Effects of Organic Loading Rate on Reactor Performance and Archaeal Community
Structure in Mesophilic Anaerobic Digesters Treating Municipal Sewage Sludge

THESIS

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Abstract

Full-scale high solid anaerobic digestion (AD) technology for wastewater treatment and renewable energy production, the first of its kind in the country, was recently installed in the city of Akron, Ohio. Currently, 1/3 (5000 dry tons/yr) of their dewatered municipal sewage sludge (MSS) is stabilized via AD, while the remainder is stabilized by composting. Expansion and sizing of future AD reactors to accommodate additional organics generated by the facility is being considered. To evaluate this option a better understanding of the effects of organics loading rate (OLR) on AD is needed because, there is a lack of information on the effects of increased OLR on reactor performance and stability. The hypothesis of this study was that increasing the OLR from 3.4 to 5.0 gVS L⁻¹d⁻¹ would not affect the biochemical or molecular properties related to reactor stability and performance. To test this hypothesis, a laboratory simulation of the full-scale AD process was conducted in a 42-day study. Six identical continuously stirred-tank mesophilic reactors with 3.5 L working volumes were used. Four different treatments were evaluated, one of which was replicated (treatment 1) to assess error and variability. The first treatment (T1) had an OLR of 3.4 gVS L⁻¹d⁻¹ equal to the current full-scale OLR. Treatment 2 (T2), treatment 3 (T3) and treatment 4 (T4) had OLR of 4.0, 4.5 and 5.0 gVS L⁻¹d⁻¹, respectively. To assess the effects of increased OLR on reactor performance and stability, biochemical and molecular parameters were used. Archaeal

community structure dynamics were determined by PCR amplification of 16S rRNA genes using fluorescently labeled archaeal primers (Ar109f and FAM labeled Ar912rt) followed by terminal restriction fragment polymorphism (T-RFLP) analyses using *TaqI*, *AluI* and *BfaI* restriction enzymes. Results showed that reactors maintained stable conditions during the experimental period. An increase in the amount of organic matter biodegradation was observed with increasing the OLR, resulting in higher organic matter removal and volumetric methane production rate. The highest VS removal and volumetric methane production results were observed for T4, $54 \pm 2\%$ and 1.4 ± 0.1 $\text{LCH}_4 \text{ L}^{-1}\text{d}^{-1}$, respectively. Moreover, the efficiency of reactor conversion of organic matter to methane was found to be similar in all the treatments with an average value of $0.57 \pm 0.07 \text{ LCH}_4 \text{ gVS}_{\text{removed}}^{-1}$. The archaeal TRFs derived from the three endonucleases used for molecular analysis, showed that predominant TRFs remained stable during the experiment accounting for an average relative abundance (RA) of $81 \pm 1\%$. The TRFs with the greatest abundance using *TaqI*, *AluI* and *BfaI* respectively had sizes of 185, 166 and 104 base pairs, respectively. Archaea consistent with combinations of the three sets of TRFs included members of both the Euryarchaeota and Crenarchaeota phyla, including acetoclastic metabolic groups which utilize acetate to produce CH_4 and hydrogenotrophs, which utilize hydrogen as an electron acceptor to produce CH_4 . Orders of Euryarchaeota consistent with the TRFs were Methanomicrobiales, Methanosarcinales and Methanobacteriales. The TRF combination that was consistent with the Crenarchaeota phylum, belonged to an uncultured *Crenarchaeote*. In conclusion, this laboratory-scale study suggests that performance and stability as well as the archaeal community structure

in this digester system was unaffected by increasing the OLR by nearly 50%. Furthermore, increasing the OLR increased the amount of methane gas generated from the same size reactor.

Dedication

This document is dedicated to my family.

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List of Abbreviations

ACF	Akron Composting Facility.
AD	Anaerobic digestion.
ALKt	Alkalinity.
BSA	Bovine serum albumin.
bp	Base pairs.
COD	Chemical oxygen demand.
CSTR	Continuously stirred-tank reactor.
DGGE	Denaturing gradient gel electrophoresis.
DNA.....	Deoxyribonucleic acid.
dNTPs	Deoxynucleoside triphosphates.
FA	Free ammonia.
FABE	Department of Food, Agricultural and Biological Engineering, The Ohio State University.
FID	Flame ionization detector.
FISH.....	Fluorescence <i>in-situ</i> hybridization.
IVFA	Individual volatile fatty acids.
LCFA	Long chain fatty acids.
MiCA	Microbial Community Analysis database, University of Idaho.

MPB..... Methane producing bacteria.

MSS Municipal sewage sludge.

MSW..... Municipal solid waste.

OARDC Ohio Agricultural Research and
Development Center, The Ohio State University.

OFMSW Organic fraction of municipal solid waste.

OLR Organic loading rate.

OTUs..... Operational taxonomic units.

PCR..... Polymerase chain reaction.

RNA..... Ribonucleic acid.

rRNA..... Ribosomal RNA.

RTSF..... Research Technology Support Facility,
Michigan State University.

SRB..... Sulfate reducing bacteria.

TC..... Total carbon.

TGGE..... Temperature gradient gel electrophoresis

TS Total solids.

T-RFLP Terminal restriction fragment length
polymorphism.

TRF..... Terminal restriction fragment.

TS Total solids.

TVFA..... Total volatile fatty acids.

UPGMA Unweighted pair group method with
arithmetic mean.

U.K. United Kingdom.

U.S. United States.

U.S. EPA..... United States Environmental Protection
Agency.

VFA Volatile fatty acids.

VS Volatile solids.

WAS Waste activated sludge.

WPCS Water Pollution Control Station.

WWTP Wastewater treatment plant.

Chapter 1. Introduction

Municipal sewage sludge (MSS) is a by-product of the physical, chemical and biological processes used during primary, secondary and tertiary treatment in wastewater plants. According to the United States Environmental Protection Agency (U.S. EPA) (1999), nearly 60% of the total annual production of 6.9 million dry metric tons of sludge produced in 1998 in the U.S. was beneficially used, including, composting, land application or used as landfill cover. The U.S. EPA (1999) estimated 7.1 million tons for 2000 with 63% used, 7.6 million tons for 2005 with 66% used and 8.2 million tons with 77% used beneficially for 2010. The use was expected to increase accompanied with the benefits from rising disposal costs and public perception issues associated with disposal.

Processing, utilization and disposal of MSS could represent the most difficult and expensive operation conducted by municipalities in the U.S. (Lue-Hing, 1998). Common MSS processing techniques include incineration, landfilling, long-term storage and land application on agricultural and non-agricultural lands; however, these are not always safe and cost-effective. Recently, many investigations have been undertaken to develop reliable management programs to meet the stringent regulations and to offset the rising costs of plant operation.

Anaerobic digestion (AD) technology is the oldest and the most cost-effective technique used for sewage sludge stabilization before its final disposal (Ponsá *et al.*, 2008). AD transforms part of the organic material into biogas, a mixture of

predominately methane, carbon dioxide and trace gases. Thereby, this technology has the potential to optimize wastewater treatment plant (WWTP) operations, while making renewable energy with reduced environmental impact. The AD process is achieved by the concerted action of several groups of bacteria involved in four distinguished steps including hydrolysis, acidogenesis, acetogenesis and methanogenesis.

A full-scale AD plant was recently installed in the city of Akron, Ohio to treat an average of 5,000 dry tons/year of dewatered MSS. According to Brian Gresser, Akron Water Pollution Control administrator, Akron's system is the first of its kind in the U.S., a high solid AD system treating sewage sludge with solid content up to 16% dry solids (City of Akron, 2007). Currently, only 1/3 of the total MSS production is stabilized via AD, while the remainder is stabilized with composting. Expansion and sizing of future AD reactors to accommodate additional organics generated by the facility requires an improved understanding of the effects of organic loading rate (OLR) on reactor performance. However, there is a lack of information on the effects of OLR on common biochemical and molecular parameters used to monitor the system's performance and stability.

Many authors have investigated the effects of OLR on common monitoring parameters in laboratory and full-scale AD systems treating a wide variety of substrates; however, there is no information available for the high solid AD of MSS in Akron's plant. To address this lack of knowledge the goals of this study were to determine the effects of increasing the OLR on biochemical and molecular parameters for the waste stream treated in Akron's high solid facility. The hypothesis of this study was that

increasing the OLR in a full-scale, continuous, high solid MSS digestion process from 3.4 to 5.0 gVSL⁻¹d⁻¹ would not affect the biochemical or molecular properties related to the stability and performance. To test this hypothesis, a laboratory simulation of the AD process was developed and a 42-day one phase study was conducted to analyze the effects of four different OLR on biochemical and microbial characteristics of the system.

The overall objective of this study was to investigate how OLR affects the stability, process performance and methanogenic consortia of a full-scale mesophilic anaerobic reactor digesting high solid content sewage sludge. To test the hypothesis, a laboratory-scale experiment was conducted of a stable, continuous digestion process fed at four different OLR. The performance of the digesters and changes in microbial community structure were characterized by collecting the following biochemical and molecular data; organic matter removal efficiency, biogas production rate, pH, free ammonia, total volatile fatty acids, alkalinity, individual volatile fatty acids and methane yield. In addition, the effects of loading rate on the archaeal community structure were determined by PCR amplification of 16S rRNA genes with labeled archaeal primers and terminal restriction fragment polymorphism analyses.

Specific objectives were:

Objective 1. Develop a laboratory-scale reactor system that simulates the operational conditions of a full-scale mesophilic anaerobic reactor treating high solid content sewage sludge.

Objective 2. Determine the influence of organic loading rate and hydraulic retention time on biochemical parameters such as, organic matter removal efficiency, biogas production rate, total and volatile solids, total carbon and nitrogen, pH, free ammonia, total volatile fatty acids, alkalinity and individual volatile fatty acids.

Objective 3. Study the influence of increasing the OLR on the archaeal community structure as revealed by 16S rRNA amplification and terminal restriction fragment length polymorphism (TRFL-P).

This thesis is divided into six chapters, beginning with the Introduction (Chapter 1). The Literature Review (Chapter 2), explains overall AD technology and the different stages of this process. Materials and Methods Chapter (3) describes the techniques and procedures used in this thesis. In the Results (Chapter 4), the findings of the characterization of the AD process by biochemical and molecular parameters are presented through the use of figures and tables. Chapter 5 (Discussion) interprets the relevant findings of this study. The main conclusion, limitations of this study and recommendations for future experiments are presented in Chapter 6. Finally, Appendix A shows an aerial image of Akron's wastewater plant and Appendix B includes the tabulated data for this experiment.

Chapter 2. Literature Review

Anaerobic digestion (AD) is a multi-step process that biologically converts organic carbon into biogas by the coordinated activity of a complex group of microorganisms under anaerobic conditions (Appels *et al.*, 2008). Biogas, the end product, is a renewable energy source that is mainly composed of a mixture of methane, carbon dioxide and trace gases such as, hydrogen, hydrogen sulfide, nitrogen and oxygen.

Anaerobic Digestion History

The biological conversion of organic matter into biogas, has been used for energy recovery for many centuries. Anecdotal evidence suggests that biogas was first used in Assyria during 10th century B.C. for heating bath water (Lusk, 1998). Scientific interest in biogas production was first attributed to Robert Boyle and Stephen Hale in the sixteenth century, when they associated the release of flammable gases by disturbing the sediments from natural streams and lakes with the decomposition of organic material (Ferguson and Mah, 1987).

Jan Baptita Van Helmont in the 17th century stated that these flammable gases could evolve from the decay of organic matter (Lusk, 1998); these observations were then confirmed by Count Alessandro Volta in 1776 (Barker and Buswell, 1956). Sir Humphry

Davy found that methane gas was generated during the anaerobic digestion of cattle manure in 1808 (Lusk, 1998). In 1856, Reiset reported methane being generated from piles containing decomposing manure and realized that the study of this phenomena could explain the organic matter decomposition in general (Buswell and Hatfield, 1938).

According to Meynell (1976) the first biogas plant was built by a leper colony in 1859 in Bombay, India, which marked the beginning of the AD application. On the other hand, the French journal Cosmos described an air-tight chamber built in 1860s in France, called “Mouras’ automatic scavenger”. In that article (Moigno, 1881; Moigno, 1882), M. Allain Targé announced the invention as “the most simple, the most beautiful, and perhaps, the grandest of modern inventions” and “a complete solution of the problem which for centuries had been an insolent menace hurled in the face of all humanity”. The development of AD technology led to research by several scientists in 1930s that identified anaerobic bacteria and the conditions that promote methane production (Buswell and Hatfield, 1936).

Anaerobic Digestion Principles

The AD process is usually divided into four important stages: hydrolysis, acidogenesis also known as fermentation, acetogenesis and methanogenesis. Complex materials are first catalyzed with excreted enzymes of hydrolytic and acidogenic (or fermentative) bacteria that degrade organic material into smaller units. Acetogens then

oxidized simple molecules such as alcohols and long-chain fatty acids (LCFA) created during acidogenesis to produce acetate as well as carbon dioxide and hydrogen. Acetate, hydrogen and carbon dioxide produced during fermentation stage can be used directly by methanogenic bacteria to produce biogas. The final stage of the process involves methanogenic microorganisms converting intermediates from proceeding stages into methane, carbon dioxide and water (figure 1).

Figure 1. Proposed substrate flow scheme for the AD process adapted from Kaspar and Wuhrmann (1978), Gujer and Zehnder (1983) Angelidaki *et al.* (2002).

Hydrolysis

Hydrolysis is considered as one of the main stages in AD, utilizing a complex group of microbial species to both solubilize insoluble particulate organic matter and decompose organic polymers into monomers or dimmers (Parawira *et al.*, 2005; Ponsá *et al.*, 2008). Particulate organic matter including, carbohydrates, lipids and proteins, as well as soluble inert material are products of disintegration of composite material. Further degradation of particulate materials by hydrolytic enzymes include, monosaccharides, amino acids and glycerol that will be further degraded into precursors for methane production, such as, volatile fatty acids (VFA) and hydrogen (Vavilin *et al.*, 2008).

The complete hydrolysis stage consists of enzyme production, diffusion, adsorption, reaction and enzyme deactivation (Batstone *et al.*, 2002). Hydrolytic enzymes include cellulase to degrade carbohydrates into simple sugars, proteases to degrade protein into amino acids and lipases for the degradation of lipids into fatty acids (Forster, 2003). Common hydrolyzing conceptual modes include:

- The organism secretes enzymes to the bulk substrate in which adsorption to the particle occurs (Jain *et al.*, 1992).
- The organism attaches to the particle to produce enzymes and benefit from products of the enzymatic reaction (Vavilin *et al.*, 1996).
- The organism has to adsorb onto the surface of the particle to use an attached enzyme that may also act as a transport receptor to the interior of the cell (Angelidaki and Sanders, 2004).

The degradation potential of an specific enzyme can be dependent on their location (Higuchi *et al.*, 2004; Parawira *et al.*, 2005), freely enzymes (cell-free) could present different activity than those that are cell-associated (Morgenroth *et al.*, 2001). In processes such as digestion and/or fermentation higher free enzymatic activity has been reported (Kosugi *et al.*, 2001; Parawira *et al.*, 2005; Sharp and MacFarlane, 2000). According to Zhang *et al.* (2007), enzymes could have different locations, which are determined by the structure of the cell wall.

On the other hand, biodegradability of a complex substrate and hence its biogas potential are dependent on the fraction of biodegradable carbohydrates (cellulose, hemicellulose and lignin), proteins and lipids (Angelidaki and Sanders, 2004), and the adsorption of hydrolytic enzymes to its surface sites (Tong *et al.*, 1990; Veeken and Hamelers, 1999); therefore, AD has been pointed to be a surface related process (Hills and Nakano, 1984), where AD occurs by a biofilm formation on the particles surface (Song, 2003).

The overall rate in processes that are composed of a sequence of reactions is determined by the slowest reaction, named the rate-limiting step (Hill, 1977). Hydrolysis has been considered to be the rate limiting stage when complex organic matter, and especially when lignin rich matter is degraded during AD (Eastman and Ferguson, 1981; Myint *et al.*, 2007; Pavlostathis and Giraldo - Gomez, 1991). The overall hydrolysis rate is therefore a function of organic material size, shape, surface area, solubility, biomass concentration and parallel enzymatic activity (Batstone *et al.*, 2000).

Acidogenesis

Acidogenesis is generally considered to be the quickest step during AD of complex organic material (Vavilin *et al.*, 2008), utilizing part of the organic molecule to be oxidized as a terminal electron acceptor (Gujer and Zehnder, 1983). During this stage, soluble sugars and amino acids are degraded into simpler products, while LCFA must be oxidized by an external electron acceptor. Acidogenesis unlike anaerobic oxidation can occur without an external electron acceptor, which, allows this reaction to occur at high hydrogen and formate concentrations (Batstone *et al.*, 2002). Additionally, more reduced metabolites such as, VFA, lactate and ethanol will be produced under this condition (Angelidaki *et al.*, 2002).

According to Ramsay and Pullammanappallil (2001) the most common fermentation pathway for amino acids is through the Stickland reaction, involving one amino acid to act as an electron donor, while another acts as an electron acceptor. On the other hand, glucose fermentative microorganisms are able to metabolize the substrate in different pathways, yielding varied amounts of energy from different fermentation products (Dolfing, 1988). Table 1 shows examples of glucose fermentation products and their stoichiometric reaction:

Products	Reaction
Acetate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$
Propionate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2COOH + 2H_2O$
Acetate + propionate	$3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O$
Butyrate	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$
Lactate	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$
Ethanol	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

Table 1. Products from glucose fermentation (Batstone *et al.*, 2002; Schink, 1997; Thauer *et al.*, 1977).

Different pathways will be used as a function of factors such as, pH, substrate concentration and hydrogen partial pressure. In an experiment conducted to model product formation from glucose, Rodriguez *et al.* (2006) reported that:

- Acetate was the main product at low hydrogen pressure, but, increases in partial pressure will cause acetate formation to be energetically expensive causing a shift to butyrate production, that will be countered by decreases in hydrogen production since formation of more reduced butyrate yields no net hydrogen.
- Acetate was the main product at high pH, but, its production decreases at lower pH due to energy requirements to transport acetic acid outwards. Butyrate production will increase proportionally at decreasing pH values until reaching a pH of less than 5, where ethanol will become the dominant product. According Hwang *et al.* (2004) fermentation processes may cease at pH lower than 4.0.

- Changes in fermentation products related with substrate concentrations follow a similar pattern than the one described for pH. As mentioned in another section, higher influent concentrations may lead to increases in intermediate production that if not consumed by final members of the food chain, intermediates would accumulate in the reactor causing potential decreases in pH.

Acetogenesis

During acetogenesis, fermentation products such as fatty acids longer than two carbon atoms, branched-chain and aromatic fatty acids and alcohols longer than 1 carbon atom are oxidized at low H₂ partial pressure to methane precursors by obligated proton reducing bacteria (Schink, 1997). Furthermore, VFA have been recognized as the most important intermediates in the AD process and proposed as a control parameter (Pind *et al.*, 2003). Examples of VFA oxidation reactions are shown in table 2.

During the acetogenesis stage propionate is mainly oxidized to acetate, bicarbonate and hydrogen via the methylmalonyl-CoA pathway (Houwen *et al.*, 1990). In addition, butyrate has been reported to follow the β -oxidation pathway of *n*-butyrate yielding acetate, and that *i*-butyrate served as equilibrium storage of butyrate (Aguliar *et al.*, 1990; Ahring and Westermann, 1987; Stieb and Schink, 1989; Tholozan *et al.*, 1988). *n*-valerate has been reported to be degraded into both acetate and propionate via the β -

oxidation pathway, while only acetate resulted from *i*-valerate degradation (Stieb and Schink, 1986; Wang *et al.*, 1999).

Substrate	Reaction
Propionate	$\text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + 2\text{H}_2$
<i>n</i> -butyrate	$\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2$
<i>i</i> -butyrate	$\text{CH}_3(\text{CHCH}_3)\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{H}_2$
<i>n</i> - valerate	$\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{CH}_3\text{CH}_2\text{COOH} + 2\text{H}_2$
<i>i</i> - valerate	$\text{CH}_3(\text{CHCH}_3)\text{CH}_2\text{COOH} + \text{CO}_2 + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_3\text{COOH} + \text{H}_2$

Table 2. Degradation reactions from Pind *et al.* (2003).

During the oxidation step of organic acids to acetate with no internal electron acceptors, formate and hydrogen have been reported to be external electron acceptors that are utilized in syntrophic relationship by acetogens and methanogens to produce carbon dioxide or hydrogen gas, respectively (Batstone *et al.*, 2002). Low concentrations of these electron acceptors are usually reported for these processes since they are being consumed by methanogenic members, which is thermodynamically favorable for oxidation reactions to be possible (de Bok *et al.*, 2004; Stams *et al.*, 2005).

Methanogenesis

The final stage of the AD process consists of using methane precursors formed during a sequence of biochemical reactions, to be finally converted into mainly methane and carbon dioxide by methanogens. Methanogenesis is as important as the other stages, maintaining stability of the process by conversion of reduced intermediates that if not removed, would accumulate causing an inadequate environment for microbial activity. According to Gerardi (2003) methanogens can benefit directly from substrates such as acetate, formate, methanol and methylamine and other one-carbon compounds.

Specifically for acetate, an important intermediate in the digestion process, two common mechanisms for conversion to methane have been described (Schnurer *et al.*, 1994). In the first one, known as the aceticlastic pathway, the methyl group of acetate will be converted to CH₄ and the carboxyl group is converted to carbon dioxide. In the second one, syntrophic oxidation of acetate to carbon dioxide and hydrogen occurs, and then reduction of carbon dioxide to methane by hydrogenotrophic methanogens. This process is considerably slower than the aceticlastic methanogenesis, with higher doubling times and lower growth rates. Reactions related to the methanogenesis stage can be observed in table 3.

Substrates that are easily degraded upstream in the process chain can often lead to the rapid buildup of metabolic intermediates, making methanogenesis the rate limiting step of the overall process (Murto *et al.*, 2004). In addition, factors such as temperature, salinity, redox potential, pH, substrate availability and nutrient concentrations are known

to influence the methanogenesis stage (Conrad, 1991). Usually temperature is recognized as the most important of these variables (Khalil *et al.*, 1998; Schutz *et al.*, 1990).

Substrate	Reaction
Aceticlastic methanogenesis	$\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2$
Methylotrophic methanogenesis	$4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O}$
Hydrogenotrophic methanogenesis	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$
Acetate oxidation	$\text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{CO}_2$
Homoacetogenesis	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$

Table 3. Reactions related to methanogenesis stage by Laloui-Carpentier *et al.* (2005).

Both methanogenic archaea and homoacetogenic bacteria are the main hydrogen consumers in the absence of other inorganic electron acceptors rather than carbonate, for example, nitrate or sulfate (Kotsyurbenko *et al.*, 2001). Furthermore, competition for hydrogen between these metabolic partners has been reported (Schink, 2002), most commonly at low temperature conditions (Conrad *et al.*, 1989; Kotsyurbenko *et al.*, 1996; Kotsyurbenko *et al.*, 1993). The general assumption is that homoacetogens can take advantage of their metabolic versatility to compete with several partners of similar metabolic types, utilizing one or two substrates simultaneously (Schink, 1997).

As a result of their competition, an influence in carbon and electrons flow, in which either acetate or CH_4 , is produced (Kotsyurbenko *et al.*, 2001). Therefore, the rate

of CH₄ production is strongly affected by the production pathway, even though that acetate will be eventually consumed by methanogens and converted to CH₄ (Hornibrook *et al.*, 2000; Shannon and White, 1996). In standard conditions, for thermodynamic reasons, the consumption of hydrogen by hydrogenotrophic methanogens is more favorable than homoacetogens, and acetate consumption by aceticlastic methanogens rather than acetate oxidation (Boe, 2006).

Petersen and Ahring (1991) studied the syntrophic acetate oxidation in a thermophilic anaerobic sewage sludge digester. The authors reported a contribution of acetate oxidation to methane formation up to 14% under such conditions, corresponding to the abundance of hydrogenotrophic communities in thermophilic digesters (Griffin *et al.*, 1998). In addition, Schink (2002) stated that the acetate oxidation pathway becomes more favorable at high temperatures (> 30°C).

Aceticlastic methanogenesis is usually favored by high acetate concentrations rather than the acetate oxidation pathway (Zinder and Koch, 1984). On the other hand, in extreme thermophilic conditions (> 65°C), out of the optimum range for aceticlastic methanogens, the acetate oxidation pathway was favored (Lepisto and Rintala, 1999). The average lower limit temperature for acetate oxidation to be effective is 37°C (Schink, 2002), and at temperatures lower than 15°C (psychrophilic) the activity of the hydrogen utilizing methanogens is very low (Boe, 2006). In such conditions, homoacetogenic bacteria take charge of the main hydrogen removal, while aceticlastic methanogens will be dominant in the methane formation process (Kotsyurbenko *et al.*, 2001).

Another factor influencing the methanogenesis process is the concentration of ammonia, where acetoclastic methanogenesis is more sensitive than the hydrogen utilizing one (Borja *et al.*, 1996). The acetate oxidation pathway was found to be the preferred methane formation route in an experiment conducted at mesophilic conditions with a triculture grown in synthetic medium at high ammonium concentrations (Schnurer *et al.*, 1994).

Factors Affecting the Anaerobic Digestion of Wastewater Treatment Sludges

Substrate and Nutrients

The composition of the substrates that are used for AD is varied and its biodegradable fraction directly determines the biogas potential. The biodegradable input is often measured as chemical oxygen demand (COD) or volatile solids (VS) in the substrate, it is important to emphasize that there is no correlation between these measures and hence they are not comparable. Furthermore, the biodegradability of a particular substrate will be mainly affected by the content or the fraction composed of recalcitrant compounds. For example, increases in one percent of lignin will drop the biodegradable fraction by 3 percent (Nijaguna, 2006).

In municipal wastewater treatment plants, the desirable VS concentration in the feed is achieved by mixing raw sludges having a low concentration of solids with thickened activated sludge or secondary sludges, since a feedstock of mainly raw sludge reduces the hydraulic retention time and consequently affect digester operation. This substrate also serves to seed the anaerobic digesters, providing facultative anaerobes, methane-forming and many organic particulates that are needed at the start-up of the process (Gerardi, 2003).

Bacterial nutrients requirement is one of the main factors influencing digestion of the organic fraction of municipal solid waste. Kayhanian and Rich (1995) reported incomplete and unstable conversion of the organic substrates. Critical nutrients for bioconversion of acetate to methane include the macronutrients nitrogen and phosphorus and the micronutrients cobalt, iron, nickel and sulfur (Gerardi, 2003). Indeed, an adequate concentration of nutrients is necessary for microbial cell growth. Moreover, nutrients such as sulfide and phosphate may decrease the metal ion bioavailability if present in high concentrations (Boe, 2006).

Temperature

The complex degradation of organic substrates into biogas involves different groups of microorganisms performing crucial activities within different steps of the AD process. Factors such as number of bacteria and activities are known to be strongly

dependent on temperature, thus, the importance of maintaining optimal reaction temperature (Cha *et al.*, 1997). Suppression of microbial activities due to transient changes in temperature has been reported to negatively affect the whole digestion process (Bull *et al.*, 1983; Peck *et al.*, 1986; Topiwala and Sinclair, 1971).

The methane formation process in natural environments occurs in a wide range of temperatures, from 0°C to 97°C (Zeeman *et al.*, 1988). The anaerobic digestion of organic wastes has been mostly studied at three temperature ranges, psychrophilic (<20°C), mesophilic (20-45°C) and thermophilic (45-60°C) (Kashyap *et al.*, 2003). Effects of temperature are reflected on physicochemical properties of the all components in the digester and in the thermodynamics and kinetics of the biological processes (Boe, 2006).

Advantages of increasing digestion temperature include increasing accessibility of microorganisms to more soluble organic compounds, accelerated biological and chemical reactions, higher microbial growth rates, accelerated interspecies hydrogen transfer resulting in increased methanogenic potential and improvements in pathogen death rates (Oropeza *et al.*, 2000; Zabranska *et al.*, 1999). The counterpart of temperature increases other than higher energy input, include increases in the fraction of free ammonia that is inhibitory for microorganisms. It also increases the pKa of the VFA that at low pH have the potential to make the process more susceptible to other inhibitory compounds (Appels *et al.*, 2008).

The effect of AD temperature on the death rate of pathogenic bacteria has also been studied. Watanabe *et al.*(1997) conducted a laboratory experiment on reactors

treating wastewater sludges. It was observed that higher pathogen inactivation was achieved at thermophilic (55°C) temperature and shorter retention times (20 days). Investigations conducted by Gavala *et al.* (2003) in AD of primary and secondary sludges showed higher efficiencies in solids removal and methane production in thermophilic processes at lower retention times than in mesophilic conditions. Consequently, sensitivity of thermophilic AD to inhibition will thus require rigorous control and monitoring.

pH, Volatile Fatty Acids and System Buffering Capacity

Variations in pH over optimum ranges have been found to have a strong influence on microbial activities, including utilization of energy sources, efficiency of substrate degradation, synthesis of materials and release of metabolic products from the cell (Sakharova and Rabotnova, 1976). Enzymatic activity is known to be influenced by the pH, since each enzyme is active within a specific pH range (Lay *et al.*, 1997). A pH range within 6.7 and 7.4 has been reported for most methanogenic archaea to function optimally. In addition, the rate of methanogenesis may decrease if pH is lower than 6.3 or higher than 7.8 (Lay *et al.*, 1997). A broader range is reported for fermentative bacteria, with values ranging from pH 4.0 to 8.5 (Hwang *et al.*, 2004).

Variations in pH influenced by organic overloads leading to reactor failure are normally related with VFA accumulation due to the kinetic uncoupling between acid

producers and consumers (Harper and Pohland, 1986; Lahav and Morgan, 2004; Pavlostathis and Giraldo - Gomez, 1991; Switzenbaum *et al.*, 1990). Furthermore, acid consumers (methanogens) species are more sensitive to a decrease in pH than the acid-producing species (Anderson and Yang, 1992).

If the production rate of organic acids is higher than the rate at which they can be converted by methanogens, stability in the digester will be dependent on the solution buffering capacity (Reinhart and AlYousfi, 1996). The main buffers in anaerobic reactors include bicarbonate and ammonium bicarbonate in the liquid phase and carbon dioxide in the gas phase (Gallert *et al.*, 1998; Turovskii and Mathai, 2006).

Quick responses in pH due to VFA accumulation are observed in systems with low buffering capacity. Furthermore, it has been reported that pH as an indicator of process instability is not recommended in a well buffered system, where variation in pH due to VFA accumulation is often minimal and slow (Bjornsson *et al.*, 2000). Under these circumstances, the pH drop will only be observed when the process is already imbalanced (Mechich and Sayadi, 2005).

Several authors have used the total volatile fatty acids (TVFA) to alkalinity (ALKt) ratio (TVFA/ALKt) as a rapid and economic indicator of process stability (Barampouti *et al.*, 2005; Fannin, 1987; Switzenbaum *et al.*, 1990; Wheatley, 1990) in which buffering capacity is considered to be sufficient at ratios below 0.4 (Zhao and Kugel, 1996).

Mixing

Distribution of bacteria, substrate, nutrients and temperature equalization by means of adequate mixing, are known to be crucial for the overall AD process (Chapman, 1989). Several investigations show that improvements in reactor performance can be achieved when changes in mixing intensity are imposed (Chen *et al.*, 1990; Chynoweth, 1987; Kim *et al.*, 2002; McMahon *et al.*, 2001; Stroot *et al.*, 2001; Vavilin and Angelidaki, 2005). According to Gerardi (2003) the main advantages of mixing in AD are: minimization of solids accumulation that may restrict reactor hydraulics, reduction of scum build up, elimination of temperature stratification and maintaining close contact between substrate particles and microbial communities.

In a sequential experiment Stroot *et al.* (2001) studied the feasibility of co-digestion of municipal solid waste, primary sludge and waste activated sludge (WAS) under mesophilic conditions in laboratory-scale continuous stirred-tank reactors (CSTRs) under different OLR and mixing conditions. Significant results from the first experiment showed that reductions in mixing intensity improved digester performance. In the second experiment observations suggested that continuously mixed digesters were unstable in terms of performance at higher OLR, while minimally mixed reactors remained stable. In a third experiment results indicated that reductions in mixing intensity were able to stabilize an unstable continuously mixed digester.

McMahon *et al.* (2001) utilized ribosomal RNA based oligonucleotide probes to track changes on abundance of syntrophic VFA degrading bacteria and methanogens in

unstable digesters treating a mixture of municipal solid waste (MSW) and sewage sludge (Part II - microbial populations). Process instability as revealed by traditional performance parameters was observed when a minimally mixed reactor was switched to continuously mixed conditions. On the other hand microbial populations as revealed by archaeal abundance were also strongly affected after imposing the change in intensity, inhibiting the syntrophic VFA oxidation by syntrophic bacteria and their metabolic methanogenic partners.

Vavilin and Angelidaki (2005) evaluated theoretically and experimentally the effects of OLR and mixing intensity on process stability during co-digestion of MSW and digested manure in mesophilic conditions. Their results indicated that at higher OLR low mixing was crucial to maintain process stability while at lower OLR mixing intensity had no significant effect on the overall AD process. Conclusions suggested mixing as an important component influencing the efficiency for AD, as hydraulic dead zones are detrimental to reaction kinetics involved in the process.

Inhibitory Substances

Inhibitory substances in wastewaters if present in high concentrations are one of the main causes of process imbalance, causing reductions in wastewaters treatment capacity and energy recovery potential (Chen *et al.*, 2008). Inhibitors can be either present on the residue or formed in the reactor during degradation processes. Guideline

values at which toxicity exist vary according to the ability of bacteria to adapt to constant concentration of the toxic and/or changes in operational conditions (Gerardi, 2003). Ammonia, sulfide, light metal ions, heavy metals and organics are the most common inhibitory substances found in anaerobic reactors treating waste activated sludge (Appels *et al.*, 2008).

As mentioned before, among the different factors influencing the stability of the AD process, ammonia has been regarded as one of the main reasons for reactor imbalance (Braun *et al.*, 1981). Among the four groups of microorganism found in anaerobic reactors, methanogens have been found to be the least tolerant and most likely to be inhibited by ammonia (Kayhanian, 1994). Ammonia is a product of bacterial activity during the degradation of nitrogenous material (Kayhanian, 1999).

Ammonium (NH_4^+) and free ammonia (NH_3) are the two principal forms of inorganic nitrogen present in aqueous solution, Kroeker *et al.* (1979) reported free ammonia (FA) to be the most toxic of both, since it is cell membrane permeable. Inside the cell, FA may cause proton imbalance and/or potassium deficiency (Sprott and Patel, 1986). However, concentrations of about 200 mg L^{-1} have been found to be beneficial for microbial activity, since nitrogen is an essential nutrient for microorganisms (Sung and Liu, 2003).

Differences in the ammonia inhibiting concentration is dependent on parameters like, temperature, pH and acclimation periods (Hansen *et al.*, 1998). It has been demonstrated that pH also affects the concentration at which total ammonia nitrogen is toxic for the AD process (Hashimoto, 1983; Hashimoto, 1984). Borja *et al.*, (1996)

observed that an increase in pH would result in an increased toxicity because of the higher shift ratio from FA to ionized ammonia (NH_4^+). Reactor instability caused by ammonia is often followed by an accumulation in VFA. In systems with low buffering capacity this accumulation will decrease pH that will eventually reduce ammonia concentration and although stability may not be affected, it will negatively impact the methane yield potential (Hansen *et al.*, 1998; Sung and Liu, 2003).

Sulfide toxicity has also been found during anaerobic treatment of sulfate containing wastewaters as an undesirable outcome of the competition for substrate between sulfate reducing bacteria (SRB) with methane producing bacteria (MPB) and acetogenic bacteria (AB) (Karhadkar *et al.*, 1987; Winfrey and Zeikus, 1977). Two stages of inhibition have been reported. The first one includes methane suppression by competition for common organic and inorganic substances (Harada *et al.*, 1994). The second one occurs when concentrations of sulfide reach up to levels that are toxic for SRB and other bacterial groups (Anderson *et al.*, 1982).

Some authors have attributed this toxicity to the reduced form of sulfate, sulfide, specifically the neutral undissociated form (hydrogen sulfide), which is capable of passing through the cell membrane (O'Flaherty *et al.*, 1998; Tursman and Cork, 1988). Inside the cell sulfide may be inhibitory by denaturing proteins, interfering with coenzyme linkages or with the assimilatory metabolism of sulfur (Chen *et al.*, 2008). On the other hand, several authors have claimed toxicity of sulfide to be dependent on factors such as concentration and pH of the wastewater system (McCartney and Oleszkiewicz, 1991; O'Flaherty *et al.*, 1998).

According to Schink (1997) the presence of sulfate affects both MPB and anaerobic bacteria due to better growth kinetics of SRB and their capacity to oxidize most fermentation products to carbon dioxide with sulfate reduction as electron acceptor. When concentrations of sulfate is low SRB will compete with MPB for acetate and hydrogen, whereas at high concentrations SRB will compete with anaerobic bacteria for propionate and butyrate (Visser *et al.*, 1993). Competition of SRB with other groups in anaerobic reactors includes:

- Hydrolytic and acidogenic bacteria: most SRB are not able to degrade natural biopolymers such as protein and lipids, depending on the activity of other microorganisms (Hansen, 1993). In addition, SRB are generally assumed not to be competitors against the fast growing fermentative microorganisms involved in monomer degradation (Postgate, 1984).
- Acetogens: from a thermodynamically and kinetic point of view, literature indicates that SRB should out-compete other anaerobes for substrate (O'Flaherty *et al.*, 1998). Previous investigations showed affinity of SRB for propionate rather than the propionate utilizing species (Parkin *et al.*, 1990; Uberoi and Bhattacharya, 1995). In addition, Visser *et al.* (1993) observed solely utilization of butyrate by SRB in a reactor fed with a mix of VFA and sucrose.
- Aceticlastic methanogens: several authors have reported SRB to be dominant for acetate utilization (Alphenaar *et al.*, 1993; Rinzema and Lettinga, 1988; Stucki *et al.*, 1993), whereas others have claimed MPB to be successful competitors (Colleran *et al.*, 1998; Isa *et al.*, 1986; Omil *et al.*, 1996).

Improving the Anaerobic Digestion Process

Anaerobic digestion (AD) of agricultural and industrial residues is a method that provides pollution control and energy recovery from biomass. Because of these benefits many investigations have been dedicated to improve process performance. The close relation between biodegradable solid content and energy yields is one of the reasons that attract researchers to find methods to reduce retention times and to increase the overall degradation efficiency (Appels *et al.*, 2008). Investigations focused on the first stage of the process, in which cells of complex polymers are ruptured and hence converted to a more readily available organic material, making clear the importance between successful pretreatments and improved performance (Mata-Alvarez *et al.*, 2000).

Pretreatment methods include biological, mechanical and physico-chemical actions. Cell degradation and partial solubilization by mechanical means are generally based on disruption of the cell wall (Neyens and Baeyens, 2003; Weemaes and Verstraete, 1998). Rivard and Nagle (1996) observed that a combination of shear stress and high temperature could result in an increase of 88-90% in substrate bioconversion potential during laboratory-scale experiments with municipal sewage sludge (MSS). Choi *et al.* (1997) conducted a laboratory-scale experiment to test the effects of high pressure jet and smash device. Pretreated waste activated sludge (WAS) resulted in volatile solids removal efficiencies of 13-50% compared to 2-35% of those in which intact WAS was

fed into the batch AD process. Baier and Schmidheiny (1997) reported enhancements by a factor of 1.2-1.5 for both overall COD degradation and net biogas production after digestion of mechanical pretreated sewage sludge.

Mechanical ultrasound methods such as sonication and induced cavitation have shown 100% cell disintegration at high power levels; however, power consumption is a serious drawback (Weemaes and Verstraete, 1998). Application of ultrasound in WAS pretreatment is reviewed in detail by Dewil *et al.* (2006). Literature data such as Shimizu *et al.* (1993) reported increases in solubilization ratios up to 80% during a kinetic study that included first order reaction kinetics of ultrasonic pretreatment of WAS. Wang *et al.* (1999) reported increases of 64% in the total amount of methane gas generated during AD of solubilized WAS; increases in bioavailable COD have also been reported by Neis *et al.* (2000).

Pretreatment with chemicals, temperature and a combination of both have also been used to hydrolyze the cell wall and rupture the membrane in order to improve solubility of organic matter. Methods such as acid and alkaline hydrolysis, ozonation and advance oxidative sludge are commonly reported in literature data. The acid and base method is carried out at ambient or moderate temperatures, avoiding the necessity of high temperatures (Neyens and Baeyens, 2003), nevertheless, re-neutralization prior digestion is a limiting factor when raw substrates are forced to extreme pH levels (Appels *et al.*, 2008).

Tanaka *et al.* (1997) studied the effects of NaOH (chemical), heating (thermo) and a combination (thermochemical) pretreatment of domestic WAS. Thermochemical

treatment gave the best results with an average increase in methane production by more than 200% and a 40-50% rate of VS solubilization. In a similar experiment Lin *et al.* (1997) pretreated WAS with NaOH, their results demonstrated improvements in gas production with increases of 75%. Tanaka and Kamiyama (2002) conducted studies with WAS in semi-continuous mesophilic AD in order evaluate the effects of thermochemical pretreatments, increases in both biodegradability as well as solubilization were reported.

In wastewater treatment of municipal sludges and biosolids, in which large quantities of cellulose, lignocellulose, polysaccharides, proteins and other organic compounds can be found (Champagne and Li, 2009), augmentation of the AD process by enzymatic hydrolysis have also been studied. Higher hydrolysis rates will eventually increase the concentration of intermediates in solution, leading to higher biogas production with higher methane contents (Bochmann *et al.*, 2007). Higgins (1986) evaluated the effects of enzyme hydrolysis for three different modes in a two-phase digester configuration, and found increases in methane production of 15 % after pretreatment of MSS with cellulases and lipases. Increases of 22% in soluble oxygen chemical demand were observed by Nagle *et al.* (1992) after four hours of enzymatic pretreatment of predigested sewage sludge before bioconversion analyzes.

Hasegawa *et al.* (2000) solubilized sewage sludge by means of pretreatment with isolated thermophilic aerobic bacteria prior to mesophilic AD. Increases in biogas production of 1.5 as compared with the untreated treatments were reported. In addition, extracellular enzymes such as proteases and amylases secreted by aerobic bacteria appeared to have much more solubilization efficiency when compared with commercial

proteinases. Other authors have also investigated the possibility of using the activity of the hydrolytic enzymes to monitor wastewater treatment processes, which provides a direct measure of the hydrolysis rate (Morgenroth *et al.*, 2001; Sarada and Joseph, 1993).

Microbiology of Anaerobic Digesters

As any other biological process, understanding the complex diversity of microbial community in anaerobic reactors is useful, since microorganisms play an important role in decomposing organic matter and subsequently in biogas productivity. Direct relation between microbial community structure and reactor performance has been previously acknowledged (Stroot *et al.*, 2001). In addition, AD is performed by several metabolic groups including hydrolytic bacteria that hydrolyze organic matter into smaller organic compounds, hydrogen-producing and consuming acetogenic bacteria that convert products from the first group into methane precursors such as acetate (or formate) and hydrogen; and methanogenic bacteria (methanogens) which are strict anaerobes that utilize products from earlier stages to produce methane.

Studies on methanogenic diversity, structure and function in mixed culture anaerobic reactors provide essential knowledge to monitor stability and to improve process efficiency (Gomec *et al.*, 2009). Investigations on optimization of the AD processes for treatment of organic waste streams have mostly focused on operational performance parameters such as digester temperature, composition of the feedstock and

substrate influent concentrations. Moreover, evaluation of shifts in the composition of the microbial structure caused by a change in one of these parameters has been rarely investigated (McMahon *et al.*, 2004).

Knowledge of the ecology and function of the microbial community in anaerobic reactors has been studied using culture-dependent and culture-independent molecular approaches (Sekiguchi *et al.*, 2001). While culture-dependent methods are time consuming and problematic because of the low growth rate and anaerobic requirements of methanogenic bacteria (Raskin *et al.*, 1995), molecular fingerprinting techniques encoding the 16S rRNA genes provide a rapid measurement and high-throughput approach of the microbial community structure and diversity of an ecosystem (Collins *et al.*, 2003). Molecular tools with rRNA gene-based approach allowing rapid identification of ribotypes from various samples include the denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998), temperature gradient gel electrophoresis (TGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Collins *et al.*, 2003).

Direct links between microbial community structure and process performance in 21 single-stage full-scale AD systems treating MSS were reported by Raskin *et al.* (1995). Structure analyses were performed by means of oligonucleotide probes complementary to conserved tracts of the 16 rRNAs of defined groups of methanogens. Results indicated that methanogens accounted for 8-12% of the total community in stable mesophilic digesters. Methanosarcinales and Methanomicrobiales constituted a majority of the total population with *Methanosaeta* species making up the bulk of the Methanosarcinales.

Other authors have focused their attention on the study of methanogenic population dynamics in situations where the anaerobic digester is unstable or under stress conditions; since stable or steady-state processes are not always the ideal scenarios for the study of variability. Griffin *et al.* (1998) observed variations in methanogenic members after an aggressive start-up of bench-top mesophilic reactors co-digesting municipal solid waste and WAS. Results obtained by rRNA-targeted oligonucleotide probes analyses revealed important fluctuations on methanogenic structure during the start-up stage. *Methanosaeta* species were found to be more abundant in the inoculum followed by decreases as acetate built up. In addition, increases in acetate levels were followed by increases in abundance of *Methanosarcina* species, constituting up to 12% of the total ribosomal RNA.

McMahon *et al.* (2004) studied microbial population dynamics during organic overload conditions in mesophilic digesters co-digesting MSW and sewage sludge reflecting U.S. production rates. The authors observed that even when biogas production rate and total archaeal rRNA abundance increased with increasing OLR, no correlation between OLR and individual microbial populations was observed. They also found that microbial populations from reactors with an unstable history were better adapted to deal under extreme organic loads.

Studies conducted by Karakashev *et al.* (2005) to assess the influence of environmental conditions (operating temperature, OLR, VFA and ammonia concentrations) in the microbial diversity of six full-scale plants reactors treating sludge from wastewater treatments plants revealed that dominant methanogens were not affected

by the OLR. Furthermore, concentrations of VFA and ammonia appeared to have more influence. Results of microbial diversity studies using fluorescence *in-situ* hybridization (FISH) are in agreement with those reported previously (McMahon *et al.*, 2001; Sekiguchi *et al.*, 1999), with Methanosaetaceae being the most abundant in mesophilic digesters treating similar waste streams.

Great efforts in understanding molecular microbial ecology have made a better characterization of the AD process possible (Griffin *et al.*, 1998; McMahon *et al.*, 2004; Raskin *et al.*, 1995), as well as improvements in reactor concept and design (Garcia *et al.*, 2000). The direct relation between microbial community structure and reactor performance has been has previously reported. However, much more knowledge of the biochemical and genetic characteristics of methanogenic bacteria is still necessary to be able to optimize AD technology to treat in a cost-effective way the increasing production of organic residues.

Anaerobic Digestion Facility in the City of Akron, Ohio

For 20 years, the City of Akron has been able to manage 100% of the sludges generated during wastewater treatment by a composting process. However, increasing operational costs and odors as a product of outside operations, still continued to be a challenge. Awareness of the existing problems challenged City of Akron officials to look on potential treatments that could help to stabilize the rising costs while treating sludges

with a futuristic and environmentally sound technology (Berger and Gresser, 2008). A two-stage mesophilic, high solid AD system was selected (figure 2). System was installed by Quasar Energy Group LLC., a U.S. company licensed to sell a technology supported by 200 AD facilities installed in Europe by Schmack Biogas AG (Germany).

The high solid anaerobic reactor installed in Akron is the first of its kind in the country, capable of digestion feedstocks with solid content up to 16% dry solids when most traditional digesters in the U.S. work in range between 3 and 8%. This system will also allow the ACF to receive residues from food processing industries to take advantage of high solid digesting ability, and hence reducing the footprint of the overall operation (Berger and Gresser, 2007).

Currently, wastewater from the City of Akron is primarily treated at the Water Pollution Control Station (WPCS), then sludges are stabilized the Akron Composting Facility (ACF). The WPCS (figure 3) is a single stage nitrification, activated sludge treatment facility with an average design flow of 90 million gallons per day to process raw primary and waste activated sludges. Sludges produced during wastewater treatment are then pumped to the ACF. In this facility, sludges are dewatered using belt filter presses to take the liquid from approximately 5 to 30% solids a hence reducing the volume of sludges to be stabilized.

Figure 2. The City of Akron AD system, A: 600 m³ plug-flow EU^{CO}[®] reactor, B: 2000 m³ round tank CO^{CCUS}[®] reactor, C: AIO[®] unit (source: http://www.kbcompost.com/From_waste_to_fuel/, retrieved: 02/08/2010).

While separated liquids in the ACF return to the WPCS, 2/3 of dewatered municipal sewage sludges are composted and the remainder is anaerobically digested in a two-stage mesophilic suspended bacterial growth digestion system. Stage I consist of a 600 m³ plug-flow EU^{CO}[®] anaerobic reactor with an average OLR of 15 kgVS m⁻³d⁻¹. At stage II, effluent from stage I is pumped at an average OLR of 3.4 kgVS m⁻³d⁻¹ to a 2000 m³ round tank CO^{CCUS}[®] reactor. Both reactors design allows gentle mixing as well as even heat distribution, moreover, the main digestion process occurs in the second stage. Attached to the CO^{CCUS}[®], the all-in-one (AIO[®]) unit houses the electricity generator, feeding pumps and a constant flow gas analyzer.

For suspended bacterial growth systems, both the substrate and the biomass are completely suspended by means of intermittent or continuous mixing action. Since the biomass is not incorporated or retained by means of a specialized process design, it will be washed out from the system during digested sludge extraction, which makes necessary to maintain HRT of 10 or more days in order to prevent wash out and maintain reasonable biomass concentrations (Pind *et al.*, 2003). This aspect was considered in Akron's facility, where part of the effluent is reintroduced in the system to ensure adequate solid content of the feedstock and biomass recuperation.

Figure 3. The City of Akron Water Pollution Control Station process flow diagram, A: influent screening, B: grit removal, C: stormwater retention, D: primary settling, E: secondary aeration, F: secondary clarifiers, G: recirculation loop, H: effluent disinfection, I: Cuyahoga River, J: recycle flow stabilization, K: recycle flow equalization, L: primary gravity thickeners, M: gravity belt thickening, N: sludge mixing and holding, O: AD system and P: Akron Composting facility (source: <http://www.ci.akron.oh.us/PubUtil/wpc/index.htm>, retrieved: 02/08/2010).

Currently, interest in expansion to treat the 100% of the MSS produced in the wastewater treatment facility has led researchers to focus on the potential outcomes of converting the whole facility to a complete AD sludge stabilization system. With this approach, many questions have arisen on whether changes on the feeding recipe proposed by the German's would affect the performance and stability of the actual full-scale system. Towards a better understanding on the potential effects on converting to 100% AD facility, Quasar Energy Group LLC. and the Department of Food, Agricultural and Biological Engineering (FABE) at The Ohio State University have joined efforts to conduct experiments that will provide useful information for modeling and design for AD facilities.

Future plans for the AD system at the ACF includes installation a of a 8,000 gallon pasteurization unit capable of bringing digested solids up to 70°C for 30 minutes. Once biosolids have been pasteurized and dewatered, they will be transported to a soil manufacturing facility for further processing and final marketing (Berger and Gresser, 2008). Akron's AD system leads the country since October of 2007, managing MSS in a virtually odorless process that converts organic matter into renewable energy.

Chapter 3. Materials and Methods

Experimental Procedure

A laboratory-scale experiment was performed to study the effect of organic loading rate (OLR) on the performance and stability of continuous stirred-tank reactors (CSTR) treating municipal sewage sludge (MSS). The aim of this work was to investigate the effects that an increase in the OLR would have in a full-scale mesophilic anaerobic reactor treating a mixture of raw primary and secondary MSS for the City of Akron, Ohio.

To assess the effects on reactor stability and performance produced by an increase in organic loading rate, four different treatments were evaluated. Because of the number of experimental units available, only treatment 1 had three independent replicates (table 4). The studied OLR during this study were in accordance to the feedstock availability for the full-scale reactor operational parameters that we strived to simulate.

Bioreactor No.	Treatment	OLR (gVS L ⁻¹ d ⁻¹)	HRT (days)
1, 2 and 3	1	3.4	29
4	2	4.0	24
5	3	4.5	22
6	4	5.0	20

Table 4. Description of the four experimental treatments organic loading rates and respective hydraulic retention times.

Reactor Operation

The anaerobic digestion (AD) assays were performed at the Quasar Energy Group LLC. research laboratory located at the Ohio Agricultural Research and Developing Center (OARDC) in Wooster, Ohio. Six identical laboratory-scale CSTRs, without solids recycling (figure 4) were used. The reactors consisted of a 4 L Pyrex[®] vertical cylinder (Wilmad-Labglass, Vineland, NJ, U.S.). Continuous mixing and homogenization was achieved using a magnetic stirring bar. Reactors were operated in a semi-continuous mode (once a day feeding and wasting). Temperature was maintained at mesophilic (36 ± 1) conditions by means of a thermostatic chamber. Each reactor had a 10 L Tedlar bag fitted to a separate port for biogas collection, produced volume and methane (CH₄) content were quantified on a daily basis before feeding the reactor.

Figure 4. Schematic diagram of the laboratory-scale CSTRs. 1. Digester reactor (3.5 L working volume), 2.feeding port, 3. valve, 4.Tedlar bag for biogas collection, 5. effluent extraction port, 6. magnetic stirring bar, 7. stirring plate.

Substrate

MSS was obtained in November of 2008 from the wastewater system at the Water Pollution Control Station (Akron, Ohio) after being dewatered at the Akron Composting Facility. Feedstocks for the experiments were prepared by mixing 1:1 parts of MSS and tap water to reach the desired solid content, the mixture was then weighed according to studied OLR and stored at -20°C in individual plastic bags, everyday bags were thawed overnight at 4°C. Before feeding operations, thawed samples were acclimated at 37°C to avoid a sudden drop in temperature inside the reactors that may have negatively affected reaction rate and subsequently reduce biogas production.

Reactor Inoculation and Start-up Strategy

All the reactors were inoculated with 3.5 L of methanogenically active sludge obtained in November of 2008 from Akron Composting Facility full-scale (3000 m³) anaerobic reactor effluent. Once inoculated, reactors were sealed to initiate the start-up phase. Start-up strategy consisted of adapting the digesters biomass to the operational conditions of the laboratory-scale reactors and to an initial feed rate of 3.4 gVS L⁻¹d⁻¹. The initial feed rate was imposed in all the digesters for a one week period, this to assure that reactor operation was correct before initiating the study with the experimental OLR.

After proper function of the reactors was confirmed, feeding with the desired OLR was initiated. A second one week adaptation period to the experimental OLR was given. Following the adaptation phase, a third one week was given until reaching steady-state conditions. The experiment was then started on (day zero) and continued for an experimental period of 42 days.

Chemical Parameters

A variety of analytical measurements were used to monitor digester performance and stability. These included biogas production, CH₄ content and pH which were measured on a daily basis and total solids (TS), volatile solids (VS), total carbon (TC), free ammonia (FA), alkalinity (ALK_t) at pH 4.4 and organic acids which were measured weekly.

pH

The pH was measured using a Fisher Scientific Accumet AP85 pH/ATC meter equipped with a Fisher Scientific Accumet AP55 pH/ATC probe (Thermo Fisher Scientific Inc., Pittsburgh, PA, U.S.).

Biogas Production and Methane Content

Volumetric production of biogas was measured using ten liter tedlar bags to collect off gas from the reactors. The bags were emptied using a vacuum pump. The gas volume was measured by pumping the gas through a drum type wet-test volumetric gas meter (RITTER®, Bochum, Germany), measurements were converted to standard temperature and pressure (STP) conditions.

The methane content in the biogas was determined using a gas chromatograph (GC) HP 6890 (Agilent Technologies, Santa Clara, CA, U.S.) series equipped with a ValcoPlot VP-Alumina (Valco Instruments Co. Inc., Ontario, Canada) column and a thermal conductivity detector (TCD) operated isothermally at 40°C, helium was the carrier gas at a flow rate of 20 ml/min.

Total and Volatile Solids

The total and volatile solids content were determined according to Standard Methods for the Examination of Water and Wastewater (APHA *et al.*, 1998). For the determination of the different solids, samples were first dried at 103-105°C (method 2540B) and then the residue was ignited at 550°C (method 2540E) to constant weight.

Total Carbon

The total carbon (combustion method with CO₂ detection) analyzes were performed according to the US Composting council (Thompson *et al.*, 2002) at the Service Testing and Research laboratory (STAR) at The Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH . A VarioMax Carbon-Nitrogen combustion analyzer (Elementar Americas Inc., Mount Laurel, NJ, U.S.) was used to carry out the analyzes.

Alkalinity and Free Ammonia

Alkalinity and free ammonia concentration in the sludges were determined according to Standard Methods for the Examination of Water and Wastewater 2320B and 4500-NH₃C (APHA *et al.*, 1998), respectively. ALKT was measured using a titration method at pH 4.4, samples were titrated with hydrochloric acid (HCL at 0.1N) using a Mettler Toledo DL22 F&B (Mettler-Toledo Inc., Columbus, OH, U.S.) titrator. The concentration of FA as ammonia nitrogen was determined using the distillation and titration procedure. The distillation process from an alkaline medium (sodium hydroxide at 33% w/v) was performed using a BÜCHI B-324 (BÜCHI Labortechnik AG,

Switzerland), distillate was absorbed in boric acid (4% w/v) and then titrated with the titrant and titrator mentioned before.

Individual Volatile Fatty Acids

Individual volatile fatty acids were measured using a GC HP 5890 (Agilent Technologies, Santa Clara, CA, U.S.) series equipped with a Stabilwax[®]-DA fused silica (Restek Chromatography Products, Bellefonte, PA, U.S.) column and a flame ionization detector (FID) operated at 50°C with a maximum oven temperature of 250°C, the carrier gas was helium on a split mode. The total volatile fatty acids concentration accounts for the sum of the detected individual acids in the sample.

A preliminary sample preparation step was needed to perform IVFA analyzes. Samples were first diluted with double deionized water at a ratio of 1:5. From the diluted mixture, 12ml were then mixed with 4ml of 20% formic acid and mixed thoroughly for one minute, mixture was then filtered through a 0.2µm pore size nylon syringe filter (Whatman plc, Maidstone, Kent, U.K.), filtrate was filled into a GC vial for gas chromatography analysis.

Archaeal Community Structure

Total Genomic DNA Extraction and Polymerase Chain Reaction Amplification

Total genomic DNA was extracted from all reactors during weeks 1, 4 and 7. Approximately 0.45 g of sample were used for extraction using a PowerSoil™ DNA Isolation kit (MO Bio Laboratories Inc., Solana Beach, CA, U.S.) according to manufacturer's protocol. DNA extractions were visualized by ethidium bromide staining after gel electrophoresis in 1% (w/v) agarose and 1x TAE buffer (Tris-Acetate – EDTA, pH 8.3 ± 0.1). Quantifications of genomic DNA were performed using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE, U.S.).

The Polymerase chain reaction (PCR) amplification of 16S rRNA was performed in duplicate for each sample with the primers set Ar109f (5' - ACK GCT CAG TAA CAC GT -3') (Grosskopf *et al.*, 1998) and 6-carboxyfluorescein (FAM) labeled Ar912rt (5'-GTG CTC CCC CGC CAA TTC CTT TA -3') (Lueders and Friedrich, 2000). Master mix methodology was used to reduce variability in results by pipetting inaccuracies during PCR and restriction digestion reaction mixtures as suggested by Osborn *et al.* (2000).

The PCR mixture contained in a total volume of 100 μ l, 10 μ l of 10x/15mM MgCl_2 , 4 μ l of 25mM MgCl_2 , 2 μ l of 10mM deoxynucleoside triphosphates (dNTPs), 1 μ l of 20 μ g/ μ l of bovine serum albumin (BSA), 2 μ l of 25 μ M for each DNA primer,

DNA extract, 1 μ l of 5 U/ μ l Taq DNA polymerase HotStarTaqR[®] (QIAGEN, Valencia, CA, U.S.) and nuclease-free water (Millipore, Billerica, MA, U.S.) to complete the volume. Amplification was performed in a T-gradient Biometra thermocycler (Biometra Biomedizinische Analytic GmbH, Göttingen, Germany), the profile consisted of an initial activation step of 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 45 sec, and extension at 72°C for 1 min and a final extension of at 72°C for 10 min.

Terminal Restriction Fragment Length Polymorphism Fingerprinting Profiling

Fingerprints of archaeal community structure dynamics and putative microorganisms were analyzed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) in conjunction with gene library. Principles of T-RFLP as a quantitative molecular tool for assessing diversity of microbial communities have already been described in detail (Liu *et al.*, 1997). Duplicated PCR amplicons from reaction mixture were pooled and purified with the Wizard[®] SV Gel and PCR Clean-Up System (PROMEGA Corporation, Madison, WI, U.S.). Products were then separated on 2% (w/v) agarose gel and visualized by ethidium bromide staining, electrophoresis conditions were previously described.

Fluorescently labeled PCR products were used for separate endonuclease restriction with three different cutting enzymes, TaqI (10 U/ μ l, PROMEGA), AluI (10

U/μl, New England BioLabs Inc., Ipswich, MA, U.S.) and *BfaI* (5 U/μl, New England BioLabs), isoschizomer of *MaeI*. Enzymes were chosen according to coverage and specificity for major groups and individual methanogens in the digestion process, as described by (Padmasiri *et al.*, 2007) and initially developed by (Klein, 2003). *TaqI* restriction digestion was performed in reactions containing 1 unit of enzyme, 10X buffer E and acetylated BSA (10 μg/μl); samples were incubated at 65°C for 1 hour. *AluI* digestion protocol contained 1 unit of enzyme, 1X NEBuffer 4 and 10 mg/ml BSA; samples were incubated at 37°C for 1 hour, followed by heat enzyme inactivation of 20 min at 65°C. *BfaI* digestion reaction was the same as described for *AluI*, in contrast, samples were incubated at 37°C for 1 hour, followed by heat enzyme inactivation of 20 min at 80°C. All reactions were performed in 50 μl volumes, containing approximately 500 ng of genomic DNA, volumes were also completed with nuclease-free water.

Digested products were purified with the kit mentioned above, samples were then sent to the Research Technology Support Facility (RTSF) of Michigan State University, there, samples were mixed with 100mM ROX standard size (BioVentures Inc., MurFreesboro, TN, U.S.). TRFs were sized using an ABI PRISM[®] 3130 capillary genetic analyzer (Applied Biosystems Inc., Foster City, CA, U.S.). Electropherogram output was quantified with Perkin Elmer's ABI GeneScan Analysis System, data consisted of TRF peak size (length) in base pairs (bp), area and height. Only peaks greater than 50 fluorescence units were considered as operational taxonomic units (OTUs).

Data Analysis

Experimental data of treatment 1 was analyzed by arithmetic means and standard deviations of three independent replications. Averages and standard deviations were applied to experimental data to assess if observed changes were caused by substrate influent concentrations. Values for treatments 2, 3 and 4 represent values of only one experimental unit.

Variability of this experiment was assessed using the experimental data obtained from the three independent replicates of treatment 1 (T1) during the six week study. Two-way analysis of variance (ANOVA) were conducted for pH, acetic acid, propionic acid, total volatile fatty acids, alkalinity and free ammonia concentrations for reactor stability whereas the variable solids removal (%), volatile solids removal (%) and total carbon removal (%) were analyzed for reactor performance. The sources of variation were reactors and weeks. The statistical analyses were performed with Minitab statistical program version 15 (Minitab Inc., State College, PA, U.S.).

Archaeal community structure dynamics were studied by means of peak area (as a function of peak height and the relative spread of the peak) relative abundance (RA), calculated as the ratio of the normalized peak area of each TRF over the total peak areas of all TRFs as presented by Padmasiri *et al.* (2007). All observed TRFs were introduced to the entire TRF data set, peaks that were detected in at least one sample were also added, and a value of zero was introduced when not present in other samples (Sait *et al.*, 2003).

Experimental data for peak area was also used to analyze similarities among treatments over time by hierarchical clustering with an agglomerative algorithm. This type of clustering considers each data point as a single cluster, then merges pairs of clusters into a single cluster containing all observations (Zhao and Karypis, 2005). Community relatedness dendrogram of hierarchical clustering was performed with Minitab statistical program version 15 (Minitab Inc., State College, PA, U.S.). Correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA) were the selected distance measure and linkage type, respectively.

Putative microorganisms corresponding to specific TRF sizes matching the three T-RFLP profiles as well as common microorganisms among treatments were compared with an in silico collection of 16s rRNA sequences. The 16s rRNA based TRFs used for the in silico analysis were downloaded from the Microbial Community Analysis (MiCA, <http://mica.ibest.uidaho.edu/>) database of the University of Idaho in September 2009 (Shyu *et al.*, 2007), an allowance of ± 3 bp was given during TRFs assignments.

Chapter 4. Results

Feedstock and Inoculum

Sewage sludge produced in wastewater treatment has been found to be suitable to be anaerobically digested, however its biogas potential will be dependent on the biodegradable organic fraction. Nontoxic organic compounds such as the combination of primary and secondary microbiological sludges are a major part of the dry matter content (60% on a dry basis); other components such as inorganic material and small amount of toxic compounds can also be found in these waste streams (Rulkens, 2007).

The feedstock used for this study was municipal sewage sludge (MSS) produced in the wastewater system at the Water Pollution Control Station and dewatered at the Akron Composting Facility located in Akron, Ohio. On the other hand, the inoculum consisted of methanogenically active sludge obtained from the Akron Composting Facility full-scale (3000 m³) anaerobic reactor effluent. Chemical compositions are presented in table 5. It should be noted that “inoculum” is equivalent to Akron’s full-scale reactor effluent.

According to Berger and Gresser (2007) Akron’s wastewater sludges analyses showed that substrate is suitable to benefit from anaerobic (AD) process with a biomethane potential value of 1.21 m³ per kilograms of solids destroyed, similar to the 1 m³ kg⁻¹ suggested by Rulkens (2007).

Parameter	Units	Inoculum	Feedstock ^a
Total solids (TS)	%	9.05 ± 0.04	13.3 ± 0.10
Volatile solids (VS)	%	64 ± 0.4	76 ± 0.2
pH	SU.	8.44 ± 0.01	6.80 ± 0.04
Total Carbon (C)	% wet	3.5 ± 0.1	6.15 ± 0.02
Total Nitrogen (N)	% wet	0.45 ± 0.03	0.650 ± 0.001
C/N ratio	-	8 ± 1	9.50 ± 0.04
Free ammonia (FA)	mgNH ₃ -N kg ⁻¹	4494 ± 141	1094 ± 57
Alkalinity at pH 4.4 (ALKt)	mgCaCO ₃ eq kg ⁻¹	15234 ± 319	1986 ± 37
Total VFA (TVFA)	mg kg ⁻¹	2845 ± 134	5167 ± 20
TVFA/ALKt ratio	-	0.19 ± 0.02	3 ± 0.04
Acetic acid (HAc)	mg kg ⁻¹	2318 ± 131	3416 ± 46
Propionic acid (HPr)	mg kg ⁻¹	527 ± 138	1168 ± 34
Isobutyric acid	mg kg ⁻¹	n.d. ^b	90 ± 5
Butyric acid	mg kg ⁻¹	n.d.	319 ± 11
Isovaleric acid	mg kg ⁻¹	n.d.	129.3 ± 6
Valeric acid	mg kg ⁻¹	n.d.	n.d

Table 5. Characteristics of the inoculum and feedstock used for the experiment. Values are averages of three determinations ± standard deviations. Samples were obtained in November of 2008. ^a1:1 dewatered MSS and water. ^bNot detected.

Monitoring Process Stability

pH

Reactor pH was measured daily. Initial values were of 7.8 ± 0.1 , 7.7, 7.7, 7.7 for T1, T2, T3 and T4, respectively (figure 5). During the experiment the pH remained between 7.6 and 8.0 for all treatments, and no substantial variations were observed within reactors over time. The average of the six week pH values for each organic loading rate (OLR) was 7.8 ± 0.1 for T1, T2 and T3, T4 pH was 7.7 ± 0.1 . The lowest pH value (7.6) was measured in T4 during week 3, while a highest value of 8.0 was observed in T2 during last week of experiment. The average pH value across treatments during the six week experiment was of 7.8 ± 0.1 .

Figure 5. Variation in pH level as a function of time and organic loading rate. Values are averages of 7 days measurements per treatment. T1 had three determinations ($n=3$) $\pm$ standard deviations per day. T2, T3 and T4 had one determination per day ($n=1$).

Individual Volatile Fatty Acids

Concentrations of acetic acid (HAc) during the first week of experiment ranged between 1381 and 1468 mg kg^{-1} across treatments (figure 6). From week 2 to week 5 concentrations remained stable in a range between 1390 and 1579 mg kg^{-1} across treatments. In the last week of study a noticeable variation in HAc concentration was observed, with increases of 613, 434, 504 and 268 mg kg^{-1} that reached final values of 2043, 1900, 1894 and 1847 mg kg^{-1} for T1, T2, T3 and T4, respectively. The lowest and highest concentrations measured during this experiment were 1381 and 2292 mg kg^{-1} , for

T3 and T1, respectively. In addition, the average concentration of HAc across treatments during the six week experiment was $1542 \pm 183 \text{ mg kg}^{-1}$.

Figure 6. Acetic acid (HAc) concentrations (mg kg^{-1}) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4.

Variation in initial propionic acid (HPr) concentrations (mg kg^{-1}) during the experimental period ranged between 231 and 522 mg kg^{-1} across treatments (figure 7). HPr concentrations of T4 were substantially different from those measured in T2 and T3 during the first week of experiment. In the second week, T4 stabilized to 484 mg kg^{-1} similar to concentrations observed in T1 and T3. Moreover, T2 HPr value presented a sharp decrease reaching a concentration of 263 mg kg^{-1} in the same week. During week 3

the measured concentrations ranged between 502 and 504 mg kg⁻¹ for T1, T2 and T4; T3 concentration was considerably different with 378 mg kg⁻¹. Values had no substantial difference for T1 and T4 during week 4 with an average concentration of 341 ± 11 mg kg⁻¹. In the same week values for T2 and T3 ranged between 540 to 560 mg kg⁻¹. Observed concentrations in the final weeks of study, ranged between 344 and 613 mg kg⁻¹ for the studied influent concentrations. The lowest and the highest HPr concentrations observed during the experimental period were measured for T4 with concentrations of 232 and 613, respectively. The average HPr concentration across treatments during the six week experiment was of 439 ± 95 mg kg⁻¹.

Although slight variations were observed for the values of IVFA, concentrations remained stable during the experimental period. The averages of the six week HAc determinations were 1576 ± 231, 1529 ± 184, 1515 ± 195, and 1547 ± 161 mg kg⁻¹ for T1, T2, T3 and T4, respectively. On the other hand, the averages of the six week HPr determinations were 439 ± 60, 460 ± 117, 437 ± 67 and 421 ± 137 mg kg⁻¹ for T1, T2, T3 and T4, respectively. Isobutyric, butyric, Isovaleric and valeric acid concentrations were below the detection limit of 50 mg kg⁻¹ during experimental period for the studied OLR.

Figure 7. Propionic acid (HPr) concentrations (mg kg^{-1}) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4.

Total Volatile Fatty Acids

Initial concentrations of total volatile fatty acids (TVFAs) across treatments ranged between 1636 and 1988 mg kg^{-1} (figure 8). A slight increase in concentration was measured during week 2 for T1 and T4, reaching final values of 1990 ± 26 and 1986 mg kg^{-1} , respectively. Considerably lower concentrations were observed for T2 and T3 during the same week with values ranging between 1670 and 1800 mg kg^{-1} . Concentrations increased substantially during week 3 of experiment, the highest concentration was observed for T4 with 2031 mg kg^{-1} . In the same week T4 was

considerably different from the lowest concentration, measured for T3 with a value of 1820 mg kg^{-1} .

An opposite change was observed for week 4 of experiment, in which a decrease in concentration was observed for T1 and T4 (1846 ± 138 and 1767 mg kg^{-1} , respectively), while an increase was measured for T2 and T3 with values that ranged between 2040 and 2090 mg kg^{-1} . It was not until week 5 when concentrations for T1, T2 and T3 were slightly similar with an average concentration of $1848 \pm 45 \text{ mg kg}^{-1}$. On the other hand, T4 differed substantially from T2 and T3 with higher concentration of organic acids (1923 mg kg^{-1}). A sharp but not considerable increase in TVFAs values was observed between treatments during the last week of the experiment, reaching final concentrations of 2492 ± 287 , 2433 , 2327 and 2460 mg kg^{-1} T1, T2, T3 and T4, respectively.

The average concentration of TVFAs during the six week experiment across treatments was $1981 \pm 234 \text{ mg kg}^{-1}$. The lowest and highest concentrations were 1636 (T4) and 2492 (T1) mg kg^{-1} and were measured during weeks 1 and 6, respectively. The averages of the six week determinations per treatment were 2015 ± 241 , 1989 ± 255 , 1952 ± 215 and $1967 \pm 282 \text{ mg kg}^{-1}$ for T1, T2, T3 and T4, respectively.

Figure 8. Total volatile fatty acids (TVFA) concentrations (mg kg^{-1}) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4.

Total Alkalinity

Initial alkalinity (ALK_t) concentrations for T1, T2 and T3 were 13788 ± 487 , 13416, and 13638 $\text{mgCaCO}_3\text{eq kg}^{-1}$ (figure 9), respectively, which differed substantially from T4 concentration ($12226 \text{ mgCaCO}_3\text{eq kg}^{-1}$). A small increase was observed during week 2 for all treatments, however, the lowest ALK_t concentration was observed for T4 with $13021 \text{ mgCaCO}_3\text{eq kg}^{-1}$, considerably different with those observed for T1 and T3 of 14871 ± 627 and $14908 \text{ mgCaCO}_3\text{eq kg}^{-1}$, respectively.

Another decrease was observed during week 3 in all treatments, the highest reduction from previous week actual value was observed for T3 with $975 \text{ mgCaCO}_3\text{eq}$

kg⁻¹. T4 concentration differed substantially from the other treatments in the same week, having the lowest concentration for three consecutive weeks (12655 mgCaCO₃eq kg⁻¹). This trend remained as T4 concentrations became closer to other treatments during week 4 of study, becoming the lowest concentration of that week too with 13102 mgCaCO₃eq kg⁻¹, a substantial difference with concentrations observed for T1, T2 and T3. Concentrations in the same week for T1 were substantially different if compared with measured concentrations of T2 and T4.

Observed alkalinity concentrations for T1, T3 and T4 were similar during week 5 of experiment with an average value of 14216 mgCaCO₃eq kg⁻¹ and less than 1% standard deviation; moreover, these concentrations were substantially higher than the one of T2 (13513 mgCaCO₃eq kg⁻¹). Similar ALK_t concentrations were observed across treatments in the last week of experiment with values ranging between 14267 and 14668 mgCaCO₃eq kg⁻¹.

The averages of the six week determinations per treatment were 14430 ± 393, 13875 ± 401, 14254 ± 471 and 13239 ± 819 for T1, T2, T3 and T4, respectively. The lowest and highest concentrations were 12226 (T4) and 14908 (T3) mgCaCO₃eq kg⁻¹, respectively. The average concentration during the six week experiment across treatments was 13950 ± 693 mgCaCO₃eq kg⁻¹.

Figure 9. Alkalinity (ALKt) concentrations (mgCaCO₃eq kg⁻¹) as a function of time and organic loading rate. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Total Volatile Fatty Acids to Alkalinity Ratio

The total volatile fatty acids (TVFA) to alkalinity (ALKt) ratio (TVFA/ALKt) is used as a rapid and economic indicator of process stability. (Barampouti *et al.*, 2005; Fannin, 1987; Switzenbaum *et al.*, 1990; Wheatley, 1990). With this method, system's buffering capacity is considered to be sufficient at ratios below 0.4 (Zhao and Kugel, 1996).

Initial TFVA/ALKt ratios did not differ substantially across treatments. Ratios were found in a range between 0.13 and 0.15 (figure 10). The ratios in T1, T2 and T3 decreased slightly during week 2, while an important increase up to 0.15 was observed in

T4. This conduct ended during the third week of study with small increases in ratios for T2 and T3. T1 remained constant in the same period and T4 was considerably different from other treatments presenting the highest ratio (0.16). Even if ratios for T2 and T3 increased 0.1 and T4 decreased 0.3 in ratios, values remained within the same range without considerable differences. Ratios remained quite stable during week 5, lowest ratios were observed for T1 and T3 (0.13) compared with those observed for T2 and T4 (0.14). A sharp increase in ratios was observed for all treatments for the last week of the experiment with ratios ranging between 0.16 and 0.17.

The averages of the six week determinations per treatment were of 0.14 ± 0.01 for T1, T2 and T3, on the other hand T4 had a higher ratio of 0.15 ± 0.02 ; although, differences in ratios were not substantial. The lowest ratio measured during this experiment was 0.12 and was observed for T2 and T3 during week 2, while the highest value was observed in T1, T2 and T4 (0.17) during week 6. The average ratio during the six week experiment across treatments was 0.14 ± 0.02 .

Figure 10. Variation in the total volatile fatty acids to total alkalinity ratio (TVFA/AlkT) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4.

Free Ammonia

Initial free ammonia (FA) concentrations were considerably different across treatments with values of 3792 ± 229 , 3380, 4121 and 3176 for T1, T2, T3 and T4, respectively (figure 11). From week 1 to week 5 of study, concentrations in FA remained stable for all treatments in a range between 3057 and 4121 $\text{mgNH}_3\text{-N kg}^{-1}$ for the studied influent concentrations. Final concentrations were observed to be higher in those treatments with higher OLR if compared to T1, however, these differences were not considerable. The lowest and highest FA concentrations were measured for T4 (3058 $\text{mgNH}_3\text{-N kg}^{-1}$) and T3 (4134 $\text{mgNH}_3\text{-N kg}^{-1}$) during weeks 2 and 6, respectively. The averages of the six

week determinations per treatment were 3678 ± 183 , 3587 ± 303 , 3676 ± 387 and 3478 ± 365 $\text{mgNH}_3\text{-N kg}^{-1}$ for T1, T2, T3 and T4 respectively. The average concentration of FA during the six week experiment across treatments was 3605 ± 310 $\text{mgNH}_3\text{-N kg}^{-1}$.

Figure 11. Free ammonia (FA) concentrations ($\text{mgNH}_3\text{-N kg}^{-1}$) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4.

Parameter	Units	Treatment			
		1	2	3	4
pH	-.	7.8 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.1
Acetic acid (HAc)	mg kg ⁻¹	1576 ± 231	1529 ± 184	1515 ± 195	1547 ± 161
Propionic acid (HPr)	mg kg ⁻¹	439 ± 60	460 ± 117	437 ± 67	421 ± 137
Total VFA (TVFA)	mg kg ⁻¹	2015 ± 241	1989 ± 255	1952 ± 215	1967 ± 282
Alkalinity (ALKt)	mgCaCO ₃ eq kg ⁻¹	14430 ± 393	13875 ± 401	14254 ± 471	13239 ± 819
TVFA/ALKt	-	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	0.15 ± 0.02
Free ammonia (FA)	mgNH ₃ -N kg ⁻¹	3678 ± 183	3587 ± 303	3676 ± 387	3478 ± 365

Table 6. Summary of the parameters used for monitoring process stability. pH value is the average of daily measurements during the six week experiment. HAc, HPr, TVFA, ALKt, TVFA/VFA and FA values are averages of six determinations.

Monitoring Process Performance

Total Solids Removal

Initial TS removals of 38 ± 1 and 39 were observed T1 and T4, respectively. Initial removals of T2 (33%) and T3 (33%) were substantially different from T1 and T4 in the same week (figure 12a). An average reduction on the TS removal of $3 \pm 1\%$ was observed for T1, T2 and T3 during week 2. The highest reduction in TS removal was observed for T4 (10%) during the same week. During week 3 of experiment, an average removal of $33 \pm 0.5\%$ was measured for T1 and T4, moreover, considerably lower values were observed for T2 (30%) and T3 (29%), respectively.

Other 1 to 2% variations in TS removal were observed during the last 3 weeks of experiment across treatments, values remained in a range between 27 and 31% of removal. Final values did not differ substantially; 31 ± 1 , 29, 29 and 31% were measured for T1, T2, T3 and T4, respectively. The average TS removal across treatments achieved during the six week experiment was $31.09 \pm 0.03\%$. The lowest and highest removals were 27 and 39% and were measured for T3 (week 5) and T4 (week 1), respectively.

The averaged TS removals of six week determinations were of 1.5 ± 0.1 , 1.6, 1.8 and 2.1 for T1, T2, T3 and T4, respectively (figure 12b). Removal rates observed in T1 were substantially different from T3 and T4. On the other hand, the average rate of T4 was substantially greater than T1, T2 and T3 rates, 28, 23 and 14% greater, respectively.

An average increase of $11 \pm 4\%$ in removal rates with increased OLR rate was observed during this study. In addition, the strong relation between increased OLR and total solids removal rates given by the best-fit line of the experimental data was observed ($r^2 = 0.9328$).

Figure 12. (a) Total solids (TS) removal (%) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4). (b) TS removal rate (g L⁻¹d⁻¹). Values are averages of six week determinations. Note: standard deviations for T1 were smaller than symbols in (a).

Volatile Solids Removal

Initial VS removals were of 54 ± 0.9 , 51, 51 and 55% for T1, T2, T3 and T4, respectively (figure 13a). A 48% removal of VS was observed for T2 during the week 2, while other removals remained within 51 and $54 \pm 0.4\%$. Similar conducts were observed during the last 4 weeks of study across treatments with values ranging between 49 and 55% removal of VS. T2 presented the lowest removal value (48%) during week 2 of experiment, while the highest was achieved by T4 (55%) during week 1. The average VS removal observed during the six week experiment across treatments was $52.02 \pm 0.02\%$.

Substantial differences in VS removal rates were observed across treatments (figure 13b). The averages of the six week determinations per treatment were 1.80 ± 0.05 , 2.02 ± 0.05 , 2.31 ± 0.07 and $2.66 \pm 0.02 \text{ g L}^{-1}\text{d}^{-1}$ for T1, T2, T3 and T4, respectively. An average increase in removal rates of $12 \pm 1\%$ was observed with the linear increases in the OLR. The regression coefficient (r^2) given by the best-fit line of the VS removal rate data was 0.9892, showing the strong relation between system VS removals capacity with increased OLR (figure 13 b).

Figure 13. (a) Volatile solids (VS) removal (%) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4). (b) VS removal rate (g L⁻¹d⁻¹). Values are averages of six week determinations. Note: standard deviations for T1 were smaller than symbols in (a).

Total Carbon Removal

Week 1 total carbon removals were of 54 ± 3 , 54, 52 and 56% for T1, T2, T3 and T4, respectively (figure 14a), moreover, differences were not substantial. Reductions ranging between 1 and 6% were observed during week 2 of experiment across treatments; however, differences in removals values were not substantial. TC removals remained stable from weeks 3 to 5 with values ranging between 49 and 53%, differences across treatments were not considerable.

Slight reductions were observed during the last week of experiment reaching an average removal of $48.0 \pm 0.3\%$ for T1, T2 and T4, while a sharp and substantial decrease was observed for T3 reaching a final removal of 46% (the lowest of this study). T4 achieved the highest TC removal during the first week of the experiment (56%). The average TC removal during the six week experiment across treatments was $50 \pm 2\%$.

Total carbon removal rates were found to increase proportional with an increase in substrate concentrations (figure 14b). Removal rates were of 1.05 ± 0.05 , 1.20 ± 0.06 , 1.33 ± 0.06 and 1.56 ± 0.05 for T1, T2, T3 and T4, respectively. Important differences were found between T1, and T3, also substantial differences between T1 and T4 were observed. An average increase in removal rates of $12 \pm 2\%$ was observed with increases in the influent concentrations. The regression coefficient (r^2) given by the best-fit line of the average of six week determinations data was 0.9811, showing the strong relation between TC removal rates with increased OLR (figure 14b).

Figure 14. (a) Total carbon (TC) removal (%) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per week ($n=1$) for T2, T3, T4). (b) TC removal rate (g L⁻¹d⁻¹). Values are averages of six week determinations. Note: standard deviations for T1 were smaller than symbols in (a).

Cumulative Biogas Production

Volumetric biogas production was measured daily. The rate of biogas cumulative production increased linearly during the experimental period (figure 15). There was a substantial increase in the rate of biogas production with increased OLR. Cumulative productions measured at the beginning of week 1 were similar for T1 and T2, however, a substantial difference was observed for T3 and T4 with other treatments in the same week (table 7). This conduct continued until week 4, where values differed substantially by 20 L or greater across treatments. Cumulative biogas production values at the end of the experiment were 230 ± 2 , 275, 302 and 316 L, for T1, T2, T3 and T4, respectively (table 7). Increasing the OLR from 3.4 to 4.0 gVS L⁻¹d⁻¹ increased the cumulative biogas production by 16%, 9% with an increase from 4.0 to 4.5 gVS L⁻¹d⁻¹, and 4% when increased from 4.5 to 5.0 gVS L⁻¹d⁻¹. A 19% increase in biogas production was obtained with increasing the OLR from 3.4 to 5.0 gVS L⁻¹d⁻¹.

Figure 15. Daily Cumulative biogas production (L) as a function of time and organic loading rate. Values are averages of three determinations ($n=3$) $\pm$ standard deviations for T1 and one determination per day ($n=1$) for T2, T3, T4). Note: standard deviations for T1 were smaller than symbols.

Week	Unit	Treatment			
		1	2	3	4
1	L	35 ± 1	37	51	50
2		70 ± 0.3	80	97	98
3		112 ± 1	133	149	152
4		152 ± 2	180	204	209
5		191 ± 1	228	257	266
6		230 ± 2	275	302	316

Table 7. Cumulative biogas production at the end of each week of experiment.

Methane Content in Biogas

The concentration of methane (CH₄) in the biogas during the anaerobic digestion period remained virtually constant among the studied treatments (figure 16). The averaged CH₄ content across treatments was $62 \pm 1\%$. The lowest and highest methane content observed during this six week study were 61% and 63%, respectively. There were no substantial differences on methane content among treatments during the six week experiment.

Figure 16. Variation in the methane content (%) in the biogas as a function of time and organic loading rate during the experiment. Values are averages of 7 days measurements per treatment. T1 had three determinations ($n=3$) $\pm$ standard deviations per day. T2, T3 and T4 had one determination per day ($n=1$).

Methane Production Rate

Methane production rate increased linearly with increased OLR (figure 17). The averages of the six determinations for each treatment were 1.0 ± 0.1 , 1.2 ± 0.1 , 1.3 ± 0.1 and 1.4 ± 0.1 $\text{LCH}_4 \text{ L}^{-1}\text{d}^{-1}$ for T1, T2, T3 and T4, respectively. Increasing the OLR from 3.4 to 4.0 $\text{gVS L}^{-1}\text{d}^{-1}$ increased the volumetric methane production rates by 16%, and from 4.5 to 5.0 $\text{gVS L}^{-1}\text{d}^{-1}$ resulted in a 4% increase. Differences in methane production rates were only substantial between T1 and T3, and T1 with T4. The regression coefficient (r^2) given by the best-fit line of the average of six week determinations data was 0.9344, showing the strong relation between methane production rate with increased OLR (figure 17).

Figure 17. Methane production rates ($\text{LCH}_4 \text{ L}^{-1}\text{d}^{-1}$) as a function of the organic loading rate. Values are averages of weekly measurements during the six week experiment.

Methane Yield

Experimental data for methane production and volatile solids removal rates were used to determine the average methane yield of the six week experimental period (figure 18). The methane yield is widely used to evaluate the efficiency of the system in important transient phases. The methane yield was calculated as the volume of gas produced divided by amount of volatile solids removed during all the experiment for each OLR. The average methane yield obtained during the anaerobic digestion period for the studied organic loading rates was $0.57 \pm 0.07 \text{ LCH}_4 \text{ gVS}^{-1}_{\text{removed}}$, no considerable differences between treatments were observed for the averaged six week determinations.

Figure 18. Methane yield (LCH₄ g VS⁻¹_{removed}) as a function of organic loading rate. Values are averages of six week determinations. T and R are treatment and reactor number, respectively.

Parameter	Units	Treatment			
		1	2	3	4
Volatile solids (VS) removal^a	%	53 ± 1	51 ± 1	51 ± 2	53 ± 0.4
Volatile solids removal rate^a	g L ⁻¹ d ⁻¹	1.80 ± 0.05	2.02 ± 0.05	2.31 ± 0.07	2.66 ± 0.02
Total carbon (TC) removal^a	%	51 ± 2	50 ± 3	49 ± 2	52 ± 2
Total carbon removal rate^a	g L ⁻¹ d ⁻¹	1.05 ± 0.05	1.20 ± 0.06	1.33 ± 0.06	1.56 ± 0.05
Cumulative biogas production^b	L	230 ± 2	275	302	316
Methane content in the biogas^c	%	62 ± 1	62 ± 1	62 ± 0.4	63 ± 1
Methane production rate^c	LCH ₄ L ⁻¹ d ⁻¹	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Methane yield^a	LCH ₄ gVS ⁻¹ _{removed}	0.57 ± 0.06	0.61 ± 0.07	0.58 ± 0.06	0.53 ± 0.06

Table 8. Summary of the parameters used for monitoring process performance. ^a Average of six determinations. ^b Cumulative value. ^c Average of daily measurements during the six week experiment.

Archaeal Community Structure Dynamics and T-RFLP Profiling

The final stage of the anaerobic digestion (AD) process consists of using methane precursors formed during a sequence of biochemical reactions, to be finally converted into mainly methane and trace gases by methanogens. To investigate the effects of the four organic loading rates (OLR) of municipal sewage sludge on microbial community structure, archaeal community dynamics was determined using terminal restriction fragment length polymorphism (T-RFLP).

After amplification with archaeal specific primers, three different restriction enzymes were used to generate terminal restriction fragments (TRFs). After *TaqI* digestion, the most prominent TRFs during week 1 had sizes of 170 and 185bp. The 170 bp TRF peak area was found to be 15 ± 9 , 16, 20 and 8% of the total relative abundance (RA) for T1, T2, T3 and T4, respectively (figure 19a). In addition, an initial RA of 81 ± 9 , 79, 76 and 90% was observed for 185 bp TRFs for T1, T2, T3 and T4, respectively. Other TRFs observed that together accounted for an average $4 \pm 1\%$ of the total RA had sizes of 89, 108, 144, 190 and 794 bp. In a similar manner, end of experiment samples showed that population dynamics in the digesters were affected minimally over the period of study (figure 19b). No pronounced changes in the peak area percent of the 185 bp TRF were observed and this peak accounted for $79 \pm 9\%$ of the total RA, followed by 170 bp with 17 ± 8 and $3 \pm 1\%$ for diverse TRFs.

Initial *AluI* restriction digestion (figure 20a) produced 4 major TRFs with sizes of 152, 166, 319 and 794 bp. Minor TRFs were observed at sizes of 62, 71, 106, 125, 239,

307 and 776 bp. Initial analysis with *AluI* revealed that the 166 bp fragment was most abundant, with an average RA across treatments of $83 \pm 2\%$, without considerable differences among the studied OLR. This similarity between treatments was also observed for TRFs 152, 319, 794 and diverse with average RA of 4 ± 1 , 8 ± 0.7 , 4 ± 1 and 1 ± 1 for the same period, respectively (figure 20a). An average variation of 1.74% in TRFs RA was observed from week 1 to 6 of experiment for all OLR, final average RA values were of 4 ± 2 , 82 ± 4 , 8 ± 1 , 4 ± 1 and $0.5 \pm 0.5\%$ for TRFs 152, 166, 319, 794 and diverse, respectively.

Figure 19. Effects of four different OLR on relative abundance of archaeal terminal restriction fragments generated by *TaqI* during anaerobic digestion of municipal sewage sludge. (a) week 1 and (b) week 6. Values are the percent of relative abundance of each observed TRF peak area obtained for digested amplified DNA with restriction enzyme *TaqI*. Only major archaeal TRFs are shown, minor TRFs have been combined as “diverse”.

Figure 20. Effects of four different OLR on relative abundance of archaeal terminal restriction fragments generated by *AluI* during anaerobic digestion of municipal sewage sludge. (a) week 1 and (b) week 6. Values are the percent of relative abundance of each observed TRF peak area obtained for digested amplified DNA with restriction enzyme *AluI*. Only major archaeal TRFs are shown, minor TRFs have been combined as “diverse”.

Figure 21. Effects of four different OLR on relative abundance of archaeal terminal restriction fragments generated by *BfaI* during anaerobic digestion of municipal sewage sludge. (a) week 1 and (b) week 6. Values are the percent of relative abundance of each observed TRF peak area obtained for digested amplified DNA with restriction enzyme *BfaI*. Only major archaeal TRFs are shown, minor TRFs have been combined as “diverse”.

The most abundant TRF generated by *BfaI* restriction digestions initiating the experiment was of 104 bp, followed by 95 bp fragment, and then diverse TRFs group (figure 21a). Diverse TRFs were composed by fragments of 70, 128, 620, 665 and 794 bp. Initial average RA values for 95, 104 bp and diverse TRFs were 12 ± 3 , 83 ± 4 and $5 \pm 1\%$, respectively. Final results were also in accordance by those observations of enzymes *TaqI* and *AluI*, in which changes in archaeal structure were not prominent. Most abundant TRF (104 bp) RA decreased by 1.7% reaching a final RA of 81 ± 6 ; 13 ± 6 and 5 ± 0.5 were detected for 95 bp and diverse TRFs, respectively. Stable RA values over time were also observed for TRFs generated by *BfaI* restriction digestion as observed during the last week of experiment (figure 21b).

Composite profiles for TRFs RA abundance were used to assess similarity between the 6 reactors (1, 2 and 3 were replicates of T1), with experimental data obtained during the three sampling times (week 1, 4 and 7). The resulting dendrogram (figure 22) generated 17 clusters within 97 and 100% of similarity; 3 major clusters were observed in a range of between 97 and 99% similarity, the other 14 clusters were 99% similar. The results are in accordance with those observed for RA analysis by individual restriction enzymes, in can be inferred that there was no substantial effect of OLR on archaeal structure dynamics. The composite dendrogram revealed a 97% of similarity across treatments.

Figure 22. Community relatedness dendrogram of TRFs composite peak areas RA generated by *TaqI*, *AluI* and *BfaI* digestions of 16S rRNA samples from anaerobic CSTRs treating municipal sewage sludge. Input data consisted of TRFs peak areas RA for each of the 6 reactors (R1, R2, R3, R4, R5 and R6) for three sampling events, weeks 1 (W1), 4 (W4) and 6 (W6). The correlation coefficient (distance) and UPGMA (linkage) were used to perform the hierarchical clustering and obtain the similarity dendrogram.

T-RFLP data was used to obtain a list of ribotypes that matched the TRFs from all three restriction digestions for each sludge sample. Matches of three different T-RFLP profiles that constituted more than 1% of the PCR product were from archaeal members belonging to the Euryarchaeota and Crenarchaeota phyla (table 9). Orders of Euryarchaeota related to the biological conversion process in this study were found to belong to Methanomicrobiales, Methanosarcinales and Methanobacteriales. The TRF combination used to find the Crenarchaeota phylum, showed that it belonged to an uncultured *crenarchaeote*.

Methanobrevibacter spp. and an uncultured *Methanosphaera* spp. correspond to the Methanobacteriales order. An uncultured *Methanocalculus* sp. and *Methanococcoides* sp. correspond to the Methanomicrobiales and Methanosarcinales orders, respectively. These combinations generated 20 matching species for the *Methanobacterium* genus, 13 for the *Methanobrevibacter*, and 2 to 1 for the other cultured and non-cultured genus (table 9).

Match #	Terminal restriction fragment (TRF) and respective size (bp)			Identified organism by restriction enzyme combination	Number of species in genus	Substrates
	<i>TaqI</i>	<i>AluI</i>	<i>BfaI</i>			
1	185	71	128	uncultured <i>Methanomicrobia</i> sp.	1	H ₂ , CO ₂ , formate
2	185	125	104	uncultured <i>crenarchaeote</i> sp.	1	*
3	185	166	104	<i>Methanosarcina</i> spp.	2	Acetate
4	185	166	620	uncultured <i>Methanocalculus</i> sp.	1	Acetate
5	794	71	128	<i>Methanobrevibacter</i> spp.	13	H ₂ , CO ₂ , formate
6	794	71	794	<i>Methanobacterium</i> spp.	20	H ₂ , CO ₂ , formate
7	794	71	794	uncultured <i>Methanosphaera</i> sp.	2	H ₂ , methanol ^b
8	794	166	104	uncultured <i>Methanococcoides</i> sp.	1	Methanol ^c

Table 9. Identified archaeal lineages observed in all four treatments in digesters treating municipal sewage sludge using terminal restriction fragment length polymorphism (T-RFLP). ^aSame TRF combination; ^b(Bank *et al.*, 1996; Fricke *et al.*, 2006; Miller and Wolin, 1985); ^c(Demirel and Scherer, 2008), *Not found or still unclear.

Analysis of Variance

Variability of this experiment was assessed using the experimental data obtained from the three independent replicates of treatment 1 (T1) during the six week study. Two-way analysis of variance (ANOVA) were conducted for each variable, these included pH, acetic acid (HAc), propionic acid (HPr), total volatile fatty acids (TVFA), alkalinity (ALKt) and free ammonia (FA) concentrations for reactor stability. For reactor performance, total solids (TS) removal (%), volatile solids (VS) removal (%) and total carbon (TC) removal (%) were analyzed. The sources of variation were reactors and weeks.

The mean pH values among reactors and weeks were compared by a two-way ANOVA. There were no significant differences among reactors for pH values ($F = 1$; $p > 0.05$; $df = 2$). On the other hand, highly significant differences were found among pH values during the experimental period ($F = 37$; $p < 0.001$; $df = 5$). Analysis of the means (95% confidence intervals -CIs-) indicated that weeks 1 and 2 were significantly different from week 3 and 4. On the other hand, weeks 3 and 4 were significantly different from weeks 5 and 6.

The analysis of HAc concentrations mean values among reactors and weeks indicated that there were no significant differences among reactors for HAc values ($F = 0.34$; $p > 0.05$; $df = 2$). On the other hand, significant differences were found among HAc values during the experimental period ($F = 13.65$; $p < 0.001$; $df = 5$). Analysis of the means (95% CIs) indicated that results were significantly different only for week 6 of

study. Analysis of HPr concentrations mean values among reactors and weeks indicated that there were no significant differences among reactors for HPr values ($F = 2.84$; $p > 0.05$; $df = 2$). In addition, no significant differences were found among HPr values during the experimental period ($F = 1.13$; $p > 0.05$; $df = 5$).

The analysis of TVFA concentrations mean values among reactors and weeks indicated that there were no significant differences among reactors for TVFA values ($F = 2.56$; $p > 0.05$; $df = 2$). On the other hand, significant differences were found among TVFA values during the experimental period ($F = 9.40$; $p = 0.002$; $df = 5$). Analysis of the means (95% CIs) indicated that results were significantly different only for week 6 of study.

The analysis of FA concentrations mean values among reactors and weeks indicated that there were no significant differences among reactors for FA values ($F = 0.78$; $p > 0.05$; $df = 2$). In addition, no significant differences were found among FA values during the experimental period ($F = 1.39$; $p > 0.05$; $df = 5$).

The analysis of the variation on TS removal mean values among reactors and weeks indicated that there were no significant differences among reactors for TS values ($F = 0.66$; $p > 0.05$; $df = 2$). On the other hand, significant differences were found among TS values during the experimental period ($F = 51.72$; $p < 0.001$; $df = 5$). Analysis of the means (95% CIs) indicated that results from week 1 were significantly different from weeks 2 and 3. In addition, weeks 4, 5 and 6 were significantly different from the first 3 weeks of experiment.

The analysis of the variation on VS removal mean values among reactors and weeks indicated that there were no significant differences among reactors for VS values ($F = 0.59$; $p > 0.05$; $df = 2$). On the other hand, significant differences were found among VS values during the experimental period ($F = 9.64$; $p = 0.001$; $df = 5$). Analysis of the means (95% CIs) indicated that results from week 6 were significantly different from the other 5 weeks. In addition, weeks 1, 2 and 3 were significantly different from weeks 4 and 6.

The analysis of TC removal mean values among reactors and weeks indicated that there were no significant differences among reactors for TC removal values ($F = 0.15$; $p > 0.05$; $df = 2$). In addition, no significant differences were found among TC removal values during the experimental period ($F = 2.12$; $p > 0.05$; $df = 5$).

Chapter 5. Discussion

The hypothesis of this study was that increasing the organic loading rate (OLR) in a full-scale, continuous, high solid content sewage sludge digestion process from 3.4 to 5.0 gVSL⁻¹d⁻¹ would not affect the biochemical or molecular properties related to the stability and performance. The performance of the digesters and changes in microbial community structure were characterized by collecting biochemical and molecular data. These included organic matter removal efficiency, biogas production rate, pH, free ammonia (FA), total volatile fatty acids (TVFA), alkalinity (ALKt), individual volatile fatty acids (IVFA) and methane yield. In addition, the effects of loading rate on the archaeal community structure were determined by PCR amplification of 16S rRNA genes with labeled archaeal primers and terminal restriction fragment length polymorphism (T-RFLP) analyses. Previous investigations on AD of similar substrates have demonstrated the effects of increased OLR, including variations in microbial diversity and lower organic removal efficiencies (McMahon *et al.*, 2004; Stroot *et al.*, 2001).

As shown in the experimental data, pH during this study remained stable with values ranging between 7.6 and 8.0, these have been considered to be within optimal ranges for adequate mixed-culture reactor stability (Moosbrugger *et al.*, 1993; Speece, 1996). Furthermore, this study demonstrated that increases in OLR up to 5.0 gVS L⁻¹d⁻¹ did not result in a decrease in pH.

One of the main factors contributing to reductions in pH is the production and build up of VFA during the conversion of soluble organic components into organic acids. This accumulation can result in complete process failure since acid consumers (methanogens) are more sensitive to pH decreases, ceasing consumption of fatty acids. During this experiment the IVFA that gave the major contribution to the TVFAs concentrations, were acetic and propionic acids. Although slight fluctuations were observed for the two measurable organic acids, the weekly average values indicated that concentrations remained stable across treatments. Acetic and propionic concentrations (average of 7 week determinations) were found to be stable at 1542 ± 183 and 439 ± 94 mg kg⁻¹, respectively. Similar results indicating acetic and propionic acids to be the most prominent during anaerobic digestion of wastewater sludges have been reported. For example, de la Rubia *et al.* (2006) observed that higher concentrations of IVFA were also accounted for acetic and propionic acids with average concentrations of 2093 and 1910 mg L⁻¹, respectively.

Since no accumulation was observed, it can be inferred that stable methanogenic conversion rates during the experiment was not affected due to substantial amounts of these acids in the reactors. For example, Fukuzaki *et al.*, (1990) evaluated the inhibition of fermentation to methane by partial pressure of hydrogen, acetate and propionate in a sulfate-free minimal medium. Their results suggested that propionate had no inhibition effect on its own conversion rate to methane when concentrations were below 3500 mg L⁻¹ and pH was 7.5. In addition Chen *et al.* (2007) reported acetic and propionic acids to be

the main products while conducting experiments to evaluate the effect of different pH on the hydrolysis and acidification stages of waste activated sludge.

Concentrations on TVFA during this study were also found to remain stable over time. The average concentrations of the six determinations across treatments ranged between 1952 ± 215 and $2015 \pm 241 \text{ mg kg}^{-1}$, with no substantial differences. The overall behavior on the TVFA will be compromised to changes on the IVFA with higher concentrations. In our study acetic acid accounted for the majority of the TVFA concentrations. Transitory increases in the volatile fatty acids have been reported during start-up of the digestion process, when the microbial consortia has not stabilized (Hartmann and Ahring, 2005). TVFA concentrations at different hydraulic retention times (HRT) were also analyzed by de la Rubia *et al.* (2006). The authors evaluated the effect of HRT on thermophilic AD of pre-thickened primary and secondary sludges. In this study concentrations were found to increase from 5435 to 6655 mgHAc L⁻¹ when the HRT decreased. In the same study, an alkaline source (sodium carbonate) was added to improve system's buffering capacity and subsequently maintain a pH between 7.4 and 7.9.

In this study, it was observed that even if TVFA concentrations increased, pH was not considerably affected. Moreover, drastic changes in pH are usually observed when system's buffering capacity is not sufficient. Stability in pH in reactors with adequate buffering capacity has been reported during AD of various waste streams (Agdag and Sponza, 2005; Borja *et al.*, 2002; de la Rubia *et al.*, 2006; Pind *et al.*, 2003; Rincon *et al.*, 2008). This conduct was also observed in this study, since optimal biological activity was

avored by high alkalinity concentrations across the studied OLR. The experimental data obtained during this laboratory-scale experiment, indicates that a total ALKt of $13950 \pm 693 \text{ mgCaCO}_3\text{eq kg}^{-1}$ was sufficient to prevent a pH decline to 7.0. Furthermore, although ALKt concentrations were substantially lower during the first 4 weeks of experiment for T4, no substantial variations on pH were observed for the same treatment.

The TVFA to ALKt has also been used as a rapid indicator of process imbalance. Ratios below 0.4 are typical of a digestion process without acidification risk. The high buffering capacity and relatively stable concentrations of TVFA observed in this experiment contributed to maintain optimal conditions for digestion process with increased OLR. In accordance with higher concentrations of acetic acid at final stages of this experiment, this ratio was found to be slightly higher for the same period. The highest TVFA to ALKt ratio (0.16-0.17) achieved during this study, were observed across treatments during the last week of experiment, favored by sharp increases in the TVFA concentrations. Furthermore, ratios for this experiment remained within the suggested ratios for digestion processes. The use of this ratio to monitor process instability was found to be adequate for this particular substrate, since variations in pH were not observed during the six week experiment.

High concentrations of FA can lead to process inhibition in AD systems, where ammonia is produced as a result of organic material degradation (Appels *et al.*, 2008). FA concentrations were found to be slightly higher at the end of the experiment for all treatments. Moreover, values remained relatively stable during the experimental period. In addition, final concentrations did not differ substantially among the four OLR. The FA

concentrations were found to range from 3176 and 4134 mgNH₃-N kg⁻¹. Furthermore, measured FA concentrations were not affected by increased in the OLR and remained within ranges for adequate reactor performance. Moreover, Koster and Lettinga (1988) observed that when ammonia concentrations ranged from 4051 and 5734 mgNH₃-N L⁻¹, methanogenic populations lost 57% of their activity during digestion of potato processing wastewaters.

In addition, results obtained by Sung and Liu (2003) showed total inhibition of acetoclastic methanogens was not reached when ammonia values reached as high as 10000 mgNH₃-N L⁻¹ during digestion of synthetic wastewaters in thermophilic conditions under various pH and acclimation conditions. In our six week experiment, measured FA levels were acceptable for AD of MSS. This could be attributed to the use of biomass already acclimatized to the substrate; or that reactor conditions that influence the level of ammonia inhibition, such as concentration of total ammonia, pH and temperature were not favorable to cause methanogenesis inhibition by this substance (Sung and Liu, 2003).

The average total solids (TS) destruction observed during this study was 31.09 ± 0.03%. On the other hand, the averaged TS removal rates in our study were observed to be improved when increasing the influent concentrations, T4 removal rates were found to be substantially different than the ones observed for T1 and T2. An increase in OLR up to 5.0 gVS L⁻¹ d⁻¹, increased TS removals by 29 ± 6 and 26 ± 4% if compared with OLR of 3.4 and 4.0 gVS L⁻¹ d⁻¹. Studies conducted by Zhang *et al.* (2008) showed TS removals of 48% during digestion of sludges in a sequencing batch experiment, they also observed that co-digestion with the organic fraction of municipal solids waste improved TS, VS

removal and biogas yield. This was attributed to the improved C:N ratios after addition of the organic fraction of municipal solids waste (OFMSW).

The VS removals (%) were found to be similar between the studied OLR, with no considerable differences in the six week average values. The average removal achieved across treatments was of $52.02 \pm 0.02\%$. Furthermore, six week averaged VS removal rates were observed to increase linearly with increased OLR. Experimental data obtained during this study, showed improvements of 11, 13 and 12% when increases in OLR from 3.4 to 4.0, 4.0 to 4.5 and 4.5 to 5.0 gVS L⁻¹d⁻¹ were compared, respectively. Generally, the biodegradability of the organic substrate is measured as the percent of VS destruction in the system. Typical reported values for VS removals are shown in table 10.

Scale	Substrate	OLR gVS L ⁻¹ d ⁻¹	Temperature (C°)	VS removal (%)	Methane yield (LCH ₄ gVS ⁻¹ _{removed})	Reference
Lab	WAS	*	35	14 - 30	0.6-0.9 ^a	a
Lab	PS + TWAS	0.99	35	25.3	0.6	b
Lab	WAS	*	35	19 - 35	0.4 – 0.5	c
Lab	WAS	0.26	35	55.4	0.3	d
Pilot	MSS	0.96 – 2.77	55	51.5	0.3 - 0.4	e
Pilot	WAS	5.28	35	42.1	0.3	f
Full	WAS	*	35	29-36	0.6	g
Full	WAS	0.8 - 1	34 - 38	13 - 27	0.5 - 0.9	h

Table 10. Performance data of different anaerobic processes applied to sludges. Substrates included WAS (waste activated sludge), PS + TWAS (primary sludge and thickened WAS) and MSS (municipal sludge). a: (Gossett and Belser, 1982); b: (Kabouris *et al.*, 2009); c: (Lin *et al.*, 1997); d: (Habiba *et al.*, 2009); e: (de la Rubia *et al.*, 2006); f: (Ai *et al.*, 2005); g: (Bixio *et al.*, 1999); h: (Bolzonella *et al.*, 2005). ^aBiogas (methane + trace gases) yield. *Reported as grams of COD.

The proportional removal of organic matter with increasing the OLR, was also reflected in terms of volumetric biogas production. Cumulative biogas productions measured during the experimental period were substantially different across treatments. The highest cumulative biogas production in this study was achieved by T4 with 316 L, which corresponds to an average daily production of 7.5 L of biogas.

The average methane content measured during the experimental period was $62 \pm 1\%$ and no substantial differences were observed across treatments over time. The values observed during this experiment are in the same range of those reported for stable digesters treating similar substrates (Rulkens, 2007). In addition, experimental data of methane content and volumetric biogas production were used to calculate the average methane production rates during the 42-day study. Values in methane production rates also increased linearly with increased OLR. The lowest rate was observed for T1 with 1.0 ± 0.1 and the highest for T4 with $1.4 \pm 0.1 \text{ LCH}_4 \text{ L}^{-1} \text{d}^{-1}$.

Experimental data on volumetric methane production and organic material removal were used to assess system efficiency by methane yields as a result of organic matter degradation. Typical values for methane yields are showed in table 10. The methane yields observed during this study were not considerably different across treatments. The average methane yield of the four OLR was $0.57 \pm 0.07 \text{ LCH}_4 \text{ gVS}^{-1} \text{ removed}$. These results suggest that improvements in performance as a result of increased OLR can be achieved during mesophilic digestion of MSS. It can be inferred that increasing reactor feeding concentration by nearly 50% will not affect digester organic matter removal efficiency, moreover, it will increase the volumetric methane production.

The strong relation between performance and microbial community present in anaerobic reactors was mentioned by Demirel and Scherer (2008), making emphasis on structure disturbances caused by environmental and operational conditions (Yu *et al.*, 2005). T-RFLP profiling of the archaeal assemblages during initial and final stages of the digestion process using labeled reverse primer suggested that there was an inherent stability within these populations in the reactors treating MSS at different organic feeding concentrations.

Despite the use of TRF information for different restriction enzymes, there was agreement in the general patterns of archaeal structure dynamics. The archaeal TRFs patterns derived from the three endonucleases used for molecular analysis over time, showed that predominant TRFs remained stable in important proportions with an average RA of $81 \pm 1.4\%$. Dominant TRFs for *TaqI*, *AluI* and *BfaI* were found at 185, 166 and 104 bp positions, respectively. Consequently, a similar behavior in lower RA proportions was also observed for those TRFs that were included in the diverse group.

Identification of putative archaeons resulting from combination of TRFs generated during DNA single restrictions resulted in the presence of members from both the Euryarchaeota and Crenarchaeota phyla (table 9). These archaea have been widely reported for this biological process, in which community dynamics during anaerobic digestion of different substrates with different operational conditions, environmental parameters and reactor configuration have also been studied (Angenent *et al.*, 2002; Demirel and Scherer, 2008; El-Mashad *et al.*, 2004; Enright *et al.*, 2009; Karakashev *et*

al., 2005; Kobayashi *et al.*, 2008; Mladenovska *et al.*, 2003; Nishio and Nakashimada, 2004; Schmidt *et al.*, 2000; Yu *et al.*, 2005).

Acetate or hydrogen and carbon dioxide or formate are the main substrates being utilized by acetotrophic and hydrogenotrophic methanogens during methanogenesis stage to produce methane (Demirel and Scherer, 2008). Thus, results obtained by T-RFLP analysis showed the obvious presence of these archaeal members during the mesophilic digestion of sewage sludge (table 9).

Acetoclastic metabolic groups have been regarded as dominant methane producers, since 70% of the gas that is produced in anaerobic digesters comes from acetate (Smith and Mah, 1966). Concerning acetotrophs dynamics, increases in reactor relative activity due to shifts from hydrogen to acetate-utilizing methanogens from start-up to steady-state conditions have been reported (Casserly and Erijman, 2003; Griffin *et al.*, 1998; Jetten *et al.*, 1992; McMahon *et al.*, 2001; Padmasiri *et al.*, 2007; Stroot *et al.*, 2001; Zheng and Raskin, 2000). On the other hand McMahon *et al.* (2004) investigated variations during laboratory-scale anaerobic co-digestion of municipal solid waste (MSW) and MSS during start-up period and overload conditions. The authors observed that digesters that started-up successfully contained high levels of archaea, being *Methanosaeta concilii* the dominant acetoclastic methanogen. Furthermore, lower levels of archaea were found in reactors that presented troubles during start-up, *Methanosarcina* spp. was found to be the abundant population.

Hydrogenotrophs, which utilize mainly hydrogen as an electron acceptor to produce methane, are also known to use formate as an electron donor to reduce CO₂ into

CH₄. Their role in maintaining hydrogen partial pressure in these environments is crucial for digester stability. In addition, maintaining adequate functioning of the syntrophic group, which are members known to take place during conversion of process intermediates to direct methane precursors (Pauss *et al.*, 1990).

In a study conducted to evaluate the effect on performance and microbial dynamics caused by an increase in temperature from 55 to 65°C in reactors treating cattle manure, the hydrogenotrophic were found to exhibit higher specific methanogenic activity (SMA). These members remained constant as revealed by cultivation method (Ahring *et al.*, 2001). On the other hand, Montero *et al.* (2008) reported that hydrogen-utilizing methanogens were dominant during start-up phase. Moreover, they were displaced by acetate-utilizing members when steady-state conditions were reached in a laboratory-scale thermophilic AD of the organic fraction of municipal solid waste.

Diversity of methanogenic consortia in full scale biogas plants have also been studied (Karakashev *et al.*, 2005), specifically, the effects of changes in environmental parameters during anaerobic digestion of sludge. The authors observed that members of the acetoclastic Methanosaetaceae were dominant in digesters with low levels of ammonia and VFA, while Methanosarcinaceae were observed to be less sensitive in digesters treating manure with higher concentrations of the same substances. McHugh *et al.* (2003) reported *Methanosaeta*-like spp. to be dominant in reactors treating a variety of low and high strength wastewaters under psychrophilic, mesophilic and thermophilic conditions, but particularly for digesters operated between 10-14°C with synthetic sewage as biomass. Crenarchaeota members have also been reported to be abundant in

anaerobic wastewater treatment systems, moreover, their roles and functions are still not clear (Collins *et al.*, 2005; Collins *et al.*, 2003).

Variability of this experiment was assessed using the experimental data obtained from the three independent replicates of treatment 1 during the six week study. Two-way ANOVA analyses indicated that there were no significant differences among the three replicates for pH, acetic acid, propionic acid, total volatile fatty acids and free ammonia concentrations for reactor stability. In addition, no significant differences among reactors were observed for total, volatile solids and total carbon removals for reactor performance.

On the other hand, significant differences between weeks were observed for pH, acetic acid, total volatile fatty acids concentrations, total solids and volatile solids removal in the same treatment over time. Furthermore, propionic acid and free ammonia concentrations, as well as total carbon removals were found to be similar in the same treatment during the six weeks of study. These results suggest that differences across reactors of the same treatment were mostly observed over time, and not between replicates.

Chapter 6. Conclusions

This study demonstrates the effect of increases in organic loading rate (OLR) on the performance and stability of mesophilic high solid anaerobic reactors treating a mixture of primary and secondary sewage sludge. Stability in the reactors was maintained during the entire digestion period, probably due to substrate composition, high buffering capacity, constant concentrations of volatile fatty acids and free ammonia, and the use of pre-adapted inoculum with well balanced microbial populations.

An increase in biodegradability was observed when increasing the OLR, with higher organic matter removal and volumetric methane production. The best results for performance were observed for T4, with an average VS removal and volumetric methane productions of $54 \pm 2\%$ and $1.4 \pm 0.1 \text{ LCH}_4 \text{ L}^{-1}\text{d}^{-1}$, respectively. Moreover, the efficiency in reactor conversion of organic matter to methane as revealed by methane yields, were found to be similar between the studied treatments with an average value of $0.57 \pm 0.07 \text{ LCH}_4 \text{ gVS}^{-1}_{\text{removed}}$.

T-RFLP analysis conducted during this laboratory-scale experiment revealed that archaeal structures remained stable among the four OLR. Microbial stability may be attributed to virtually constant values in common parameters that have been reported to cause population variations, including pH and inhibitory substances. This stability may be an effect of biomass well acclimatized to feedstock studied during this experiment.

In conclusion, this laboratory-scale study that simulated the operational conditions of a full-scale reactor, suggests that performance and stability may not be negatively impacted increased OLR. Furthermore, greater OLR increased the potential for renewable energy production in the form of methane gas. These results indicate that municipalities like Akron can increase their digestion of sewage sludge and production of energy without expanding their reactors, but simply by increasing the OLR.

Limitations of this study were directly related with the experimental period. An increase in the weeks of experiment is always positive for experiments on reactor stability during anaerobic digestion experiments. For example, slight increases in pH and the concentration of organic acids was observed during the last week of experiment. Thus, the effect of OLR on reactor performance and stability after six weeks of experiments still needs to be investigated. This aspect is one of the recommendations for future experiments, conducting a similar study for a longer period. In addition, a similar study on pilot scale digesters (50 gallon) will produce additional data on the effects of a variation on the operational conditions. As many other investigations, a long-term study of the full scale reactor should be undertaken.

Knowledge of many investigations has revealed the direct link between microbial community structure and operational parameters in anaerobic reactors, and hence the importance of coupling these analyses in order to improve process performance while maintaining reactor stability. In our study, combinations of T-RFLP profiles generated during single endonuclease restriction were primarily used to study community dynamics analyses and identification of putative microorganism. Moreover, these TRFs

combinations were not assigned to specific archaeal microorganisms. Further molecular analyses involving 16S rRNA cloning and sequencing are recommended to perform such assignments.

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Appendix A. Akron's Wastewater Treatment Plant Aerial Image

Figure 23. Akron's wastewater treatment plant and anaerobic digestion system.

Appendix B. Tabulated Data

Week	Parameter	Units	Treatment			
			1	2	3	4
1	pH	-	7.8 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1
2			7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	7.7 ± 0.1
3			7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.04	7.60 ± 0.04
4			7.7 ± 0.1	7.70 ± 0.05	7.7 ± 0.1	7.7 ± 0.1
5			7.8 ± 0.1	7.9 ± 0.1	7.80 ± 0.04	7.80 ± 0.05
6			7.9 ± 0.1	8.00 ± 0.04	7.9 ± 0.1	7.90 ± 0.05

Table 11. Tabulated data for pH variation during the experimental period. Values are averages of three determinations (n=3) per week $\pm$ standard deviation for four treatments.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	Acetic acid	mg kg ⁻¹	1468 ± 68	1466	1381	1404
2			1518 ± 89	1410	1432	1502
3			1487 ± 55	1445	1442	1529
4			1512 ± 32	1485	1552	1418
5			1430 ± 13	1466	1390	1578
6			2043 ± 213	1900	1894	1847

Table 12. Tabulated data for acetic acid (HAc) concentrations (mg kg⁻¹) during the experimental period. Values are averages of three determinations (n=3) $\pm$ standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	Propionic acid	mg kg ⁻¹	410 ± 111	522	483	232
2			472 ± 96	263	367	484
3			503 ± 54	504	378	502
4			333 ± 170	563	543	349
5			467 ± 31	374	417	344
6			450 ± 143	532	433	613

Table 13. Tabulated data for propionic acid (HAc) concentrations (mg kg⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TVFA	mg kg ⁻¹	1879 ± 177	1988	1864	1636
2			1990 ± 26	1673	1799	1986
3			1989 ± 75	1949	1820	2031
4			1845 ± 138	2049	2095	1767
5			1897 ± 28	1840	1807	1922
6			2492 ± 287	2433	2327	2460

Table 14. Tabulated data for total volatile fatty acids (TVFA) concentrations (mg kg⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	Alkalinity	mgCaCO ₃ eq kg ⁻¹	13788 ± 487	13416	13638	12226
2			14871 ± 627	14221	14908	13021
3			14814 ± 1065	14158	13932	12655
4			14356 ± 236	13622	14086	13102
5			14323 ± 205	13513	14294	14167
6			14425 ± 506	14321	14678	14267

Table 15. Tabulated data for alkalinity (ALKt) concentrations (mgCaCO₃eq kg⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TVFA/ALKt		0.14 ± 0.01	0.15	0.14	0.13
2			0.13 ± 0.01	0.12	0.12	0.15
3			0.13 ± 0.01	0.14	0.13	0.16
4			0.13 ± 0.01	0.15	0.15	0.13
5			0.130 ± 0.003	0.14	0.13	0.14
6			0.17 ± 0.02	0.17	0.16	0.17

Table 16. Tabulated data for total volatile fatty acids (TVFA) to alkalinity (ALKt) ratio during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	Free ammonia	mgNH ₃ -N kg ⁻¹	3792 ± 229	3380	4121	3176
2			3485 ± 124	3360	3417	3057
3			3440 ± 107	3277	3191	3415
4			3649 ± 78	3855	3716	3549
5			3866 ± 53	3618	3477	3573
6			3832 ± 586	4030	4134	4095

Table 17. Tabulated data for free ammonia (FA) concentrations (mgNH₃-N kg⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TS removal	(%)	38 ± 1.0	33	33	39
2			35 ± 1.0	29	31	29
3			34 ± 1.0	30	29	33
4			31 ± 1.0	29	28	30
5			30.0 ± 0.50	28	27	30
6			31 ± 1.0	29	29	31

Table 18. Tabulated data for total solids (TS) removal (%) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TS removal rate	(g L ⁻¹ d ⁻¹)	1.68 ± 0.03	1.76	1.96	2.54
2			1.55 ± 0.03	1.52	1.83	1.92
3			1.51 ± 0.04	1.57	1.73	2.17
4			1.37 ± 0.03	1.50	1.66	2.00
5			1.33 ± 0.02	1.49	1.62	1.94
6			1.37 ± 0.03	1.53	1.75	2.02

Table 19. Tabulated data for total solids (TS) removal rate (g L⁻¹d⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	VS removal	(%)	54 ± 1.0	51	51	55
2			54 ± 0.4	48	51	52
3			54 ± 1.0	52	52	55
4			52 ± 0.1	51	51	53
5			52 ± 0.2	52	51	53
6			51 ± 0.3	49	50	51

Table 20. Tabulated data for volatile solids (VS) removal (%) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	VS removal rate	(g L ⁻¹ d ⁻¹)	1.84 ± 0.03	2.03	2.31	2.80
2			1.83 ± 0.02	1.91	2.31	2.61
3			1.84 ± 0.03	2.10	2.33	2.74
4			1.800 ± 0.002	2.10	2.31	2.65
5			1.80 ± 0.01	2.10	2.32	2.70
6			1.73 ± 0.01	2.00	2.30	2.60

Table 21. Tabulated data for volatile solids (VS) removal rate (g L⁻¹d⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TC removal	(%)	53 ± 3	54	51	56
2			52 ± 3	47	48	52
3			51 ± 1	50	49	52
4			51 ± 1	49	49	50
5			51 ± 0.1	49	49	51
6			48 ± 1	48	46	48

Table 22. Tabulated data for total carbon (TC) removal (%) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	TC removal rate	(g L ⁻¹ d ⁻¹)	1.10 ± 0.07	1.31	1.39	1.70
2			1.08 ± 0.06	1.15	1.32	1.58
3			1.05 ± 0.01	1.22	1.34	1.59
4			1.04 ± 0.03	1.19	1.34	1.51
5			1.040 ± 0.002	1.19	1.34	1.54
6			1.00 ± 0.02	1.16	1.25	1.47

Table 23. Tabulated data for total carbon (TC) removal rate (g L⁻¹d⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Day	Units	Treatment				
		1	S.D. T1	2	3	4
1	L	5	1	6	7	7
2		10	1	10	15	14
3		15	1	16	21	21
4		20	1	21	29	29
5		25	1	26	38	36
6		30	1	31	44	43
7		35	1	37	50	50
8		40	1	43	57	57
9		45	0.4	49	64	62
10		50	0.4	56	71	70
11		54	0.3	62	77	77
12		59	1	67	83	84
13		65	0.2	73	90	91
14		70	0.3	80	97	98
15		76	0.3	88	105	107
16		83	1	96	114	115
17		88	1	103	120	121
18		94	1	111	127	129
19		99	1	118	134	135
20		106	1	126	142	145
21		112	1	133	149	152
22		118	1	140	158	161
23		124	1	147	166	169

Continued

Table 24. Tabulated data for cumulative biogas production (L) during the experimental period. Values are averages of three determinations (n=3) $\pm$ standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Table 24 continued

24	130	1	153	174	177
25	136	1	160	182	186
26	141	1	167	189	193
27	146	1	174	197	201
28	152	2	180	204	209
29	157	2	187	211	218
30	162	2	193	218	224
31	168	2	201	227	234
32	173	2	208	234	241
33	179	2	215	242	249
34	185	2	222	250	259
35	191	1	228	257	266
36	196	1	233	263	273
37	201	2	238	268	278
38	206	2	245	273	284
39	211	2	252	279	293
40	217	3	260	286	302
41	223	2	266	293	308
42	230	2	275	302	316

Week	Parameter	Units	Treatment			
			1	2	3	4
1	CH ₄ content	%	61 ± 2	61 ± 1	62 ± 1	62 ± 1
2			62 ± 3	62 ± 3	62 ± 2	62 ± 2
3			62 ± 1	62 ± 1	63 ± 1	63 ± 1
4			62 ± 1	62 ± 1	63 ± 1	62 ± 1
5			63 ± 1	62 ± 1	63 ± 2	63 ± 2
6			62 ± 3	62 ± 3	63 ± 2	63 ± 3

Table 25. Tabulated data for methane (CH₄) content (%) in the biogas during the experimental period. Values are averages of three determinations (n=3) per week ± standard deviation for four treatments.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	CH ₄ production rate	LCH ₄ L ⁻¹ d ⁻¹	0.90 ± 0.01	1.10	1.21	1.23
2			1.04 ± 0.02	1.30	1.33	1.38
3			1.01 ± 0.01	1.21	1.40	1.47
4			0.98 ± 0.02	1.20	1.35	1.44
5			1.03 ± 0.01	1.21	1.22	1.33
6			1.18 ± 0.02	1.41	1.56	1.62

Table 26. Tabulated data for methane (CH₄) production rate (LCH₄ L⁻¹d⁻¹) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Parameter	Units	Treatment			
			1	2	3	4
1	CH ₄ yield	LCH ₄ gVS ⁻¹ _{removed}	0.49 ± 0.01	0.54	0.52	0.45
2			0.57 ± 0.01	0.68	0.58	0.53
3			0.55 ± 0.01	0.59	0.60	0.54
4			0.55 ± 0.01	0.58	0.58	0.54
5			0.58 ± 0.01	0.58	0.52	0.50
6			0.69 ± 0.01	0.71	0.68	0.63

Table 27. Tabulated data for methane yields (LCH₄ gVS⁻¹_{removed}) during the experimental period. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
1	<i>TaqI</i>	170	%	15 ± 9	16	20	8
		185		81 ± 9	79	76	90
		Diverse		4 ± 1	5	4	2

Table 28. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *TaqI* for week 1. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
6	<i>TaqI</i>	170	%	9 ± 6	24	12	25
		185		88 ± 7	72	85	71
		Diverse		3 ± 2	4	3	5

Table 29. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *TaqI* for week 6. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
1	<i>AluI</i>	152	%	3 ± 1	5	3	5
		166		83 ± 3	82	85	80
		319		8 ± 1	8	7	9
		794		4 ± 1	3	3	6
		Diverse		1 ± 1	1	1	0.3

Table 30. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *AluI* for week 1. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
6	<i>AluI</i>	152	%	4 ± 1	2	7	5
		166		82 ± 3	87	78	81
		319		8 ± 1	7	9	8
		794		6 ± 1	3	5	5
		Diverse		0.3 ± 1	0	1	1

Table 31. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *AluI* for week 6. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
1	<i>BfaI</i>	95	%	14 ± 5	16	8	12
		104		80 ± 4	79	87	85
		Diverse		6 ± 1	5	5	3

Table 32. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *BfaI* for week 1. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.

Week	Restriction enzyme	TRF size (bp)	Unit	Treatment			
				1	2	3	4
6	<i>Bfal</i>	95	%	9 ± 1	8	19	18
		104		85 ± 1	87	75	76
		Diverse		6 ± 0.3	5	6	6

Table 33. Tabulated data for relative abundance of archaeal terminal restriction fragments generated by *Bfal* for week 6. Values are averages of three determinations (n=3) ± standard deviations for T1 and one determination per week (n=1) for T2, T3, T4.