic analysis of methanogens should be determined for two-stage anaerobic digesters.

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APPENDICES

A. Calibration curves

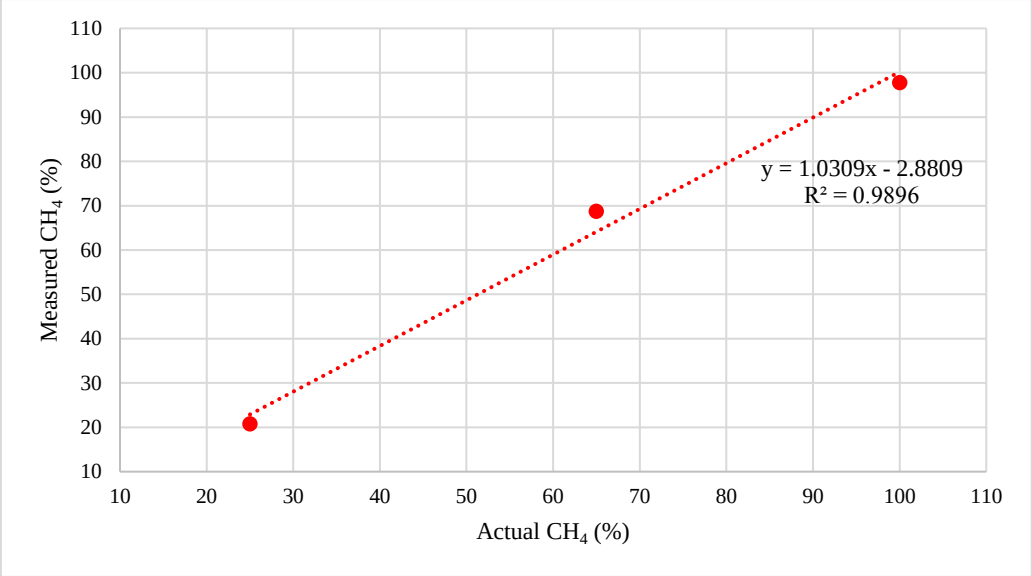

Figure A.1 GC calibration curve

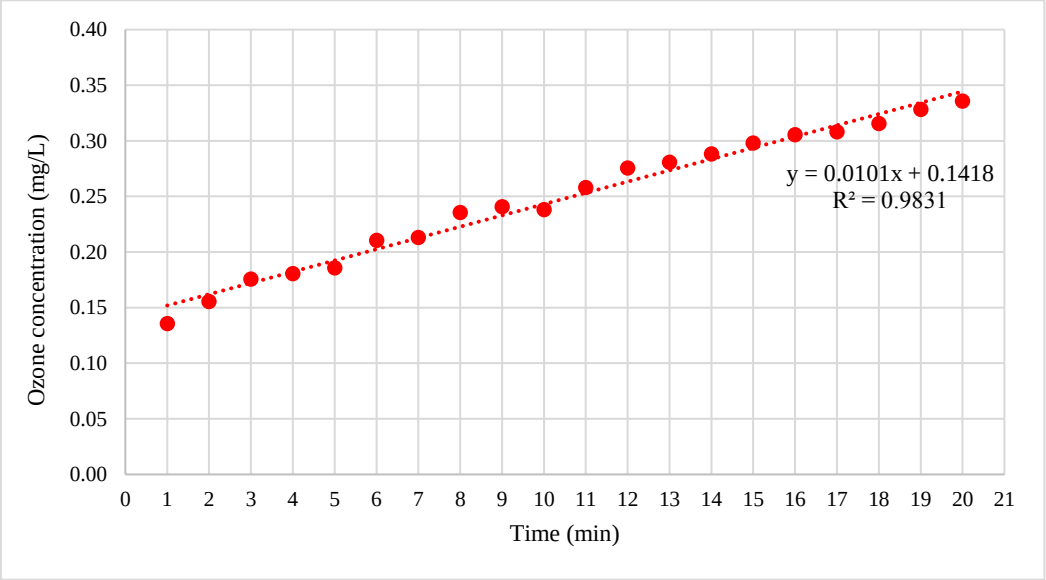

Figure A.2 Ozone calibration curve

Table B.1 Analytical analyses of control digester

Day	pH	T (°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	7.77±0.11	36.4±0.50	38.55±0.03	2.48±0.06	28.12±0.13	20.22±0.06	20.81±0.07	13.95±0.09	12.01±0.13	610	51.99±4.23
1	7.76±0.08	36.9±0.30	21.40±0.06	2.88±0.01	20.67±0.08	13.52±0.02	16.54±0.03	7.42±0.12	12.13±0.08	665	51.49±4.57
2	7.75±0.03	37.4±0.80	24.25±0.01	3.01±0.05	20.59±0.05	13.90±0.09	15.24±0.06	6.07±0.03	12.24±0.03	880	53.43±5.26
3	7.75±0.06	37.2±0.60	22.30±0.06	2.95±0.11	20.87±0.04	14.52±0.07	14.18±0.05	8.55±0.07	12.26±0.07	540	42.51±2.81
4	7.75±0.09	35.8±0.20	21.09±0.04	2.87±0.03	20.75±0.09	13.97±0.08	16.05±0.03	9.02±0.02	12.22±0.02	400	51.42±5.73
5	7.71±0.16	37.1±0.30	19.00±0.07	2.75±0.07	20.69±0.06	12.20±0.02	13.63±0.08	7.94±0.01	13.01±0.14	360	57.86±5.79
6	7.75±0.04	37.2±0.50	21.60±0.12	2.80±0.07	19.73±0.01	11.51±0.10	12.48±0.04	8.08±0.04	12.03±0.08	350	60.15±1.94
7	7.75±0.05	36.6±0.80	19.25±0.05	2.78±0.04	19.93±0.03	10.98±0.07	14.04±0.02	7.85±0.08	12.16±0.05	310	62.63±0.49
8	7.70±0.09	35.5±0.10	19.20±0.09	3.33±0.09	21.17±0.07	9.98±0.14	14.34±0.14	6.65±0.02	12.48±0.03	340	62.37±3.82
9	7.68±0.03	36.4±0.40	20.10±0.03	2.06±0.05	19.73±0.01	11.88±0.12	13.83±0.01	5.63±0.05	12.97±0.09	340	65.88±2.24
10	7.75±0.12	36.8±0.70	19.75±0.16	3.02±0.03	19.07±0.08	11.03±0.17	13.65±0.09	6.73±0.03	10.21±0.02	60	69.68±5.02
11	7.65±0.04	35.2±0.10	17.75±0.02	2.17±0.07	19.47±0.06	12.97±0.04	14.86±0.05	7.07±0.09	12.75±0.01	600	66.45±4.22
12	7.74±0.05	36.5±0.60	16.25±0.03	2.36±0.02	15.89±0.12	13.36±0.06	14.09±0.07	7.75±0.06	11.54±0.08	370	75.46±3.72
13	7.78±0.03	36.7±0.50	19.87±0.07	2.64±0.07	18.04±0.09	10.34±0.01	13.05±0.04	7.37±0.01	11.12±0.04	585	75.89±6.60
14	7.78±0.08	34.9±0.80	19.54±0.01	2.55±0.08	17.09±0.02	11.74±0.08	13.61±0.05	8.32±0.17	11.08±0.10	790	77.55±1.58
15	7.79±0.05	36.4±0.30	19.85±0.08	2.35±0.15	16.94±0.06	10.32±0.06	12.38±0.03	5.37±0.08	10.89±0.07	820	67.42±3.59
16	7.66±0.18	36.5±0.30	20.40±0.04	3.01±0.09	17.54±0.08	11.49±0.03	11.74±0.02	6.51±0.03	12.77±0.02	405	51.98±3.51
17	7.76±0.02	36.4±0.70	19.90±0.04	2.57±0.03	17.62±0.10	10.80±0.02	10.21±0.12	6.83±0.05	11.58±0.06	800	45.70±3.40
18	7.75±0.07	36.8±0.20	17.48±0.07	2.19±0.08	16.97±0.08	9.04±0.05	9.41±0.02	6.51±0.02	11.65±0.02	770	54.41±5.33
19	7.75±0.01	35.9±0.10	18.75±0.06	2.84±0.16	17.05±0.14	8.80±0.07	10.31±0.08	6.12±0.06	11.78±0.09	910	50.42±3.71
20	7.74±0.05	37.0±0.50	17.65±0.04	3.72±0.03	17.67±0.08	9.76±0.07	12.42±0.07	6.07±0.06	11.81±0.03	900	40.08±5.57

T, Temperature; 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 measurements.

Table B.1 cont'd

Day	pH	T (°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 (%)
21	7.72±0.13	35.8±0.30	19.20±0.02	2.52±0.07	16.07±0.03	10.87±0.11	11.40±0.05	6.74±0.08	12.07±0.07	600	40.32±0.55
22	7.81±0.08	35.8±0.70	18.65±0.04	2.46±0.06	19.64±0.07	10.19±0.03	13.05±0.01	7.10±0.07	9.88±0.01	215	51.99±5.25
23	7.70±0.05	35.0±0.40	18.25±0.09	2.36±0.04	18.40±0.09	10.73±0.08	14.65±0.16	7.70±0.02	10.45±0.02	450	46.27±4.46
24	7.62±0.15	36.2±0.90	18.80±0.05	2.15±0.03	18.64±0.05	8.94±0.09	11.07±0.04	5.67±0.11	10.87±0.05	770	45.23±1.87
25	7.66±0.01	35.3±0.20	17.95±0.08	2.34±0.06	18.08±0.20	8.08±0.05	19.21±0.01	6.04±0.05	10.45±0.05	290	47.41±3.55
26	7.55±0.04	35.8±0.50	17.85±0.03	2.39±0.02	17.65±0.01	10.90±0.02	11.08±0.03	6.32±0.10	11.93±0.13	220	40.29±3.48
27	7.67±0.07	37.3±0.50	18.01±0.02	3.52±0.05	17.08±0.04	9.73±0.06	9.48±0.10	7.12±0.04	11.48±0.06	350	42.96±2.65
28	7.65±0.09	36.1±0.40	18.74±0.06	2.34±0.01	16.99±0.06	8.98±0.04	10.08±0.02	6.57±0.07	11.51±0.08	340	42.92±6.44

T, Temperature; 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 measurements.

Table B.2 Analytical analyses of ozonated digester

Day	pH	T (°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	7.76±0.08	36.8±0.30	38.55±0.08	2.67±0.05	23.12±0.02	20.22±0.06	20.81±0.11	13.95±0.07	12.54±0.01	590	49.02±0.05
1	7.74±0.03	37.4±0.20	24.75±0.05	2.58±0.06	19.70±0.08	11.78±0.03	17.39±0.08	8.97±0.06	14.07±0.09	440	29.24±0.06
2	7.74±0.04	37.1±0.60	23.80±0.14	3.73±0.08	21.02±0.06	13.97±0.06	16.94±0.04	8.56±0.03	14.79±0.05	565	21.13±0.07
3	7.76±0.01	37.9±0.20	25.45±0.09	2.28±0.18	21.16±0.14	12.47±0.11	16.22±0.07	8.97±0.04	13.25±0.04	165	39.24±0.03
4	7.74±0.09	34.9±0.10	23.80±0.02	2.82±0.09	20.73±0.11	10.47±0.03	17.84±0.06	7.97±0.08	12.57±0.07	310	59.63±0.06
5	7.58±0.05	35.8±0.30	17.60±0.03	2.97±0.05	19.98±0.07	9.89±0.05	15.30±0.04	8.01±0.03	12.86±0.02	760	63.78±0.04
6	7.61±0.05	37.6±0.90	21.90±0.07	3.09±0.07	19.33±0.08	9.97±0.06	12.79±0.03	8.30±0.05	13.04±0.13	1055	65.78±0.03
7	7.70±0.11	37.1±0.10	21.50±0.05	2.31±0.01	16.38±0.03	8.93±0.09	14.37±0.06	6.09±0.12	12.79±0.08	485	69.32±0.02
8	7.63±0.02	34.9±0.70	17.85±0.03	2.41±0.04	14.73±0.05	9.93±0.02	12.97±0.02	5.07±0.08	12.81±0.08	640	70.00±0.06
9	7.59±0.07	37.5±0.30	13.75±0.06	2.19±0.02	12.13±0.06	8.55±0.07	10.57±0.05	5.95±0.15	13.26±0.06	425	71.81±0.03
10	7.67±0.03	37.4±0.60	14.87±0.02	2.80±0.03	13.98±0.04	10.07±0.06	11.03±0.10	5.93±0.06	12.57±0.01	710	73.38±0.04
11	7.67±0.17	37.1±0.40	14.75±0.08	2.83±0.07	14.23±0.09	11.62±0.01	10.82±0.03	6.07±0.04	12.56±0.03	995	84.87±0.08
12	7.71±0.08	35.7±0.30	14.85±0.13	2.99±0.01	13.07±0.02	9.17±0.01	10.83±0.05	7.48±0.02	12.077±0.05	990	85.87±0.03
13	7.66±0.06	37.2±0.20	13.74±0.03	2.57±0.12	15.50±0.07	11.84±0.17	11.50±0.06	5.47±0.05	12.59±0.07	890	89.03±0.15
14	7.65±0.05	35.8±0.10	12.98±0.05	2.80±0.05	15.77±0.02	9.27±0.09	12.21±0.02	6.45±0.07	11.475±0.02	1050	93.06±0.08
15	7.68±0.01	36.7±0.30	17.10±0.10	2.83±0.08	15.41±0.02	9.435±0.03	11.43±0.09	7.50±0.05	12.97±0.07	870	80.36±0.05
16	7.70±0.04	35.7±0.60	18.50±0.20	2.97±0.02	14.31±0.06	8.06±0.08	12.87±0.01	5.92±0.05	12.07±0.05	800	57.27±0.09
17	7.69±0.09	36.8±0.80	18.74±0.01	2.49±0.04	12.54±0.05	8.63±0.11	11.01±0.03	5.97±0.09	12.10±0.03	1135	59.97±0.01
18	7.63±0.08	36.0±0.40	15.75±0.15	2.68±0.07	12.89±0.03	9.21±0.06	9.90±0.07	6.83±0.01	12.98±0.08	795	65.65±0.07
19	7.69±0.06	37.1±0.70	15.00±0.09	2.67±0.03	16.89±0.08	11.61±0.05	13.55±0.11	6.27±0.12	12.44±0.06	975	68.21±0.02
20	7.78±0.01	35.9±0.50	16.45±0.08	3.48±0.08	15.84±0.10	9.17±0.01	11.06±0.16	5.81±0.08	11.59±0.24	1190	62.41±0.06

T, Temperature;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 measurements.

Table B.2 cont'd

Day	pH	T (°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 (%)
21	7.77±0.07	36.7±0.20	18.55±0.05	2.44±0.06	18.37±0.01	9.45±0.04	10.01±0.03	6.93±0.05	11.47±0.06	1100	63.23±0.04
22	7.79±0.07	36.5±0.90	17.65±0.04	2.31±0.05	17.49±0.08	11.41±0.07	14.78±0.05	6.08±0.07	11.45±0.03	350	62.74±0.03
23	7.77±0.04	36.7±0.80	17.50±0.07	2.21±0.20	16.65±0.13	8.96±0.04	13.20±0.02	65.63±0.01	10.96±0.04	640	60.05±0.01
24	7.69±0.02	36.0±0.30	15.77±0.02	2.08±0.02	15.41±0.03	11.73±0.22	11.83±0.08	7.56±0.04	11.12±0.08	990	60.40±0.08
25	7.67±0.09	36.1±0.10	16.65±0.03	2.51±0.07	16.57±0.05	10.15±0.06	9.66±0.08	6.84±0.02	11.26±0.03	500	61.16±0.12
26	7.62±0.05	35.9±0.10	17.08±0.01	2.40±0.05	15.36±0.07	9.03±0.05	9.06±0.09	6.73±0.03	12.45±0.05	470	63.65±0.18
27	7.68±0.06	35.9±0.60	14.80±0.06	2.97±0.05	15.52±0.02	8.87±0.03	10.97±0.01	6.11±0.08	11.00±0.02	515	59.23±0.05
28	7.66±0.01	35.5±0.40	14.31±0.12	2.55±0.09	14.4±0.04	8.73±0.08	10.86±0.06	5.76±0.05	11.70±0.09	510	60.11±0.04

T, Temperature;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 measurements.

Table C.1 Intensity analyses of control digester

		Raw data obtained from ImageJ* (%intensities)			
Microbial community	**Target microorganism and probe**	**Day 1**	**Day 7**	**Day 15**	**Day 21**
Acidogens	*Acidobacteria* (HoAc1402)	53.06%±0.23	80.98%±0.09	55.16%±0.48	73.67%±0.05
	Acidobacteria (SS_HOL1400)	67.05%±0.21	74.49%±0.13	64.85%±0.15	73.05%±0.23
	Clostridium spp. (Clost I)	76.76%±0.35	61.57%±0.13	64.18%±0.16	61.95%±0.11
	Actinobacteria (Actino221)	36.95%±0.16	46.77%±0.35	73.72%±0.27	69.43%±0.32
	Flavobacterium (CFB563)	67.68%±0.18	77.82%±0.26	79.45%±0.12	69.48%±0.17
Acetogens	*Syntrophobacterales* (DSBAC355)	56.18%±0.19	63.26%±0.18	76.79%±0.15	61.82%±0.26
	Thermacetagenium (GTAG992)	50.61%±0.11	66.46%±0.09	40.40%±0.24	36.86%±0.23
	Syntrophobacter (SYN835)	79.10%±0.09	77.98%±0.15	66.78%±0.24	83.10%±0.13
	Tepidanaerobacter (GTE1002)	81.21%±0.13	60.37%±0.26	70.23%±0.22	63.57%±0.15
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	74.46%±0.27	76.78%±0.23	63.93%±0.29	74.40%±0.18
	Desulfobacter spp. (DSB129)	84.66%±0.15	55.99%±0.20	45.50%±0.18	46.10%±0.16
	Desulfobulbus spp. (DBB660)	54.52%±0.14	57.79%±0.12	29.96%±0.23	60.74%±0.28
	Desulfosarcina variabilis (DSC193)	78.31%±0.10	80.75%±0.13	64.14%±0.14	84.98%±0.13
Denitrifiers	*Pseuodomonas* spp. (Pae997)	62.44%±0.21	90.61%±0.28	69.12%±0.34	50.65%±0.23
	Bacillus spp. (Bmy843)	86.86%±0.32	79.92%±0.11	60.55%±0.29	58.50%±0.29
	Acetate-denitrifying cluster (DEN124)	52.73%±0.08	83.66%±0.21	59.41%±0.17	71.07%±0.14
Methanogens	*Methanosarcina* spp. (MS1414)	56.02%±0.16	66.47%±0.12	39.70%±0.08	63.74%±0.12
	Methanomicrobiales (MG1200)	81.75%±0.37	70.47%±0.27	74.32%±0.16	86.08%±0.15
	Methanosaeta spp. (MX825)	68.60%±0.25	63.14%±0.14	67.86%±0.22	72.68%±0.41
	Methanobacteriales (MB1174)	55.99%±0.16	48.85%±0.20	41.09%±0.29	66.73%±0.26
Archaea	*Archaea* (ARC915)	71.77%±0.28	65.75%±0.04	44.73%±0.18	56.24%±0.12
Bacteria	*Bacteria* (EUBmix)	55.87%±0.39	59.22%±0.19	57.42%±0.14	64.98%±0.15
	Negative control (NONEUB)	19.45%±0.10	17.30%±0.34	18.08%±0.26	19.76%±0.17

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; ±, standard deviation

Table C.2 Relative abundancy (%) of control digester

		Relative abundancy from processed data*			
Microbial community	**Target microorganism and probe**	**Day 1**	**Day 7**	**Day 15**	**Day 21**
Acidogens	*Acidobacteria* (HoAc1402)	1.84%±0.02	3.44%±0.01	3.18%±0.05	4.13%±0.01
	Acidobacteria (SS_HOL1400)	2.60%±0.02	2.09%±0.00	3.01%±0.06	3.08%±0.01
	Clostridium spp. (Clost I)	1.30%±0.01	2.39%±0.00	3.96%±0.02	3.23%±0.00
	Actinobacteria (Actino221)	0.96%±0.01	1.59%±0.04	4.77%±0.02	3.80%±0.01
	Flavobacterium (CFB563)	2.63%±0.01	3.27%±0.00	5.27%±0.04	3.81%±0.02
Acetogens	*Syntrophobacterales* (DSBAC355)	2.01%±0.03	2.48%±0.02	5.04%±0.01	3.22%±0.01
	Thermacetagenium (GTAG992)	1.70%±0.01	2.66%±0.01	1.91%±0.01	1.31%±0.01
	Syntrophobacter (SYN835)	3.26%±0.00	3.28%±0.01	4.18%±0.03	4.85%±0.01
	Tepidanaerobacter (GTE1002)	3.37%±0.01	2.33%±0.01	4.47%±0.04	3.35%±0.00
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	3.00%±0.01	3.21%±0.02	3.93%±0.03	4.18%±0.00
	Desulfobacter spp. (DSB129)	3.56%±0.02	2.09%±0.01	2.35%±0.08	2.02%±0.00
	Desulfobulbus spp. (DBB660)	1.92%±0.00	2.19%±0.01	1.02%±0.01	3.14%±0.03
	Desulfosarcina variabilis (DSC193)	3.22%±0.00	3.43%±0.00	3.95%±0.01	4.99%±0.02
Denitrifiers	*Pseuodomonas* spp. (Pae997)	2.35%±0.03	3.96%±0.03	4.38%±0.02	2.36%±0.02
	Bacillus spp. (Bmy843)	3.68%±0.01	3.38%±0.01	3.64%±0.01	2.96%±0.00
	Acetate-denitrifying cluster (DEN124)	1.82%±0.01	3.59%±0.02	3.55%±0.01	3.93%±0.02
Methanogens	*Methanosarcina* spp. (MS1414)	11.68%±0.00	14.67%±0.01	5.79%±0.05	9.34%±0.01
	Methanomicrobiales (MG1200)	19.90%±0.10	15.86%±0.04	15.08%±0.04	14.09%±0.01
	Methanosaeta spp. (MX825)	15.70%±0.03	13.67%±0.01	13.34%±0.05	11.24%±0.01
	Methanobacteriales (MB1174)	11.67%±0.01	9.41%±0.01	6.17%±0.02	9.98%±0.01
Archaea	*Archaea* (ARC915)	58.96%±0.02	53.61%±0.02	40.38%±0.01	44.65%±0.03
Bacteria	*Bacteria* (EUBmix)	41.04%±0.01	46.39%±0.02	59.62%±0.01	55.35%±0.01
	Negative control (NONEUB)	19.45%±0.01	17.30%±0.00	18.08%±0.03	19.76%±0.01

*Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation

Table C.3 Intensity analyses of ozonated digester

		Raw data obtained from ImageJ* (%intensities)			
Microbial community	**Target microorganism and probe**	**Day 1**	**Day 7**	**Day 15**	**Day 21**
Acidogens	*Acidobacteria* (HoAc1402)	63.09%±0.10	62.57%±0.06	49.69%±0.18	70.33%±0.21
	Acidobacteria (SS_HOL1400)	57.73%±0.13	71.06%±0.11	65.38%±0.17	53.65%±0.25
	Clostridium spp. (Clost I)	62.73%±0.19	85.33%±0.12	51.37%±0.27	69.20%±0.26
	Actinobacteria (Actino221)	50.01%±0.26	50.84%±0.33	58.34%±0.10	67.76%±0.20
	Flavobacterium (CFB563)	72.36%±0.18	73.44%±0.21	67.72%±0.22	70.37%±0.11
Acetogens	*Syntrophobacterales* (DSBAC355)	73.41%±0.26	39.34%±0.23	73.84%±0.41	66.12%±0.43
	Thermacetagenium (GTAG992)	70.15%±0.04	58.85%±0.22	80.99%±0.33	71.13%±0.29
	Syntrophobacter (SYN835)	64.12%±0.12	68.46%±0.26	59.04%±0.09	63.05%±0.15
	Tepidanaerobacter (GTE1002)	70.76%±0.14	49.58%±0.20	65.86%±0.15	75.85%±0.17
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	74.29%±0.22	67.66%±0.11	89.06%±0.11	67.35%±0.08
	Desulfobacter spp. (DSB129)	53.55%±0.12	85.36%±0.17	73.24%±0.27	68.09%±0.12
	Desulfobulbus spp. (DBB660)	52.27%±0.27	68.46%±0.06	64.41%±0.26	52.88%±0.14
	Desulfosarcina variabilis (DSC193)	65.62%±0.29	57.49%±0.20	74.83%±0.36	74.78%±0.29
Denitrifiers	*Pseuodomonas* spp. (Pae997)	66.10%±0.36	71.59%±0.18	73.41%±0.14	75.46%±0.28
	Bacillus spp. (Bmy843)	68.31%±0.15	61.01%±0.29	63.72%±0.16	53.90%±0.10
	Acetate-denitrifying cluster (DEN124)	70.76%±0.06	70.27%±0.25	84.48%±0.15	77.33%±0.35
Methanogens	*Methanosarcina* spp. (MS1414)	48.48%±0.11	61.30%±0.38	54.33%±0.23	70.20%±0.16
	Methanomicrobiales (MG1200)	66.89%±0.17	77.12%±0.24	67.01%±0.24	79.10%±0.22
	Methanosaeta spp. (MX825)	59.65%±0.18	84.57%±0.11	78.01%±0.29	75.18%±1.47
	Methanobacteriales (MB1174)	69.84%±0.15	73.11%±0.12	68.72%±0.10	65.33%±1.21
Archaea	*Archaea* (ARC915)	62.26%±0.24	61.66%±0.06	70.76%±0.35	64.40%±0.94
Bacteria	*Bacteria* (EUBmix)	75.90%±0.25	47.54%±0.13	59.00%±0.17	58.99%±0.03
	Negative control (NONEUB)	19.78%±0.22	23.12%±0.25	20.33%±0.30	22.87%±0.41

*Mean ratios of FITC and DAPI pixel areas for the microscopic images from 10 representative areas; ±, standard deviation

Table C.4 Relative abundancy (%) of ozonated digester

		Relative abundancy from processed data*			
Microbial community	**Target microorganism and probe**	**Day 1**	**Day 7**	**Day 15**	**Day 21**
Acidogens	*Acidobacteria* (HoAc1402)	3.43%±0.02	2.28%±0.00	1.65%±0.00	3.10%±0.00
	Acidobacteria (SS_HOL1400)	3.01%±0.02	2.77%±0.00	2.54%±0.01	2.01%±0.01
	Clostridium spp. (Clost I)	3.40%±0.00	3.59%±0.01	1.75%±0.02	3.03%±0.00
	Actinobacteria (Actino221)	2.39%±0.00	1.60%±0.00	2.14%±0.02	2.94%±0.00
	Flavobacterium (CFB563)	4.16%±0.00	2.91%±0.00	2.67%±0.01	3.11%±0.00
Acetogens	*Syntrophobacterales* (DSBAC355)	4.25%±0.01	0.94%±0.00	3.02%±0.01	2.83%±0.01
	Thermacetagenium (GTAG992)	3.99%±0.01	2.06%±0.00	3.42%±0.01	3.16%±0.01
	Syntrophobacter (SYN835)	3.51%±0.01	2.62%±0.02	2.18%±0.00	2.63%±0.02
	Tepidanaerobacter (GTE1002)	4.04%±0.00	1.53%±0.01	2.57%±0.00	3.46%±0.01
Sulfate reducers	*Desulfovibrio* spp. (DSV687)	4.32%±0.01	2.57%±0.01	3.87%±0.00	2.91%±0.00
	Desulfobacter spp. (DSB129)	2.67%±0.10	3.60%±0.01	2.98%±0.00	2.96%±0.00
	Desulfobulbus spp. (DBB660)	2.57%±0.10	2.62%±0.00	2.48%±0.00	1.96%±0.00
	Desulfosarcina variabilis (DSC193)	3.63%±0.01	1.99%±0.01	3.07%±0.02	3.39%±0.00
Denitrifiers	*Pseuodomonas* spp. (Pae997)	3.67%±0.01	2.80%±0.01	2.99%±0.02	3.44%±0.00
	Bacillus spp. (Bmy843)	3.84%±0.01	2.19%±0.02	2.45%±0.01	2.03%±0.01
	Acetate-denitrifying cluster (DEN124)	4.04%±0.01	2.72%±0.01	3.62%±0.01	3.56%±0.02
Methanogens	*Methanosarcina* spp. (MS1414)	7.46%±0.00	11.48%±0.00	10.31%±0.01	12.76%±0.01
	Methanomicrobiales (MG1200)	12.25%±0.00	16.23%±0.02	14.15%±0.01	15.16%±0.00
	Methanosaeta spp. (MX825)	10.36%±0.05	18.47%±0.11	17.48%±0.01	14.11%±0.08
	Methanobacteriales (MB1174)	13.01%±0.12	15.03%±0.23	14.66%±0.01	11.45%±0.66
Archaea	*Archaea* (ARC915)	43.08%±0.57	61.21%±0.04	56.60%±0.02	53.48%±0.04
Bacteria	*Bacteria* (EUBmix)	56.92%±0.09	38.79%±0.96	43.40%±0.01	46.52%±0.03
	Negative control (NONEUB)	19.78%±1.7	23.12%±2.4	20.33%±0.02	22.87%±0.76

*Scaled data in Excel after subtracting NONEUB probe; ±, standard deviation