Microbial communities in an anaerobic membrane bioreactor (AnMBR) treating domestic wastewater at ambient temperatures in a temperate climate

by

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B.S., Kansas State University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Civil Engineering
College of Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

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Abstract

The ever-increasing demand for water, food, and energy and the simultaneous diminishment of our planets' ecosystems wrought by humans have prompted a more sustainable approach to engineering the built environment. Wastewater treatment systems stand at the interface that connects the built and natural environment where potential solutions for resource and environmental issues exist. Wastewater treatment technologies can address issues involving water, food, energy, and environmental regulation when resources are properly captured from the wastewater while it's being treated. This way of thought allows wastewater to be perceived as a source of valuable products rather than an obligate waste stream. For this reason, anaerobic wastewater treatment is progressively being considered because of its ability to improve energy and resource recovery, while reducing costs and environmental impacts associated with conventional domestic wastewater treatment. More specifically, anaerobic membrane bioreactors (AnMBRs) hold promise to effectively treat wastewater at low temperatures with low energy and nutrient requirements, low sludge production, while having the benefit of generating methane-rich biogas suitable as an energy source and the potential to capture nutrients used to fertilize cropland. But, at low temperatures the microbial communities that control anaerobic digestion (AD) face biochemical obstacles. Elucidating the microbial community dynamics within AnMBRs with respect to seasonal temperatures will give insight on how to efficiently operate AnMBRs with the goal of energy-neutral wastewater treatment. DNA based tools such as advanced high-throughput sequencing was coupled with AnMBR process data to explicate the mechanism of methane production in the suspended biomass of an AnMBR from a mesophilic startup leading into psychrophilic conditions, and then returning to mesophilic temperatures.

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I would like to thank the Department of Civil Engineering at Kansas State University and the Environmental Security Technology Certification Program (ESTCP) as sources of financial funding for this research opportunity.

I would like to thank Cody Delaney, the lab technician for the Department of Civil Engineering at KSU for his work and assistance in the environmental labs. I also want to thank Harshad Kulkarni for his guidance and assistance in the lab during my first semester as a graduate student. I would like to thank the undergraduate and graduate student research assistants: Kahao Lim, Barry Schmidt, Chad Olney, Keri Brown, and Kristen Jones. This research wouldn't have been possible without their hard work on the project site and assisting with water analyses. I want to thank Dr. Mathew Kirk for his consultation on DNA sequencing and data processing and Dr. Alina Akhunova for her help with DNA quantification. I would also like to greatly thank Dr. Pat Evans for guiding this project with his superior knowledge of AnMBR operation along with my major professor, Dr. Prathap Parameswaran for his mentorship during my time at Kansas State University.

Finally, I would like to thank my graduate committee members, Dr. Stacy Hutchinson, Dr. Matthew Kirk, and Dr. David Steward, for their insights and suggestions on this work.

Chapter 1

Introduction

1.1 Literature Review

The ever-increasing demand for water, food, and energy and the simultaneous diminishment of our planets' ecosystems wrought by humans have prompted (or induced) a more sustainable approach to engineering the built environment. Wastewater treatment systems stand at the interface that connects the built and natural environment where potential solutions for resource and environmental issues exist [1]. Wastewater treatment technologies can address issues involving water, food, energy, and environmental regulation when resources are properly captured from the wastewater while it's being treated. This way of thought allows wastewater to be perceived as a source of valuable products rather than an obligate waste stream. For example, environmental biotechnologies have the capacity to produce potable water, nutrients such as nitrogen (N) and phosphorus (P) as crop fertilizers, and energy in the form of methane rich biogas. However, only a fraction of the resource potential of domestic wastewater (DWW) is captured through the conventional, energy-intensive practice of aerobic wastewater treatment [1]. Traditional wastewater treatment commonly uses the Activated Sludge Process. The energy intensive aerobic wastewater treatment consumes about 4% of the total US energy consumption [2] [3], while the influent wastewater has three times the amount of energy that is required to treat it [4]. For this reason, anaerobic wastewater treatment is progressively being considered because of its ability to improve energy and resource recovery, while reducing costs and environmental impacts associated with conventional DWW treatment.

Growing intrigue in the development of sustainable wastewater treatment technologies have led the environmental engineering community to further examine the effectiveness of anaerobic membrane bioreactors (AnMBRs) for the treatment of municipal wastewater at ambient temperature conditions with minimal heating energy input. Compared to the most common practice of treating wastewater with aerobic biologically activated sludge, AnMBRs hold promise to not only effectively treat wastewater at low temperatures with low energy and nutrient requirements, low sludge production, but also provide the valuable benefit to generate

methane-rich biogas suitable as an energy source and the potential to capture nutrients used to fertilize cropland. Being able to treat wastewater at low temperatures is significant considering the majority of earth's population resides in a temperate climate [8] where low temperatures occur during the winter months. But, at low temperatures the microbial communities that control anaerobic digestion (AD) face biochemical obstacles. Under psychrophilic conditions, biodegradation reactions of organic matter require more energy, so chemical and biological reactions proceed much slower, which leads to a decrease in the maximum specific growth and substrate utilization rates of microbes [9].

AnMBRs can have various configurations depending on where the membrane modules are placed. Permeable membranes act as filters that separate the fraction of solids that are larger than the pore size of the membrane filter from the water. Figure 1.1 represents a typical AnMBR setup where the membranes are placed in a separate bioreactor. This separate secondary membrane bioreactor allows time for the wastewater to be treated in the primary bioreactor by creating physical separation from the sludge and effluent with the membrane. This physical separation allows absolute biomass retention, thereby providing process stabilization and control by uncoupling the solids retention time and hydraulic retention time. The ability to dictate the time the biomass is retained in the bioreactors and the flowrate through the system allows for continuous performance optimization.

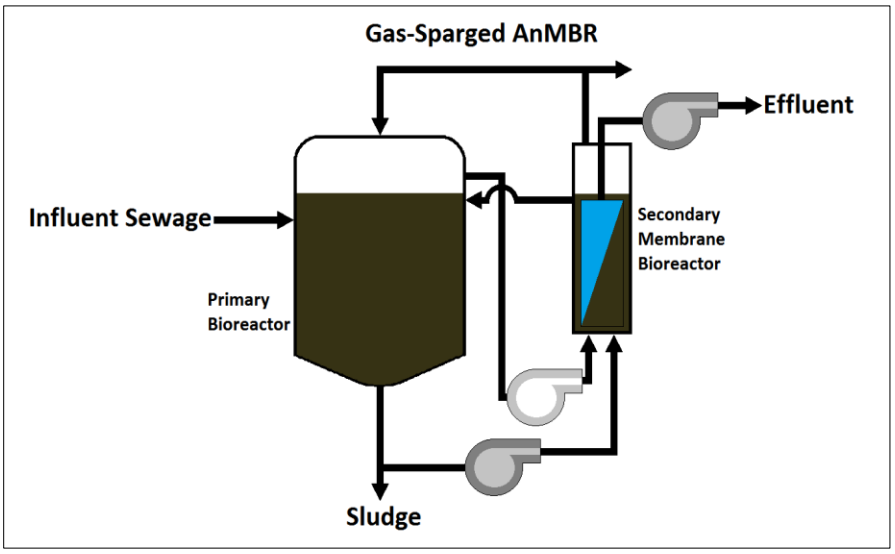

Figure 1.1. AnMBR diagram.

Anaerobic wastewater treatment at psychrophilic temperatures can prove to be a challenge when low organic loading rates (OLRs) of domestic wastewater is compounded with lowered microbial activity. Even though low operational temperatures hinder the various metabolic pathways of anaerobic digestion, AnMBRs have been reported to be fully operational at temperatures as low as 3^{o}C [7], while still being able to generate energy-rich biogas suitable for cogeneration purposes. The use of membrane filtration in AnMBRs makes it possible to decouple hydraulic retention times (HRTs) from solids retention times (SRTs). Short HRTs are required to treat large volumes of DWW while long SRTs are needed to sustain the slow growing microbial communities that perform AD. But, long SRTs can lead to membrane fouling when elevated biomass concentrations form membrane fouling products, such as SMP and EPS. Reversible and irreversible fouling of membranes in AnMBRs pose a significant problem when fouling leads to a decrease in permeate flux and an increase in transmembrane pressure (TMP) [1]. The main lines of defense against membrane fouling are membrane relaxation, back flushing, back flushing with cleaning agents, recovery cleans, and gas-sparging.

Increased methane solubility at low temperatures presents another challenge due to methane oversaturation in the permeate water. Dissolved methane recovery from the permeate and its subsequent use as a fuel source proves to be an asset that has the potential to drive AnMBRs toward being energy-neutral, or even energy-positive. Progress towards energy-neutral wastewater treatment confronts rising concerns of negative impacts that electricity generation has on our environment.

Nutrient recovery is another goal that is accomplished with coagulation/flocculation to remove phosphorus and sulfide while nitrogen can be sequestered from the water in the form of ammonia (ammonium ion) in a column containing clinoptilolite. Reuse of the treated water can provide additional revenue, reduce impacts associated with drinking water production, and alleviate stress on drinking water reservoirs [2]. With the overarching goal of environmental sustainability, this AnMBR project addresses issues present at the nexus of the valuable resources: energy, nutrients, and water.

Table 1.1. Summary of key performance parameters for AnMBR configurations treating domestic, agricultural, and industrial wastewater.

Reactor Type	Scale, Size / Time	UF/MF, Memb. Info.	Influent	Temp. (°C)	COD (mg/L) / OLR (kg COD/m³ d)	TS, MLSS or MLVSS (g/L)	TMP (kPa*) / Flux (L/m2/h)	HRT (h) / SRT (d)	COD removal/Methane Production**	Reference
AnMBR	Bench, 4 L / 129 d	MF Backflush, chem. clean	Modified Syntho	15, 25	500 / 1 (g/l/d)	6-13 MLSS	6.9–55.2 / 5	12 / 112	85%, 2.5-67.8 ml CH_4/g VSS d	Ho and Sung (2010) [8]
AnMBR	Pilot, 120 L / 120 d	UF	Brewery Waste	36	80,000–90,000 / 0.7–2.9	8.5-10 MLVSS	- / -	- / -	98%, 50 ml CH_4/g VSS d	Ince et al. (1995) [9]
AnMBR	Bench, 5 L / 30 d	MF	Municipal WW	35	480; 350–500 / -	1.05-2.41 MLSS	20-125 / 80-450	16.67 / -	98.10%	Kocadagistana & Topcu (2007) [10]
AnMBR	Bench, 5L / 70 d	MF Backflush, chemical clean	Alcohol distillery WW	55	38,400 / 3–3.5	2 MLSS	0.6 bar / 140-400	13 / ∞	>90%	Kang et al. (2002) [11]
AnMBR	Pilot, 1,500 L / 1 d, 85 d	MF Backflush, chemical clean	Piggery WW	20, 35	5,000-6,000 / -	-	0-37 / 150-825	1-2 d / -	80%, 0.32 m^3 CH_4/kg COD removed	Lee et al. (2001) [12]
AnMBR	Bench, 10 L x2 / 3.5 mo.	MF Biogas sparged, physical clean, relaxation	Kraft evaporator condensate + methanol	55; 37	10,000 / 3.1; 12.2	~9 MLSS	0-30 / 2.4; 7.2	- / 230 d	97–99%, 0.35 L CH_4/g COD / 97–99%, 0.35 L CH_4/g COD	Lin et al. (2009) [13]

AnMBR; IAFMBR	Bench, 10 L; Lab-scale, 5.8 L	UF GAC fluidized	Artificial sewage (cat food); Domestic WW	30; 35	500; 320	-	N/A; 0-30	1 d; 4, 6, 8	>96%; 80%, 200-1,600 mL CH_4/d	Gao et al. (2010) [14]; (2014) [15]
	82 d; 160 d				5; 1.29		4-12; 0.27 m^3/m^2 d	50; -		
AnMBR	Pilot, 400 L	UF Backflush, chemical clean	Food factory WW	33-39	2-15	6-8 MLSS	0.2 MPa	60	81–94%, 0.136 m^3 CH_4/kg COD	He et al. (2005) [16]
	110 d				2.0–4.5		13.1-18.9	50		
AnMBR+ Hydrolytic Reactor	Bench, 3 L	UF Biogas sparged	41% Kitchen, 48% Paper Waste	35, 21.5; 35, 20, 10	4,000-26,000	0.83-2.54; 7.2-10.8 MLVSS	-	0.37-5.7; 1.1-12 d	96.1%, 0.11–0.18 L CH_4/g COD; 95.5% sCOD removal, 0.015-0.28 L CH_4/g COD	Trzcinski & Stuckey (2009) [17]; (2010) [18]
	200 d; 30, 300 d				0.5-19.8; 4-14.1		0.5-0.8; 0-7	∞; 30, 300		
AnMBR	Bench, 50 L	UF	Municipal WW	37	685	4.3 to 4.9 MLVSS	1 to 2 bars	60-15	94%, 0.27 L CH_4/g COD	Saddoud et al. (2007) [19]
	170 d				0.23-2		3.5-13	-		
AnMBR	Bench, 50 L	UF Chemical clean	Landfill leachates	37	15, 30, 41	3 MLVSS	1-2 bar	7 d	92%	Zayen et al. (2010) [20]
	85 d				1-6.27		3-8.2	-		
AnMBR	Bench, 20 L	UF Chemical clean	Sewage sludge	70	77,500-94,000	12.76-21.8 MLSS	1.5–2.0 bar	7.8-943.4	96.5–99%, 0.19-0.54 L CH_4/g COD d	Abdullah et al. (2005) [21]
	45 d				0.1 - 10		6.9-62.1	16.1-1250		
AnMBR	Bench, 5 L	MF Biogas sparged, backflush	(SYNTHES), Domestic WW	15	440	6-10.6 MLVSS	10–45	16-24	92%	Smith et al. (2013b) [22]
	351 d				0.44-0.66		7-8	300		

AnMBR	Pilot-scale, 350 L	UF Gas sparged, relaxation, backwash; backwash	Municipal WW; WW w/glucose	35-20; 20	630; 600	9-16 MLVSS; 9.5-17.3 MLSS	177 mbar; >2 mbar/min	17-26; 0.74-1.1 d	90%; 94%	Martinez-Sosa et al. (2011) [23]; 2013 [24]
	100 d; 90d				0.6-1.1; 0.52-0.81		7; 7, 10, 12	680 d; ∞		
AnMBR	Pilot-scale, 990 L	UF GAC fluidized, relaxation	Domestic WW	8-30	207-424	0.59-1.03 MLVSS	0.06-0.5 bar	2-11.1	94%	Shin et al. (2014) [25]
	485 d				-		4.1–7.5	6.2-36		
AnMBR	Bench, 5 L	MF Biogas sparged	Domestic WW	25-30	426.8	6-9.9 MLVSS	0-30	10	83%, 0.01 L CH_4/g MLVSS	Huang et al. (2013) [26]
	90 d				1.02		-	30, 60, 90		
AnMBR	Pilot, 2,500 L	UF Biogas sparging, relaxation, backflush	Municipal WW	33	350-540	25.5 MLVS	0-0.08 bar	6	87%, 0.069 L CH_4/g COD	Gimenez et al. (2011) [27]
	150 d				-		8-13	70		
AnDMBR	Bench, 45 L	MF Physical clean	Municipal WW	10~15	302.1	5.9-19.8 MLVSS	0-24	8	57.7%	Zhang et al. (2010) [28]
	100 d				-		65	-		
AnMBR	Bench, 180 L	MF Chemical clean, backwash	Domestic WW	25	540	14-80 MLSS	15-35	4.5, 6 and 12	88%	Lew et al. (2009) [29]
	365 d				1.08, 2.16, 4.32		3.75, 7.5, 11.25	∞		
AnMBR	Pilot, 630 L; Bench, 13 L	UF biogas sparging, FeCl3 dosing	Screened sewage	23	3.4-388; 412	5.8-21.3 MLSS	1.5-21.5	8.5; 12.5	97.3%, 72-115 mL CH_4/g COD	Dong (2015) [30]
	536; 120 d				539-673; 9.4		17	40-100		

UASB w/ submerged membrane	Bench, 17.7 L 110 d	UF Chemical clean	Domestic WW	12-27	100-2,600 0.39-12.5	16-21.5 MLVSS	0-70 5-10	4, 6 150	97%, 0.13-0.42 m^3 CH_4/m^3 d	Wen et al. (1999) [31]
AnMBR	Bench, 10 L; Bench, 10 L 266 d, 440 d	MF Chemical clean, backwash	Primary effluent; municipal WW	32; -	84; 38-131 sCOD 0.03-1.64; 0.03-0.16	1-7.3 MLSS	0.1-10 psi, <1-10 psi - ; -	12, 16, 24, 48; 0.5-2 ∞; 19-217	55-68% sCOD removal; 55-72%	Baek and Pagilla (2006) [32]; (2010) [33]
AnMBR	Bench, 7 L 313 d	MF Biogas sparged, backflush	Domestic WW	15, 12, 9, 6, 3	440 0.37-0.63	-	3 °C decrease = 20 kPa increase 1.2-3.0	16-32.2 300	>95%	Smith et al. (2015) [7]
AnMBR	Pilot, 630 L 160 d	UF Biogas sparged, relaxation, chemical clean	Sewage from WW plant	22	224 0.58	13.4 MLSS	2.5 17	8.5 80-100	79%	Dagnew et al. (2012) [34]

UF/MF = Ultra Filtration/Microfiltration, Memb. Info. = Membrane Information, Temp. = Temperature, COD = Chemical Oxygen Demand, sCOD = Soluble COD, OLR = Organic Loading Rate, TS = Total Solids, MLSS = Mixed Liquor Suspended Solids, MLVSS = Mixed Liquor Volatile Suspended Solids, TMP = Transmembrane Pressure (kiloPascal), PSI = Pounds Per Square Inch, Flux = Membrane Flux, WW= wastewater, IAFMBR = Integrated Anaerobic Fluidized-bed Membrane Bioreactor, UASB = Upflow Anaerobic Sludge Blanket. Semicolons denote published research from the same authors but for a different study in most cases and in some cases, multiple reactor setups in one publication. * denotes that pressure is reported in kPa unless otherwise stated. **COD removal/methane production quantities represent the highest values obtained for that research.

Table 1.1 displays a summary of anaerobic membrane bioreactor treatment technology literature that was reviewed for this study. The research literature chosen all use actual wastewater, synthetic wastewater with complex organics, or an influent with adjuncts that create a complex organic mix. Wastewater treatment has been successfully demonstrated at psychrophilic temperatures of 15^{o}C (Ho and Sung (2010)), 12^{o}C [Wen et al. (1999)), 10-15^{o}C (Zhang et al. (2010)), 8^{o}C (Shin et al. (2014)], and even as low as 3^{o}C (Smith et al. 2015). Their success is marked by COD removals, at the above-mentioned temperatures, of 85%, 97%, 57.7%, 81%, and 86%, respectively. It is important to note that a majority of the configurations employed biogas sparging for membrane cleaning and fouling control, except Shin et al. (2014) who used Granular Activated Carbon (GAC)-fluidized reactors to clean the membranes. GAC-fluidized AnMBR operation did not provide further higher COD removals, but yielded lower HRT as low as 2 hours. None of the above-mentioned configurations had either employed dissolved methane removal or integrated nutrient recovery. Moreover, very few trials listed above have operated their pilot scale AnMBR under ambient temperature conditions subject to natural seasonal fluctuations. Table 1.1 puts the previous research in perspective to the challenges described in the previous paragraph that plague AnMBR implementation. The current study addresses these challenges effectively, as explained in the next chapter.

Significance of microbial communities for AnMBR treatment

Many anaerobic environments require diverse microbial metabolic cooperation for the complete degradation of organic matter. These thermodynamically driven relationships form catalytic units with multifarious microbial species in close adjacency to each other, working in a syntrophy [36]. This extraordinary phenomenon of syntrophy is necessary to combat product inhibition and to obtain vital energy needed for microbial metabolism that otherwise would not be produced in an energy limited environment. In syntrophic metabolism, critical oxidation-reduction reactions result in a loss of energy (thermodynamically unfavorable), which solicits the necessity for reverse electron transfer [36]. The membrane components that generate ion gradients are predicted to be key features that help in the syntrophic degradation of organic compounds such as alcohols, fatty acids, aromatic acids, organic acids such as lactate and glycolate, many amino acids, sugars, and hydrocarbons including methane under anaerobic conditions. Methanogens

that consume and hold hydrogen/formate at low concentrations enable the degradation of said compounds to be thermodynamically favorable [36].

To find operational strategies to improve AnMBR performance given the complexity of anaerobes involved in degrading complex mixtures of organics present in domestic wastewater, it is crucial to evaluate the metabolic pathways and response of microbial populations to temperature variations, challenges due to the development of distinct microbial communities in the membrane biofilm versus the biomass suspension, distribution of methane, and the impact of temperature on the availability of hydrogen for various anaerobic microbial groups [37]. The dominant phyla found in anaerobic systems are *Proteobacteria, Bacteroidetes, Firmicutes* [38][39], along with *Chloroflexi* [40].

Methanogenic archaea found in AnMBRs are of key importance because of the energy-rich biogas they generate that has the potential to push this technology towards energy-neutrality, with the hopes of producing net energy [41]. Methanogen community abundance and composition are closely related to the biogas and methane yield in the reactor [42]. Having said that, little is known about microbial interactions in psychrophilic engineered anaerobic environments, making it difficult to determine which parametric constraint (temperature or substrate availability) controls functional methanogenic communities [43]. It is also difficult to quantify archaea activity with regards to which metabolic pathways are being used in an anaerobic treatment system, and membrane biofilm communities add another grade of complexity to these metabolic relationships. Observations during temperature decreases of high COD removal from hydrogenotrophic methanogens in biofilm suggests a metabolic advantage, possibly due to proximity with supporting syntrophic exchanges and to a greater flux of organics into the biofilm due to suspended biomass inhibition during the temperature decreases. This observation is part of a study that provided evidence of the hydrogenotrophic methanogenic pathway being favored in the biofilm while the acetoclastic pathway is favored in suspended biomass [43].

McKeown and Scully et al. (2009) explained that within low-temperature engineered systems, such as anaerobic bioreactors, the successful development of stable methanogenic communities

can be accredited to hydrogenotrophic methanogenic activity. Shifts towards hydrogenotrophic methane production have been observed in bioreactors operating under psychrophilic conditions and have been reported on numerous accounts [38][44][45–54]. These findings aren't surprising when considering that hydrogen is a more thermodynamically favorable substrate than acetate at lower temperatures [55]. In opposition to those findings, acetoclastic methanogenesis has been found to be the primary methanogenic pathway at low temperatures [39].

Furthermore, specific methanogenic activity (SMA) assays indicate that psychrophilic hydrogen- and propionate-utilizing populations that developed in bioreactors are putatively considered the rate-limiting metabolisms [44]. Smith (2015) suggested that this shift towards hydrogenotrophic methanogenesis can be explained by the fact that acetoclastic methanogens seem to be more strongly affected by low temperatures than their hydrogenotrophic counterparts that might also be exposed to more hydrogen due to its increased solubility in lower temperature waters. Furthermore, the role of psychrophilic homologues, such as syntrophic acetate-oxidizing bacteria (AOB), within engineered bioreactors, remains unclear and needs further investigation [44].

At higher temperatures and low concentrations of acetate, syntrophic acetate oxidation becomes energetically favorable, but is overall an energetically unfavorable reaction that can proceed if hydrogenotrophic methanogenesis eliminates hydrogen. Only until hydrogen elimination occurs does the overall reaction become exergonic then showing the same stoichiometry as acetoclastic methanogenesis. *AOR* ('Reversibacter'), *Clostridium ultunense* BS, *Thermacetogenium phaeum* strain PB, and *Thermotoga lettingae* TMO are acetate-oxidizing syntrophs and acetogens (homoacetogens), except for *Thermaotoga lettingae* not having acetogenic qualities. A mutualism between these strains and hydrogenotrophic methanogens have been observed [56]. But, hydrogenotrophic methanogenesis, homoacetogenesis, and hydrogenotrophic sulfate reduction are more exergonic at low temperatures because of the increased solubility of hydrogen, which might also prompt an increase of net biomass yield of methanogens or acidogenic sludge [55]. At lower temperatures, these syntrophs might be active as acetogens, and therefore suppling acetate to what would be their main competitors, acetoclastic methanogens, if they were playing the role of AOB [56].

Considering syntrophic AOB, when a beneficial relationship develops with one group of methanogens and not the other, competition can occur. Another group of bacteria that can pose a competition for substrate and problems with anaerobic reactor operations are sulfate-reducing bacteria (SRB). High sulfate concentrations in DWW can impede methane generation and energy recovery of AnMBR systems when SRB outcompete methanogens for substrates and when methane is substituted for hydrogen sulfide gas (toxic and corrosive gas) in the biogas generated [57]. In anaerobic reactors with sufficient sulfate present, SRB can outcompete acetogenic and hydrogenotrophic methanogens. SRB that utilize hydrogen are able to obtain more energy from it and they also have a higher substrate affinity, growth rate, and cell yield than hydrogenotrophic methanogens and SRB that utilize acetate have a higher energy yields and growth rates than acetogenic methanogens [58]. Biogenic sulfide corrosion can damage the pipework and machinery of an AnMBR and its expensive to scrub it out of the biogas. Sulfide produced from the microbial reduction of sulfate can also be detrimental when it induces the precipitation of non-alkali metals which reduces their availability for the microorganisms, but can also be beneficial when it precipitates toxic heavy metals such as Co, Cu, Ni, Pb, and Zn [48]. Even though the presence of sulfate has been reported to inhibit methanogenesis for various anaerobic digesters, Lettinga et al. (2001) reported that anaerobic digestion is an effective treatment method for wastewaters containing sulfate concentrations up to 1,700 mg SO_4^{2-}/L without any deleterious effects on methane production and Szendrey et al. reported the highest sulfate level of 6,000 mg SO_4^{2-}/L, his work used a downflow, fixed-bed reactor [59].

Microbial community structure analysis through traditional methods based on phylogenetic analyses are insufficient to lend greater understanding about the anaerobic microbial interactions leading to methane production in AnMBRs under mesophilic or psychrophilic start-up conditions. The experimental approach to elucidate AnMBR microbial community structure is to quantify the microbial population in the suspended biomass of the AnMBR for the pilot scale AnMBR using DNA based tools. To do this, parametric correlation of molecular analyses along with a combination of advanced high-throughput sequencing of the 16S rRNA gene of bacteria and archaea is used to evaluate microbial community in the biomass suspension during the startup phase of an AnMBR operating at ambient temperatures while successfully treating domestic wastewater and generating methane. There is also a need to explicate the hierarchical

and syntrophic relationships bacteria have with methanogens as a function of temperature to better understand the pathways for methane production through hydrogen and acetate.

1.2 Overview/Thesis Structure

The focus of this study is to assess the change of microbial community structure and function during start-up of an anaerobic membrane bioreactor (AnMBR) in mesophilic conditions leading into psychrophilic conditions and then back to mesophilic conditions. The AnMBR will operate at sub-ambient temperatures harmonized with seasonal changes in a temperate climate. In the U.S., the annual mean temperature of domestic wastewater varies from 3 to 27°C, with a nationwide average of 15.6°C [35]. Operating this AnMBR at sub-ambient temperatures requires considerably less energy compared to methods of anaerobic wastewater treatment where bioreactors are held at a constant temperature [39]. The biomethane produced will be used to quantify the potential for sustainability of this project. The methane will be factored into the energy budget for the entire system with the goal of approaching energy-neutral operation.

The thesis is organized into five chapters with references at the end of each chapter. Chapter 2 describes the integrated AnMBR treatment train system that works to treat wastewater to remove pollutants per water standards. This chapter also provides the AnMBR operational parameters and data that proves its successful performance. Chapter 3 explores microbial ecology characterization during the start-up of the AnMBR beginning in late summer, through the winter, and into the spring season. The relative abundance of microbe populations was analyzed using Illumina MiSeq based high throughput DNA sequencing platform and the data was processed with QIIME (Quantitative Insights Into Microbial Ecology). This chapter also synthesizes the importance of AnMBR technology and the findings of this study in one summary. Chapter 4 gives suggestions for future research related to AnMBRs and for microbial ecology characterization.

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Chapter 2

Anaerobic Membrane Bioreactor System Performance

2.1 Introduction

The Department of Defense (DoD) currently uses aerobic treatment processes, such as activated sludge and aeration basins, to treat domestic wastewater generated at DoD facilities. Some undesirable characteristics of these aerobic treatment processes are energy-intensive aeration requirements to oxidize organic material in the wastewater, generation of a substantial amount of sludge, and production of the greenhouse gas carbon dioxide which cannot be used as a fuel source.

An alternative to conventional aerobic treatment processes is anaerobic treatment, which has a lower energy demand versus aerobic processes because it doesn't require aeration to oxidize organic material in the wastewater, they also produce less sludge while producing methane-rich biogas that can be used to generate electricity, heat, or vehicle fuel. The energy content of the biogas can offset the energy used by the treatment process, and potentially make the process energy-neutral or energy-positive.

One type of anaerobic treatment process that is of particular interest for implementation at DoD facilities is the AnMBR treatment process. In addition to the benefits described above, this process produces an effluent that can meet reuse standards, therefore implementation of this treatment technology could increase the amount of water recycled at DoD facilities while decreasing the operational costs of water treatment.

The wholistic, integrated AnMBR treatment system works to treat wastewater to remove all pollutants per EPA secondary standards and American National Standards Institute (ANSI)/National Sanitation Foundation (NSF) 350 reuse standards for five-day biochemical oxygen demand (BOD_5) and total suspended solids (TSS). Fully treating the wastewater allows further examination into reusing the water for applications such as cleaning vehicles and irrigating green spaces.

An overarching goal of this project is to compare the performance of this gas-sparged AnMBR with its counterpart project in Bucheon, Korea, which employs a granular activated carbon (GAC)-fluidized AnMBR that is also operating at ambient temperatures in a comparable climate. The GAC-fluidized AnMBR differs from the gas-sparged AnMBR by not having water reuse, nutrient capture, and dissolved methane recovery as performance goals.

2.2 AnMBR System Inoculation and Configuration

The entire AnMBR system is contained in a process trailer that is 8-ft. wide, 40-ft. long which was constructed by Intuitech, Inc. The volume of the primary bioreactor in the AnMBR system is 485 gallons, and including the secondary membrane bioreactor, the AnMBR was inoculated with 360 gallons of primary anaerobic digester sludge from the Oakland, Topeka Wastewater Treatment Plant (Topeka, Kansas, USA) on July 13th, 2016. The primary bioreactor operated at ambient temperatures that ranged from 12.7-29.3°C.

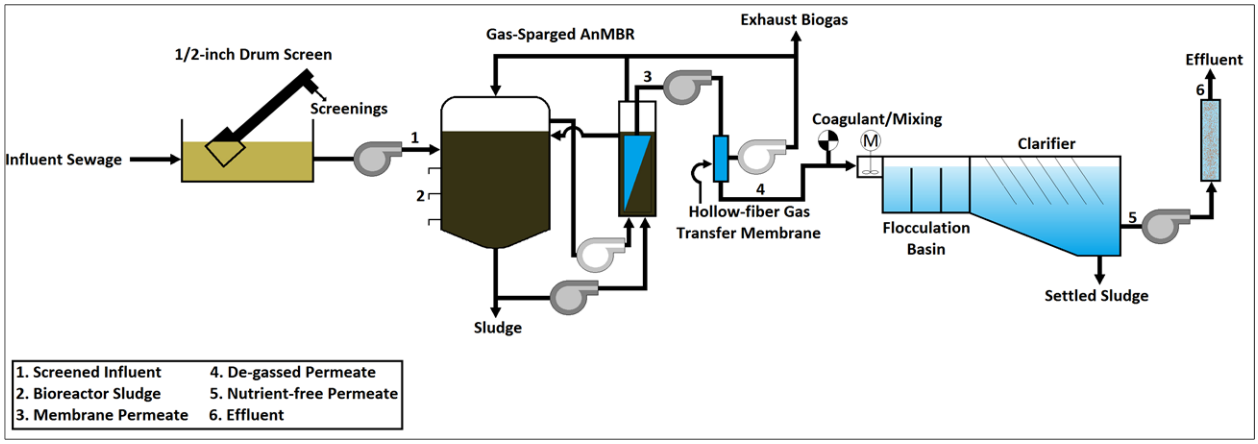

Figure 2.1. Gas-sparged AnMBR system flow diagram

As seen in Figure 2.1, the primary bioreactor receives domestic wastewater that has passed through a single drum screen with approximately 1/2-inch diameter openings. The sludge and produced biogas in the primary bioreactor is continuously circulated through the secondary membrane bioreactor. The secondary membrane bioreactor separates microorganisms and other suspended solids from the treated permeate. This physical separation process serves to maintain a desired mixed liquor volatile suspended solids (MLVSS) concentration in the bioreactor while producing a suspended solids-free permeate. The head-space biogas formed in the primary

bioreactor is pumped to the bottom of the secondary membrane bioreactor where it is sparged, creating bubbles that shear off membrane foulants. The secondary membrane bioreactor has a volume of 0.17 m^3 and contains hollow-fiber ultrafiltration (UF) membrane made of polyvinylidine fluoride (PVDF) on woven polyester (GE: ZeeWeed 500d) that exclude solid particles larger than 0.04 microns (μm). The three membrane modules have a membrane area of 12.9 m^2. The membrane modules are subjected to intermittent chemical cleanings with 500 mg/L NaOCl and 2000 mg/L citric acid. The membrane permeate is then pumped through a hollow-fiber gas transfer membrane that extracts all the produced biogas in the permeate. Next, the de-gassed permeate gets coagulated with a combination of ferric chloride, alum, and an organic anionic polymer. Phosphate and sulfide is removed from this solution after its allowed to flocculate and settle. Finally, the clarified water enters an ion exchange column where ammonia binds to the clinoptilolite contained in the column. A potential, additional source of energy from ammonia saturated clinoptilolite will be assessed by Dr. Kathryn Guy at the Construction Engineering Research Laboratory (CERL) for the feasibility of clinoptilolite regeneration and ammonia electrolysis for hydrogen and electricity production. Reusing the effluent permeate represents the final effort to capture all resources and lessen energy demand associated with water treatment. Permeate reuse examination involves the water analysis for BOD_5, TSS, ammonia, nitrate, nitrite, total phosphorus, sulfide, turbidity, pH, specific conductivity, and *Escherichia coli* and total coliforms counts to quantify chlorine demand.

The overall performance goals of the project were divided into three tiers: production of high quality effluent for reuse, energy neutrality, and implementability. Effluent quality was the most critical characteristic. The American National Standards Institute (ANSI) defines an effluent quality goal of less than 10 mg/L five-day biochemical oxygen demand (BOD_5) maximum and a 2 NTU turbidity maximum for reuse.

Implementability goals were secondary objectives that revolved around the viability of full scale operation. Such parameters as hydraulic retention time (HRT), organic loading rate (OLR), and the net flux were monitored. Additionally, the system's ability to perform when subjected to ambient temperatures as low as 10°C was to be evaluated.

The stated objectives are the goals during continuous operation, but several parameters key to water quality, implementability, and energy optimization goals were also monitored during startup.

2.3 Methods and Materials

An array of chemical analyses was used to characterize the water after each stage of the AnMBR system to monitor its performance. Organic loading rate (OLR) was calculated from COD values. Biogas quantity and composition is measured continuously on site from the bioreactor exhaust, hollow-fiber gas transfer membrane (vacuum pump discharge), and combined gas exhaust using a variable gas flow meter (Alicat Scientific). Methane content of biogas was measured using an online biogas sensor (Nova Analytical Systems Inc). Total chemical oxygen demand (TCOD) was assayed with HACH® kits using a HACH DR3900 (Loveland, CO, USA) spectrophotometer absorbance at wavelengths of 620. BOD_5 was calculated using Method 5210B of the Standard Methods for the Examination of Water and Wastewater with the use of a YSI MultiLab 4010-2 DO Meter to read initial and final dissolved oxygen (DO) concentrations. Sulfate and sulfide was measured by ion chromatography using a DIONEX ICS 1000 unit fitted with an anion exchange column and an electrochemical detector. Total solids (TS) and total suspended solids (TSS) were calculated using the protocols 2540B and 2540D, respectively, and the fixed and volatile portions of those solids were calculated using 2540E of the Standard Methods for the Examination of Water and Wastewater, 22nd Edition. The volatile portions are volatile solids (VS) and volatile suspended solids (VSS). Volatile fatty acids (VFAs) are measured by Kansas State University (KSU) using a high-performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H column and a photo diode array and refractive index detectors as described previously [1]. pH was measured according to Standard Methods 4500 immediately after sampling. All chemical analyses utilized ultra-pure water having a resistivity of approximately 18.2 MΩ-cm when necessary.

2.3.1 AnMBR operational parameters and data

The seed inoculum sample collected from the Topeka WWTP mesophilic anaerobic digester on July 13th, 2016 and had a temperature of 25.2°C after being placed in the bioreactor. July 15th, 2016 represents day 0. Bioreactor samples for microbial analysis were collected on days 157, 203, 222, 229, 243, 257, 262, and 271 and are denoted on figures 2.3 and 2.4 as vertical, pale orange lines that run from the permeate to the temperature line. The bioreactor operated at various flowrates of 1.6, 2.7, and 5.5 m^3/d (minimum, intermediate, and maximum flow, respectively); OLRs in the average range of 0.6 to 1.4 kg COD/(m^3*d) (Figure 2.2); HRTs were in the range of 11-15 hours (Figure 2.2); and SRTs of 30-150 days. The OLR and HRT goals of ≥ 0.6 kg COD/(m^3*d) and ≤ 20 hours, respectively, were largely met.

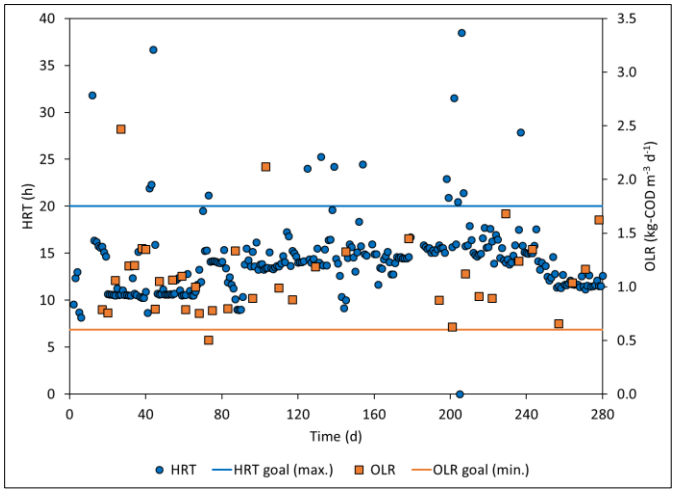

Figure 2.2. AnMBR hydraulic retention times and organic loading rates.

In the start-up period alone, only 14 days showed permeate COD values larger than the EPA secondary standard goal of ≥60mg/L (Figure 2.3) and only 9 days showed permeate BOD_5 values larger than the EPA secondary standard goal of ≥30mg/L (Figure 2.4). AnMBR permeate quality indicated that the effluent BOD_5 consistently met the ANSI reuse standard goal of ≤ 10 mg/L. The average COD and BOD_5 removal rates were 88.8% and 69.1%, respectively. DNA sample dates are represented by pale orange lines that link their metadata.

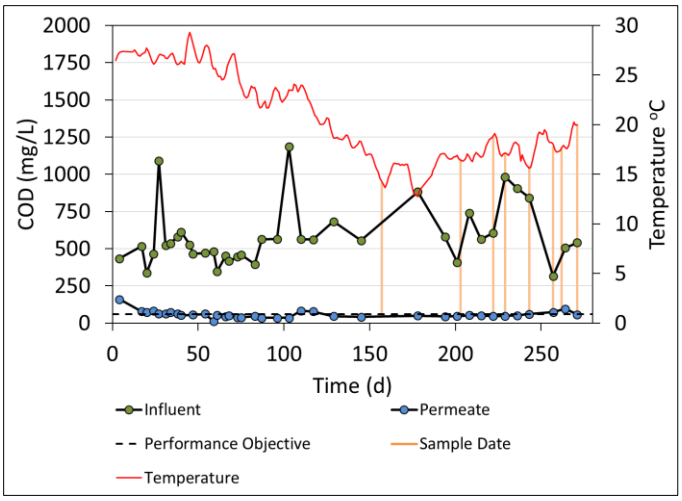

Figure 2.3. COD, temperature, and sample dates.

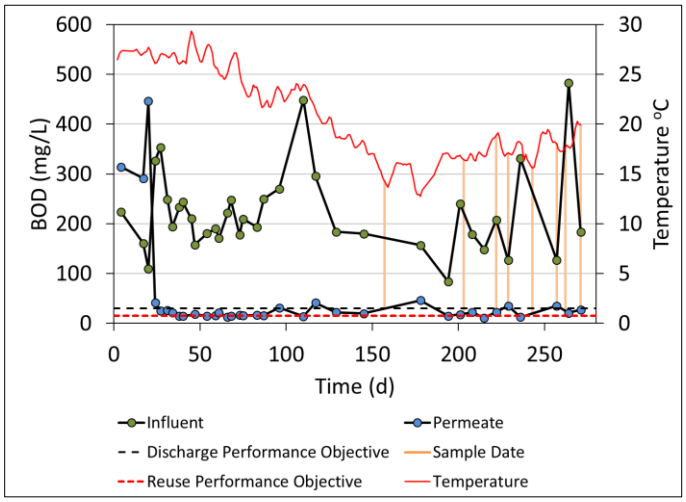

Figure 2.4. BOD, temperature, and sample dates.

The biogas generated was composed of roughly 75% methane on average with methane production rates ranging from 50 to 250 grams per day (Figure 2.5). Sample dates are represented as purple diamonds in Figure 2.5. The methane portion of the biogas might be high due to the dissolution of CO_2 back into the mixed liquid from gas sparging, thereby enriching the gas for the energy rich methane which is less soluble than CO_2. A small decrease in methane production rate to around 75 g/d with decrease in temperature likely indicated a slightly compromised Specific Methanogenic Activity (SMA), while the decreased methane composition % was due to errors in the online gas analyzer calibration, especially between 190 and 210 days.

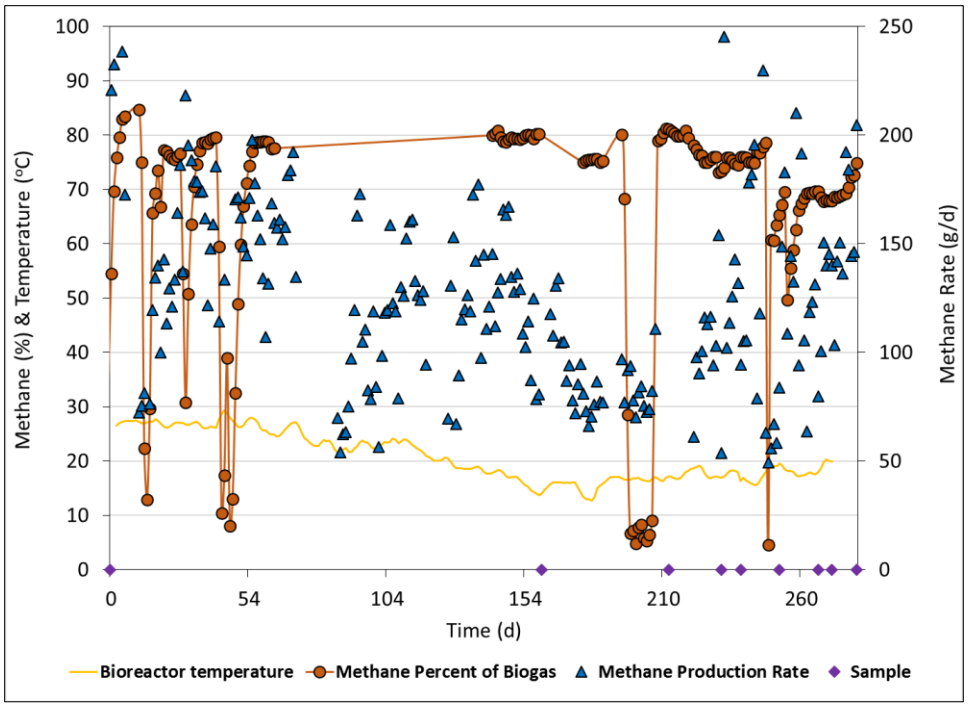

Figure 2.5. Bioreactor temperatures, methane production rates, and methane biogas percentages.

Figure 2.6 presents the total solids concentration in the bioreactor along with the volatile solids and total solids ratio. After the first 45 days, the total solids concentration lowered to an average of about 8,000 mg/L. The decrease was not associated with a system controlled solids wasting, rather it is indicative of hydrolysis of the anaerobic digested sludge, and stratification of the solids to the bottom of the bioreactor due to poor mixing. The latter is the most likely explanation, since efforts to improve bioreactor mixing by lowering reactor level on days 58 and 94 did bring back the bioreactor solids levels up. High VS/TS ratios (100%) occurred during lab procedure optimizations and may not truly represent the actual VS/TS ratios. High VS/TS concentrations indicate a greater proportion of microbial matter and is comparable to primary wastewater fed anaerobic systems [2].

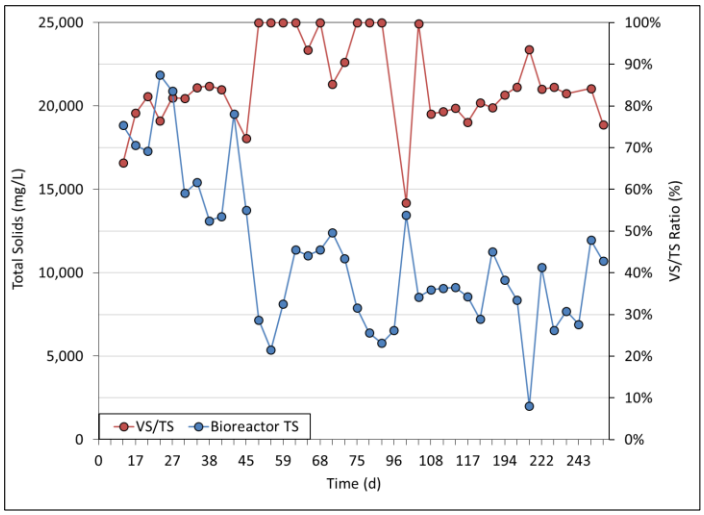

Figure 2.6. Total solids of bioreactor and volatile solids, total solids ratio.

The total VFA concentrations tended to be low throughout the entire duration of operation (Figure 2.7). Low accumulation of VFAs is an indication of stable anaerobic bioprocess. Besides a couple of peaks of membrane permeate VFA concentrations, VFAs were consumed in the bioreactor resulting in lower concentrations in the membrane permeate. The two high VFA peaks likely corresponded to membrane cleaning with citric acid, which is a VFA by itself and can also be fermented further to simpler VFAs such as acetic or propionic acids.

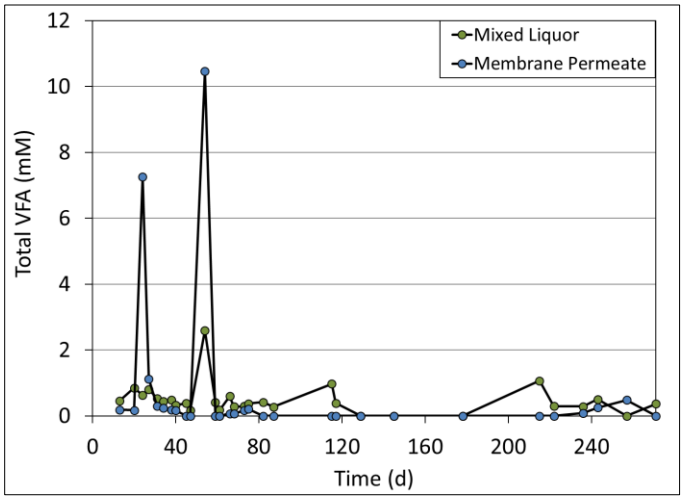

Figure 2.7. Total volatile fatty acid concentrations in millimoles/L.

Figure 2.8 shows the how the concentration of the sulfate ion decreases from the bioreactor to the membrane permeate. The presence of SRB in the bioreactor can be deduced from the sulfate data that shows a reduction in sulfur concentrations and the production of sulfide. The ratio of sulfur normalized sulfide to sulfur normalized sulfate produced is always within the range of 100% ± 10%. This is a clear indication that sulfate is getting reduced to sulfide and SRB is the most likely factor, as supported later by the microbial community data.

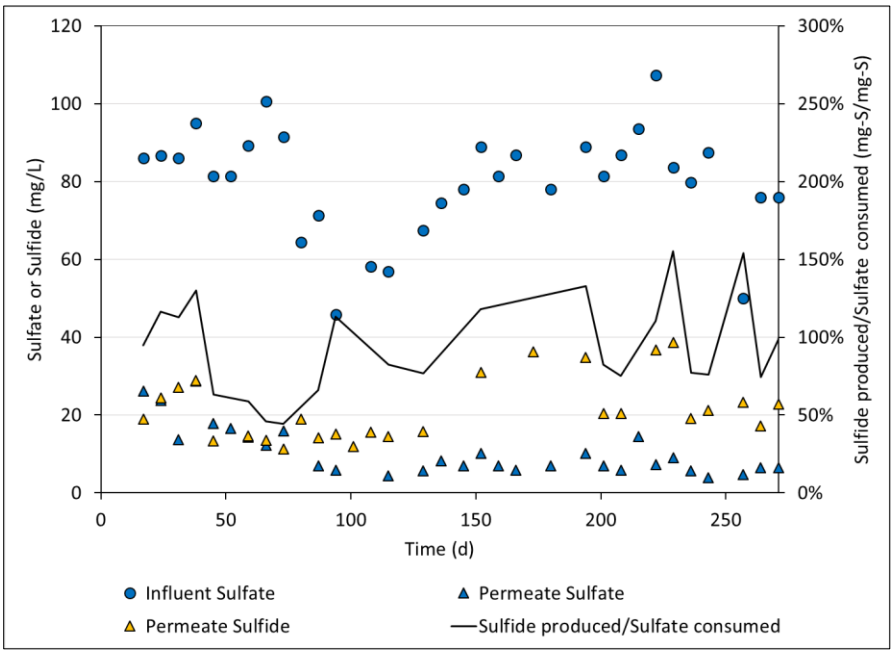

Figure 2.8. Concentration of influent and membrane permeate sulfate, permeate sulfide, and the ratio of sulfur from sulfide and sulfate.

2.4 References

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Chapter 3

Anaerobic Membrane Bioreactor Microbiome Analysis

3.1 Methods and Materials

3.1.1 DNA extraction

A sample was saved from the seed inoculum and bioreactor samples were collected from the middle sample port of the primary bioreactor. The seed inoculum sample was saved July 13th, 2016 and had a temperature of 25.2°C after being placed in the bioreactor. The bioreactor samples were collected on days 157, 203, 222, 229, 243, 257, 262, and 271; the inoculum and bioreactor samples correspond to daily average bioreactor temperatures of 25.2, 13.9, 16.4, 18.9, 17.1, 15.6, 18, 17.7, and 20°C, respectively. Bioreactor sludge samples were centrifuged in an Eppendorf centrifuge 5920 R (Hauppauge, New York, USA) at 21,000 RCF (Relative Centrifugal Force) to concentrate the biomass so that the excess water could be easily excluded. In Table 3.1, the results of three different DNA kits, MO BIO PowerSoil DNA Isolation Kit (Carlsbad, California, USA), E.Z.N.A.® Water and Soil DNA Kit (Norcross, Georgia, USA), that were used to compare the effectiveness of each one using the same sample are shown. Higher nucleic acid concentrations and a ratio of 1.8-2.2 is considered adequate for the 260/280 and 260/230 ratios which are ratios of the nucleic acid to contaminants. DNA was extracted from roughly 0.5 g (wet weight) biomass samples using the most effective DNA kit, the E.Z.N.A.® Water DNA Kit (Norcross, Georgia, USA) and samples were stored at -20°C. The Thermo Scientific NanoDrop™ 2000c (Wilmington, Delaware, USA) was used to quantify nucleic acid concentrations and quality of DNA samples.

Kit Type	Nucleic Acid Concentration (ng/ul)	260/280	260/230
MoBio Soil	51	1.78	1.44
E.Z.N.A. Soil	228	1.87	1.56
E.Z.N.A. Water	694	1.9	1.58

Table 3.1. Comparison of three different DNA extraction kit results using the same sample.

3.1.2 High-throughput microbial community analysis

To determine the structure of the Bacterial and Archaeal community during startup of the AnMBR, DNA was sequenced at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq (Illumina, USA). 16S rRNA universal prokaryotic primers 519F and 806R [1], with barcode on the forward primer, were used to amplify the V3 and V4 hyper-variable region of this highly conserved gene [2]. The reads were paired-end sequenced with DNA fragments consisting of 2 × 300 bp reads using an Illumina MiSeq with the MiSeq Reagent Kit v3.

MR DNA provided sequencing data in fasta, mapping, and quality files that were processed using the QIIME v. 1.9.1 pipeline [3]. The data set was first demultiplexed by barcode decoding and the sequences were filtered to remove low-quality reads using the script, split_libaries.py. The total sequence count is 760,810 with a minimum of 74,698 for sample 6 and a maximum of 91,511 for sample 2. Next, the sequences were aligned and binned into OTUs in a BIOM-formatted OTU table at 97% similarity and the taxonomy was assigned with UCLUST consensus taxonomy assigner using the script, pick_de_novo_otus.py. This script uses the16S rRNA gene database, Greengenes 13_8 [4]. Finally, the singletons were removed, and taxonomy charts and tables were created using the scripts, filter_otus_from_otu_table.py and summarize_taxa_through_plots.py.

3.2 Results and Discussion

3.2.1. Phylum level distribution of bacterial communities in AnMBR

Out of 639 bacterial OTUs recognized, a core group of bacterial phyla *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and to a lesser extent, *Chloroflexi* and *Synergistetes*, were observed throughout the period of AnMBR sampling from the bioreactor (Figure 3.1). Phylum *Bacteroidetes* accounted for roughly 20 to 35% of the relative abundance and did not significantly change from summer startup through winter operation. *Proteobacteria* increased in relative abundance from 14.7% at startup to 26.9% and *Firmicutes* from 8% to 16.6%, respectively, when the average effective bioreactor temperature decreased from 25°C to 16°C (7/13/2016 - 3/15/2017). Temperatures are shown on top of the graphs in figures 3.1-3.4. On the other hand, *Chloroflexi* to decrease from 29.3% to 9.1% during the same period. *Synergistes*

exhibited a minor increase in relative abundance, more noticeably from startup to winter, before achieving stable but low relative abundance levels.

It is likely that members of *Bacteroidetes* performed proteolysis in the AnMBR, which is the degradation of proteins into smaller polypeptides or amino acids (acidogenesis), and can also ferment amino acids to acetate [5][6]. The *Proteobacteria* were mainly composed of *Betaproteobacteria* and a higher abundance of *Deltaproteobacteria. Betaproteobacteria* are also likely involved in the first steps of the degradation and are the main consumers of propionate, butyrate, and acetate [5][7]. The *Deltaproteobacteria* members present are SRB and microorganisms involved in syntrophic activity, such as the genus *Syntrophus*. *Firmicutes* are another group of syntrophic bacteria that were present in increasing abundance with temperature. They are known to degrade volatile fatty acids such as butyrate and its analogs, which produces H_2 that can be degraded by hydrogenotrophic methanogens, along with acetate that can be consumed by acetoclastic methanogens. The metabolic capacities of *Chloroflexi* are still unclear, but several studies have showed their potential role in the degradation of carbohydrates [5]. They did decrease in relative abundance with the bioreactor operation and temperature decrease. *Synergistetes* convert amino acids into short-chain fatty acids and sulfate that terminal degraders, such as SRB and methanogens, can use [5]. Predominant phyla in mesophilic anaerobic reactors matches the trends observed here, except for *Chloroflexi* which underwent a marked decrease in relative abundance, which warrants further investigation on the effects of psychrophilic conditions on this *Chloroflexi* [8][9].

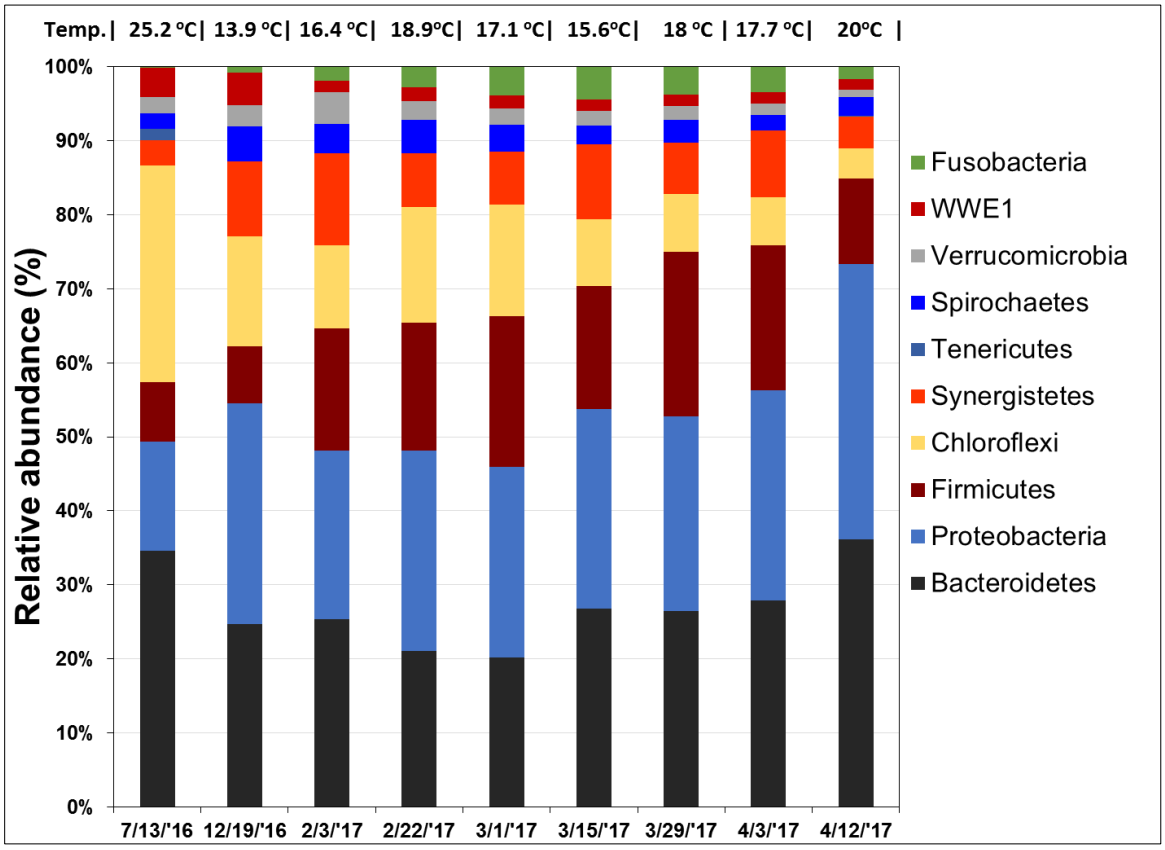

Figure 3.1. Phylum level relative abundance of bacteria with ≥1% relative abundance for at least one sample date.

3.2.2. Family/genus level distribution of bacterial communities in the AnMBR

Besides other *Bacteroidetes* and *Clostridiales; Synergistaceae, Anaerolinaceae,* and *Syntrophaceae* exhibited the highest abundances on the family rank (Figure 3.2). The *Synergistaceae* family showed the single largest abundance in the *Synergistetes* phylum (*Synergistia* class, *Synergistales* order) and is known to have the ability to degrade amino acids into volatile fatty acids and contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens [16]. Their abundance seems to have benefited from the drop in temperature experienced in the autumn, but then it declines into the spring.

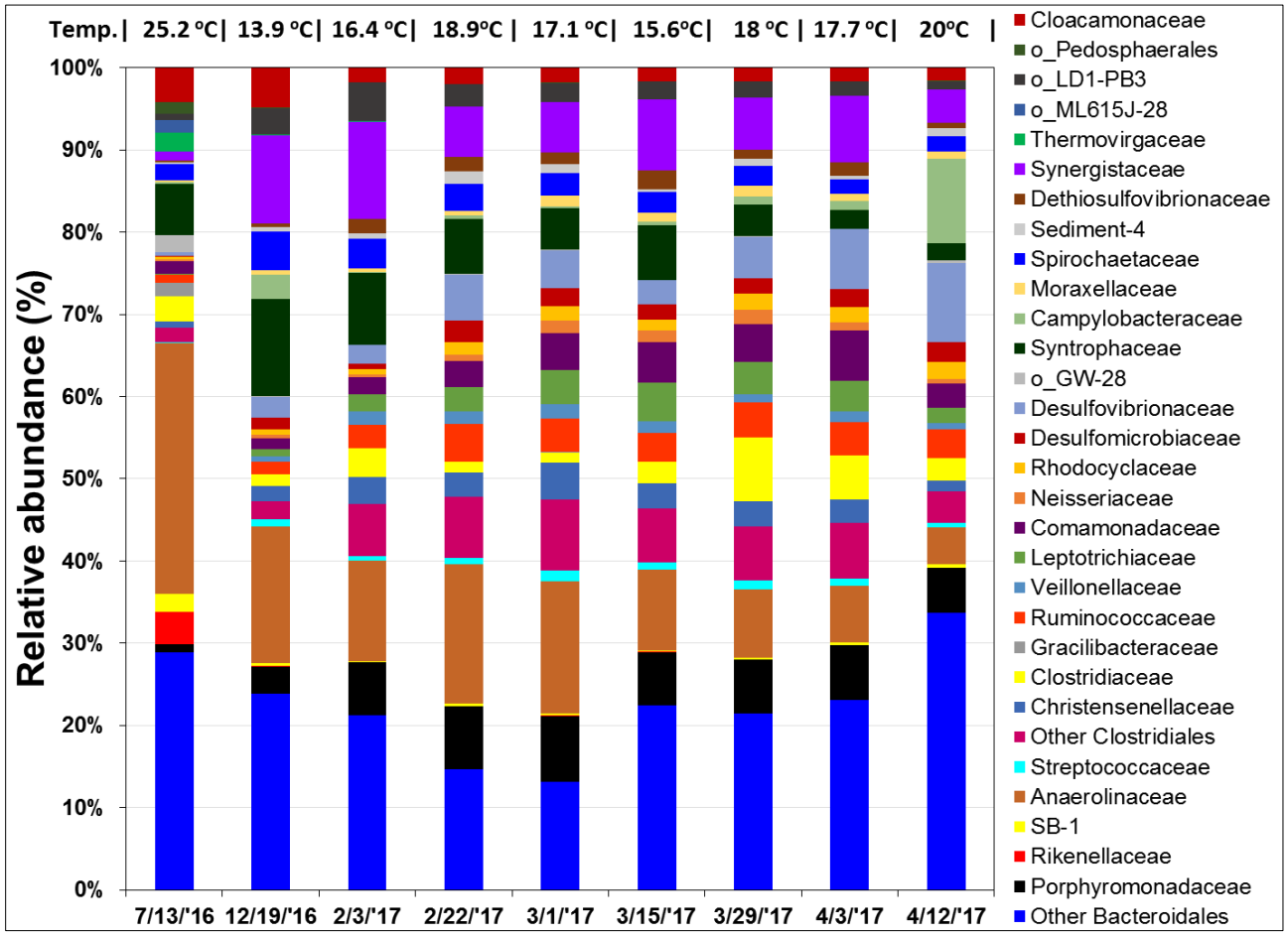

Figure 3.2. Family level relative abundance of bacteria with ≥1% relative abundance for at least one sample date. o_ indicates order level.

Three bacterial genera varieties (SHD-231, T78, WCHB1-05) found in the *Anaerolinaceae* family (*Chloroflexi* phylum, *Anaerolineae* class, *Anaerolineales* order) comprised a significant portion (30.46%) of the relative abundance of bacteria for the first sample date but their presence declines through the winter and spring months (down to 4.47%) (Figure 3.3). *Anaerolineae* is identified as one of the core populations, as primary and secondary fermenting groups, in methanogenic bioreactors and most often comprises a dominating proportion of anaerobic digestive systems. *Anaerolineae* are considered to be anaerobic semi-syntrophic organisms, degrading carbohydrates and conducting reverse electron transfer via tightly coupled mutualistic interactions with hydrogenotrophic methanogens, and in comes cases, posing the genetic

potential to metabolize ethanol to acetate, implying their reputed role as anaerobic syntrophs with acetoclastic methanogens. The adhesive feature of *Anaerolineae* enabled by active pilA expression (active type VI pili (Tfp) assembly) might serve as the adhesive matrix for the aggregation of fermentative populations in sludge granules and the causative agent of filamentous flocs in UASBs. Observations of this advantageous bonding capacity in *Anaerolinales* may provide an explanation for its ubiquity and accumulation in anaerobic digestive systems [12][13].

Along with *Anaerolineae*, the genera *Syntrophus* (*Syntrophaceae* family) also performs reverse electron transfer in mesophilic anaerobic environments and shows a similar trend of abundance as *Synergistaceae* (*vadinCA02* genus) because of their apparent increase in abundance on the coldest sample date and their abundance waning into warmer temperatures (Figure 3.3). *Syntrophus*, as the name implies, is syntrophic bacteria capable of degrading important intermediates in the methanogenic decomposition of organic matter, such as benzoate, fatty acid chains, and aromatic compounds in a symbiotic relationship with methanogens [13]. This anaerobic bacterium ferments alcohols, fatty acids longer than two carbon atoms, and benzoate to acetate, CO_2 and H_2 in the presence of hydrogen-utilizing methanogenic partners that in turn produce methane and CO_2 [14]. The hydrogen-consuming populations that maintain low H_2 partial pressures in anaerobic environments allow the conversion of benzoate to H_2, acetate, and CO_2 to be thermodynamically feasible, which are otherwise unfavorable at standard conditions [15].

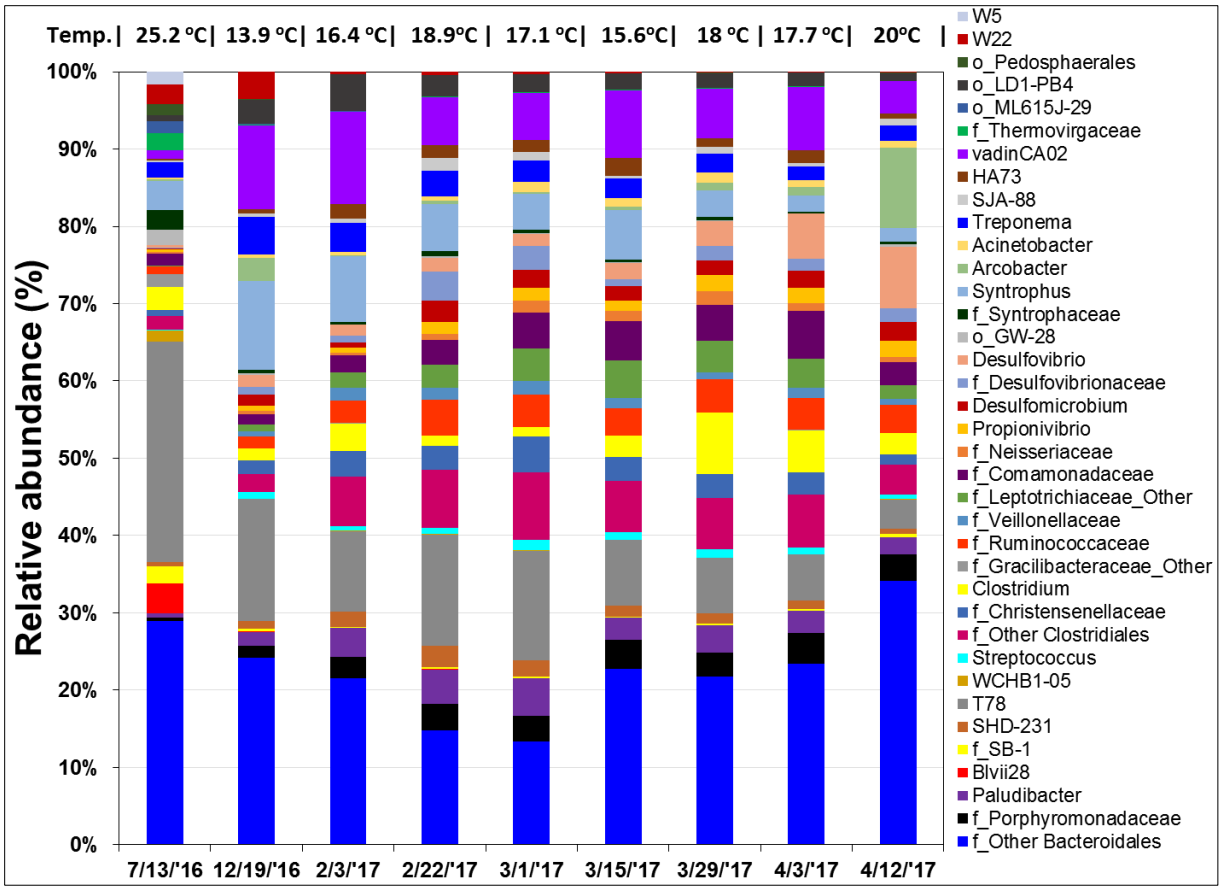

Figure 3.3. Genus level relative abundance of bacteria with ≥1% relative abundance for at least one sample date. o_ indicates order level, f_ indicates family level.

The *Desulfovibrio* genus *(Desulfovibrionaceae* family) showed increasing abundance throughout the experiment with the largest abundance on the last sample date. This SRB utilizes sulfate as a terminal electron acceptor and derive their energy for growth from the oxidation of H_2, formate, ethanol, and lactate (incompletely to acetate) and hydrogen gas [17]. Particular species perform sulfur disproportionation with elemental sulfur (S), sulfite (SO_3^{-2}), and thiosulfate ($S_2O_3^{2-}$) to produce both hydrogen sulfide (H_2S) and sulfate (SO_4^{2-}) [18]. The SRB *Desulfomicrobium* genus that utilizes H_2 as an electron donor and acetate as carbon source also showed increasing abundance but to a lesser extent [19]. The increasing abundance of *Desulfovibrionaceae* with a subsequent increase in hydrogen sulfide production causes concern because of the potential of microbially induced sulfide corrosion that degrades the inner workings of the AnMBR system. The presence of SRB also correlates with sulfate reduction that actively occurred in the AnMBR with concomitant generation of sulfide, as shown in Chapter 2.

The potential enteric human pathogen *Arcobacter* showed the second highest abundance for the last sample date [20]. This curious spike in abundance might be explained by its inoculation from influent wastewater microbiota that has changed microbial community composition within systems in other studies [4].

3.2.3. Order/genus level distribution of archaeal communities in the AnMBR

The high-throughput sequencing reveals low populations of methanogens and archaea altogether. The relative abundance of the total archaea population never amounts to >2% of the entire microbial community population (Figure 3.4). This observation is in accordance with findings on other methanogenic ecosystems that are typically comprised of <2% relative abundance of methanogens [10].

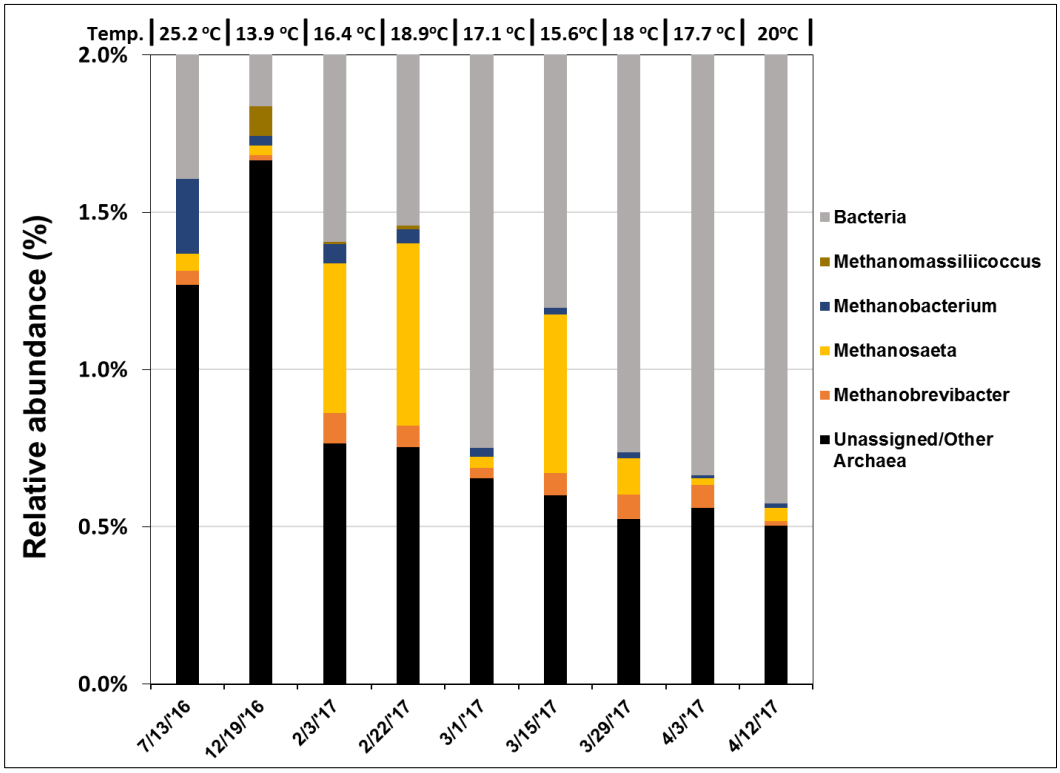

Figure 3.4. Relative abundance of archaea compared to bacteria.

Out of 12 archaeal OTUs recognized, the core Archaea group was composed of the methanogens in the order *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales* (Figure 3.5a). The obligate aceoclast, *Methanosaeta* genus (*Methanosarcinales* order, *Methanosaetaceae*

family) represents the pathway for acetoclastic methanogenesis and showed the overall highest abundance and higher abundances for more sample dates. *Methanosaeta* looks to have gained a delayed advantage in the bioreactor after the drop in temperature. *Methanobacteriales* and *Methanomicrobiales* represent the pathway for which hydrogenotrophic methanogenesis takes place. *Methanobacteriales* order, *Methanobacterium* genus' dominance for the first sample might be due to its selection in the digester from which it originates. Anaerobic digesters treating municipal wastewater are known to be predominated by the acetoclastic *Methanosaeta*, although several studies indicate hydrogentophic predominance as well, especially if the influent wastewater exhibits unusual composition [8][21][22]. It shows predominance that lasts through the fall and into the beginning of winter (Figures 3.5a and 3.5b), after which a sharp shift in predominance takes place towards the acetoclastic genera.

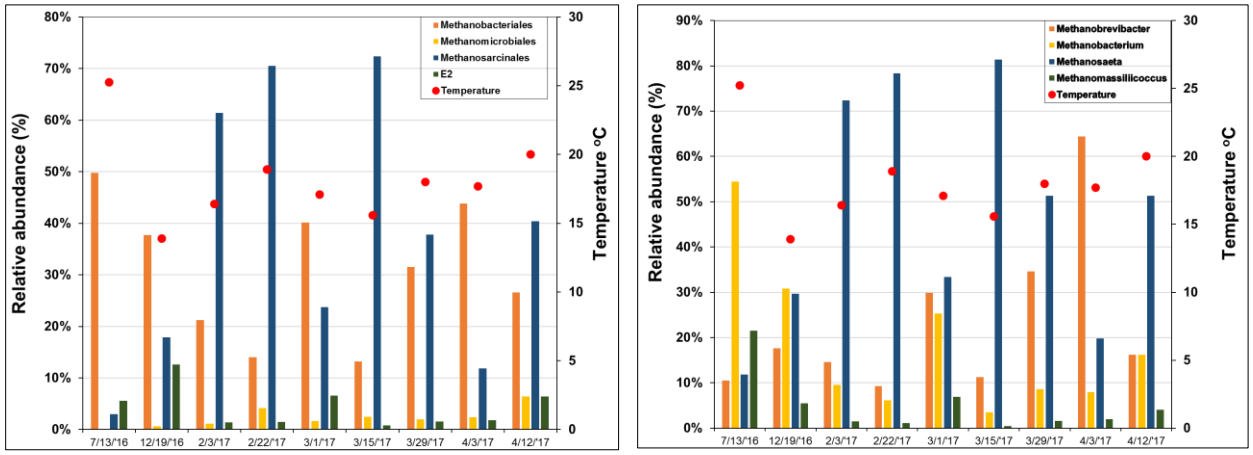

Figure 3.5a and 3.5b. Relative abundance of key methanogen orders and genera compared to the total abundance of the set.

Methanosarcina (*Methanosarcinales* order, *Methanosarcinaceae* family) wasn't present in the inoculum and is suspected to not be present in the source sludge anaerobic digester. If it were present, the generalist *Methanosarcina* would be a better competitor for acetate, instead we observed high abundances of the acetate specialist, *Methanosaeta*, that is favored in systems with a low acetate concentration, such as this one [11].

Proposed microbial interactions in the AnMBR with decreasing temperature

It is hypothesized that *Anaerolinaceae*'s provides the bonding capacity that builds an adhesive matrix that aggregates key archaea and bacteria [12], including *Synergistaceae and Syntrophaceae* altogether. This relationship is thought to couple the reactions of *Synergistaceae* degrading amino acids into volatile fatty acids with the metabolism of *Syntrophaceae*, which further converts volatile fatty acids into acetate and H_2 that are syntrophically tied to methanogens such as *Methanosaetaceae* and *Methanobacterium*, respectively (Figure 3.6). *Methanosaeta*, that comprise the entire *Methanosaetaceae* population in this study, might contribute in this adhered relationship because they are commonly found in methanogenic biomass due to their filamentous morphology and granulogenesis ability in forming biofilms in bioreactors [4][23]. This points to the fact that direct acetate utilization by acetoclastic methanogens might downplay the occurrence of acetate oxidation, often considered a preferred pathway under thermodynamically and metabolically unfavorable conditions for acetoclastic methanogenesis. On the other hand, the high shear environment created by biogas sparging and sludge circulation might disrupt these syntrophic relationships found in suspension. An interesting factor that needs to be further examined is the role of decrease or increase in bioreactor temperature in forging these microbial community interactions, as shown below.

A fascinating perspective can be gleaned about Direct Interspecies Electron Transfer (DIET) in these systems under sub-ambient temperature conditions as well, based on our current results. Recent studies have repeatedly suggested syntrophic cooperation between *Methanosaeta* and metallic Fe reducing bacteria such as *Geobacter* [24][25]. The microbial community results do show the possibility of the increasing proportion of *Methanosaeta* to be a component of aggregates, which is not however matched by a corresponding increase in *Desulfuromonodales* (to which *Geobacteracea* belong). This does raise an intriguing research question on the mechanism of acetate uptake by *Methanosaeta* in the AnMBR system at low temperatures.

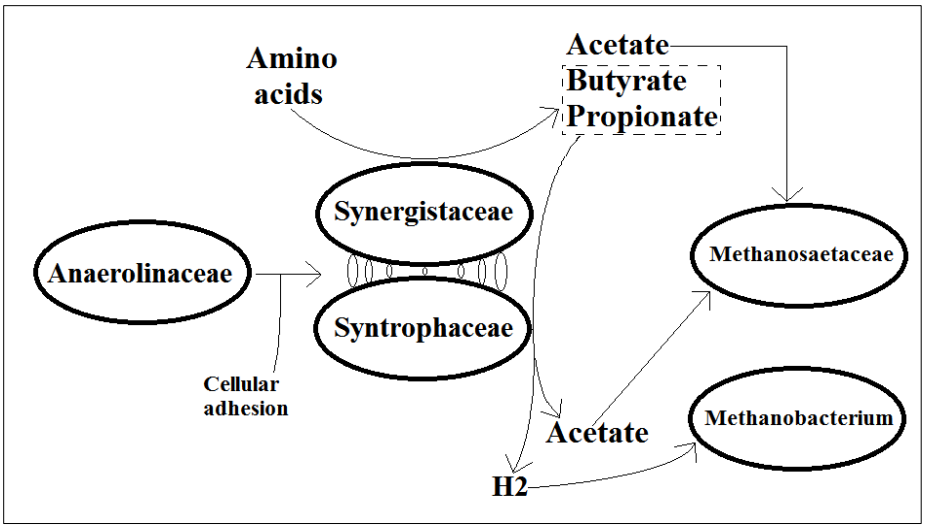

Figure 3.6. Diagram of hypothesized relationships of key microbes.

3.2.4 Statistical significance of data

R (RGui) v. 3.4.1 was used to test the statistical significance of correlations in our dataset based on Pearson correlations and Spearman's Rho rank correlation. The significance of the Pearson Correlation Coefficient, R, was checked by comparing the P values from each dataset pair with alpha, α, equal to 0.05 meaning at least 95% certainty is needed to prove that the correlation is not random. $P < 0.05$ was considered to be significant. Furthermore, a Pearson correlation coefficient is significant if the absolute value of R is greater than R Critical which is dependent on α. Additionally, Spearman's rho rank correlation using two-tailed tests were also performed on the dataset and $P < 0.05$ was considered to be significant.

Pearson Correlation Test

Only the relative abundance of *Synergistaceae* showed a strong negative correlation with temperature, R = -0.894, P = 0.001, meaning its relative abundance generally increased with decreasing temperatures. *Syntrophus* exhibited the same trend, R = -0.603, P= 0.084, but did not exhibit a 95% certainty. The relative abundances of *Methanobacterium, Bacteroidetes, Anaerolinaceae* and *Chloroflexi* in general exhibited a moderate positive correlation with temperature, R = 0.571, P = 0.109, R = 0.622, P = 0.074, R = 0.528, P = 0.146, R = 0.558, P = 0.119, respectively. Again though, these correlations did not exhibit a 95% certainty. The correlation seen with *Methanobacterium, Anaerolinaceae* and *Chloroflexi* in general can be

attributed to the initial abundance in the inoculum followed by ever decreasing abundances throughout the sample time range.

Spearman's Rho Rank Correlation Test

Only the relative abundances of *Synergistaceae* and *Syntrophus* showed a strong negative correlation with temperature, P = 0.0061 and P = 0.0311, respectively. This is in line with the Pearson correlation test and can be seen in Figures 3.2 and 3.3 where it seems that the relative abundance of *Synergistaceae* and *Syntrophus* grow and reach peak abundance during the coldest temperature period.

3.3 Future Recommendations

- DNA/RNA influent wastewater sampling to monitor the potential effect of continuous inoculation from wastewater microbiota.

- Sequencing of the 16S rRNA gene (rDNA) along with sequencing the 16S rRNA for relative abundance and relative activity, respectively, to get a better understanding of the roles that microbes play and not just their presence.

- There is a need to verify microbe abundances with the use of other technologies, besides DNA sequencing. This can be done by using reverse transcription quantitative PCR (RT-qPCR) targeting the methanogen orders *Methanomicrobiales*, *Methanobacteriales*, and *Methanococcales*; and the families *Methanosaetaceae* and *Methanosarcinaceae* along with the methyl coenzyme-M reductase (mcrA) gene which is a biomarker for methane yield. To clarify specific methanogenic contributions, acetoclastic methanogen metabolic activity can be traced by their acetate kinase and phosphotransacetylase gene, hydrogenotrophic methanogens can be traced by carbon monoxide dehydrogenase and formylmethanofuran dehydrogenase. To explicate syntrophic relationships that methanogens have with bacteria, qPCR can also be done for crucial bacteria groups such as AOB, homoacetogens, SRB, and syntrophic fatty acid fermenters. Syntrophic acetate-

oxidizing bacteria can be identified by the transcriptional profiling of formyltetrahydrofolate synthetase (FTHFS) gene, an ecological biomarker engaged in reductive acetogenesis.

- Investigation into the ability of mesophilic sludges to become psychrotolerant or the necessity for psychrophilic or psychrotolerant sub populations for high-rate psychrophlic anaerobic wastewater treatment.

- Investigation into AnMBR inoculation strategies to enrich bioreactors with particular microbiomes or using substrates to selectively enrich key microorganisms.

- Study on the syntrophic mechanism and direct interspecies electron transfer (DIET) involvement between key syntrophic microbes.

- Examination on the effects of materials such as GAC, biochar, or other materials in AnMBRs that could enhance syntrophy by enhancing biofilm, granular architectures, or by providing an electrically conductive media that enables sufficient treatment.

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Chapter 4

Conclusions

The AnMBR has successfully demonstrated the ability to treat domestic wastewater to EPA secondary standards and, in some occurrences to ANSI/NSF 350 reuse standards, even during low temperature conditions during the winter months. The AnMBR system has mostly met the performance goals set for HRT, OLR, COD, and BOD. The high proportion of methane in the generated biogas indicates attainable AnMBR treatment technologies that require less energy expenditures than previous technologies. The high methane content in the permeate that's captured by the hollow-fiber transfer membrane drives this system toward energy-neutral operations. The sulfide that is being generated in the bioreactors are a cause for concern if corrosion starts to deteriorate the inner components of the system, but the coagulation/flocculation system will work to capture sulfide and phosphate from the treated wastewater. None the less, these findings are encouraging for the future of AnMBR biotechnologies.

The common but diverse microbial community structure needed for the complete breakdown of complex organic molecules in the dilute domestic wastewater influent are present in the bioreactors of this study. *Bacteroidetes* and *Proteobacteria* exhibited similar abundances to each other throughout the sampling period, and when combined composed almost three quarters of the relative abundance on the last date. *Firmicutes* also showed a trend of increasing abundance while *Chloroflexi* showed a decrease. The elevating abundances of SRB are concerning because of the ability for their metabolic byproducts to deteriorate the inner AnMBR machinery. The order and genus graphs show trends of dominant species enrichment as more limited microbe groups with functional redundancies start to wan at the end of the sample period.

The 16S rRNA gene sequencing revealed changes in the methanogenic community structure as temperature decreased through the seasons. The shift to acetogenic methanogenesis inferred from relatively higher abundances of *Methanosaeta* that occurred after the initial winter temperature drop are surprising to see based on previous research findings that show results of hydrogenotrophic methanogens' ability to outcompete acetoclastic methanogens in colder

temperatures. *Methanosarcina* might have been absent from all samples because of its absence in the inoculum or because of the fact that the bioreactor VFA concentrations were consistently low and the AnMBR was run at temperatures below the mesophilic optimum of 35^{o}C. The absence of *Methanosarcina* indicates that acetoclastic methanogenesis was achieved primarily by *Methanosaeta*. Even though the trend of increasing relative abundance during low temperatures for *Methanosaeta* are evident on figure 3.5b, the statistical significance of this observation could not be calculated. *Synergistaceae* and *Syntrophus* did show a strong correlation with temperature, $P = 0.0061$ and $P = 0.0311$, respectively, according to the Spearman's rho rank correlation test. The relative abundances of *Methanobacterium, Bacteroidetes, Anaerolinaceae* and *Chloroflexi* in general exhibited a moderate positive correlation with temperature using Pearson's correlation test. The significant correlations between temperature and relative abundance fluctuations found can not be completely considered as causation because of many other factors, but temperature seems to be the biggest influential factor for microbial population fluctuation considering the near consistent chemical composition of influent wastewater and operating conditions.